A biaxial rotating bioreactor for the culture of fetal mesenchymal stem cells for bone tissue engineering

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ABSTRACT

The generation of effective tissue engineered bone grafts requires efficient exchange of nutrients and mechanical stimulus. Bioreactors provide a manner in which this can be achieved. We have recently developed a biaxial rotating bioreactor with efficient fluidics through in-silico modeling. Here we investigated its performance for generation of highly osteogenic bone graft using polycaprolactone-tricalcium phosphate (PCL–TCP) scaffolds seeded with human fetal mesenchymal stem cell (hMSC). hMSC scaffolds were cultured in either bioreactor or static cultures, with assessment of cellular viability, proliferation and osteogenic differentiation in vitro and also after transplantation into immunodeficient mice. Compared to static culture, bioreactor-cultured hMSC scaffolds reached cellular confluence earlier (day 7 vs. day 28), with greater cellularity (2×, p < 0.01), and maintained high cellular viability in the core, which was 2000 μm from the surface. In addition, bioreactor culture was associated with greater osteogenic induction, ALP expression (1.5×, p < 0.01), calcium deposition (5.5×, p < 0.001) and bony nodule formation on SEM, and in-vivo ectopic bone formations in immunodeficient mice (3.2×, p < 0.001) compared with static-cultured scaffolds. The use of biaxial bioreactor here allowed the maintenance of cellular viability beyond the limits of conventional diffusion, with increased proliferation and osteogenic differentiation both in vitro and in vivo, suggesting its utility for bone tissue engineering applications.

1. Introduction

Bone tissue engineering provides a promising approach to address the significant drawbacks of existing bone grafts, which include: firstly, the limited availability of bone tissue and donor-site morbidity associated with autografts [1,2]; secondly, the significant risk of disease transmission and immune reaction arising from the use of allografts [3]; and lastly, the lack of remodeling and subsequent fatigue-associated graft failure with the use synthetic grafts [4]. In bone tissue engineering, biodegradable porous scaffolds are seeded with osteogenic cell types to develop an in-vitro-matured engineered cellular bone graft, which can be fashioned into different shapes and sizes, stimulate bone healing and remodel accordingly [5].

Three dimensional (3D) scaffolds provide the necessary support for cells to attach, proliferate and differentiate, and define the overall shape of the tissue engineered transplant [6]. Scaffolds made of polycaprolactone (PCL) have recently been shown to possess favorable properties for load bearing bone tissue engineering application compared with other materials such as PLGA [7]. They have a slower degradation kinetics of only 7% over a six month period in vivo [8], maintaining a sustained period of mechanical support. In addition the slow degradation kinetics would result in a reduced risk of acidosis from the rapid accumulation of acidic by-products, suggesting its utility for bone tissue engineering applications.

Mesenchymal stem cells (MSCs) are increasingly being used as a cellular source for osteogenic tissue engineering applications, due to their ease of isolation and well defined osteogenic differentiation pathways [9,10]. However, the use of human adult MSC has been
handicapped by their low frequencies, slow proliferation time and generally limited proliferation capacity [11]. More recently, human fetal MSC (hfMSC) has been characterized, with significantly higher proliferation capacity and reduced immunogenicity when compared to their adult counterparts [12–15]. In a head-to-head comparison, we have showed that hfMSC demonstrates significant proliferative and osteogenic advantages over MSC types derived from the umbilical cord, adult adipose tissue or adult bone marrow in 3D culture systems both in vitro and in vivo [16].

The simple loading of osteogenic cell sources to suitable scaffolds to generate a bone graft however has largely been limited by the challenge of maintaining cellular viability at the core where larger grafts are concerned. This is due to the limits of diffusion of both nutrients and waste products, generally accepted to be around 1–200 μm [17,18]. In addition, homogenous cellular seeding and extracellular matrix distribution become inefficient when larger grafts are concerned [19,20]. One way of overcoming these limitations is with the use of bioreactors. Bioreactors enable improved flow perfusion of cellular scaffolds, which in turn improves mass transfer efficiencies. In addition, they provide a mechanical stimulation which triggers the mechano-transduction signaling pathway important for osteogenic differentiation in MSC cell types [19,21,22].

While most of these commercially available bioreactors are uniaxial in design, we have recently shown that a biaxial design which rotates simultaneously in two independent orthogonal axes resulted in improved fluidics over an uniaxial design through in-silico simulations [23]. Here we investigated the use of this biaxial bioreactor system for the in-vitro maturation of high-porosity PCL/TCP scaffolds seeded with hfMSC to generate bone tissue engineered grafts. We report that the incorporation of a biaxial bioreactor maturation step resulted in enhanced homogenous cellular proliferation, ECM distribution and osteogenic differentiation, and maintained high cellular viability in the core of the scaffolds.

2. Materials and methods

2.1. Samples, animals and ethics

Fetal tissue collection was approved by the Domain Specific Review Board of National University Hospital, Singapore in compliance with international guidelines regarding the use of fetal tissue for research [24]. Pregnant women gave separate written consent for the clinical procedure and for the use of fetal tissue for research purposes. And fetal tissues were collected from fetuses after clinically indicated termination of pregnancy. Fetal gestational age was determined by crown-rump length measurement. Two fetal samples at 16–18 and 14–22 (weeks + days) gestations were utilized for this study. Eight weeks old immunodeficient male NOD/SCID mice were acquired through Charles Rivers, Australia, and all procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at National University of Singapore. All materials used were purchased from Sigma–Aldrich (Singapore) unless otherwise stated.

2.2. Isolation and culture of hfMSCs

Bone marrow derived hfMSCs were isolated as previously described [25]. Briefly, single-cell suspensions of fetal bone marrow were prepared by flushing the bone marrow cells out of the humeri and femurs using a 22-gauge needle into Dulbecco's modified Eagle's medium (DMEM, Sigma, USA)–Glutamax (GIBCO, USA) supplemented with 10% fetal bovine serum (FBS), 50 U/ml penicillin, and 50 mg/ml streptomycin (GIBCO, USA), which will be referred to as D10 medium, and plated on 100 mm dishes at 10^4 mono-nuclear cells/ml in the D10 medium. Medium was changed every 2–3 days and non adherent cells were removed. Cells were trypsinized and replated at 10^6/cm^2 at sub-confluence. hfMSCs at Passage 3 were used for both characterization and the experiments described here. Both samples yielded similar results, and data presented here are representative of the experiments.

2.3. Characterization of hfMSC

2.3.1. Immunophenotype

hfMSCs were characterized by immunocytochemistry for CD14, CD34, CD45, CD51, von Willebrand factor (vWF) (Abcam, USA), CD105 (SH2), CD73 (SH3, SH4) (Abcam, USA), vimentin, laminin, CD29 (Chemicon, USA), CD44 (BD, USA), CD 106, CD 90 (Chemicon, USA), HLA I, HLA II (Dako, USA);Oct-4 (Abcam, USA) and Nanog (Abcam, USA) while flow-cytometry was used to screen for Sca-1 (Chemicon, USA) as previously described [16,25].

2.3.2. Tri-lineage mesenchymal differentiation

In order to assay the tri-lineage differentiation of the hfMSC, standard osteogenic, adipogenic and chondrogenic differentiation protocols were utilized and the results were confirmed with von Kossa staining for osteogenic differentiation, Oil Red staining for adipogenic differentiation and Safranin O staining for chondrogenic differentiation, as previous reported [16,26].

2.4. Scaffold manufacturing and surface treatment

We have previously described the generation of a high-porosity bioactive PCL-tricalcium phosphate (TCP) composite scaffold which would allow for rapid vascularization and maintenance of the structural integrity through a honeycomb structure, while allowing customisation of the scaffold to fit any particular shape and size [26]. PCL–TCP 3D bioactive scaffold specimens were fabricated using the fused deposition modeling techniques as previously described [27]. A lay-down pattern of 0/60/120° was utilized to give a honeycomb-like pattern of triangular pores with a porosity of 70% and average pore size of 0.523 mm. A scaffold size of 6 × 6 × 4 mm was used in these experiments (Fig. 2A).

The scaffolds were surface treated in 5% NaOH for 3 h to enhance their hydrophilicity. After rinsing 3 times with PBS, they were sterilized in 70% ethanol for 24 h, and rinsed twice in PBS, and then transferred into an incubator at 37 °C for 24 h for drying.

2.5. Seeding hfMSC to PCL–TCP scaffold

hfMSCs (Passage 3) were seeded onto the porous scaffolds by adding 50 μl of cell suspension media with 5 × 10^5 cells to each scaffold (5 × 10^5/μl) in a dropwise manner, placed in 24-well culture plates, and incubated for 3 h in an incubator. Thereafter, an additional 2 ml of D10 medium was slowly added to each well and hfMSC cellular scaffolds were incubated in a humidified atmosphere at 37 °C and 5% CO₂ for one week with D10 medium changed 3 times a week to allow for cell attachment to scaffolds (Fig. 2A).

2.6. Biaxial rotating bioreactor culture vs. static culture

After one week of static culture, scaffolds were randomly divided into two groups: (A) a bioreactor group where 45 scaffolds were transferred to the biaxial rotating bioreactor loaded with a total of 500 ml of osteogenic inductive medium which fills the vessel and the reservoir. This medium was changed once a week for a total of four weeks, with 11.3 ml of medium per scaffold per week; and (B) the static culture group where scaffolds were transferred to new 24-well plates with 3.5 ml of osteogenic inductive medium per well which was changed three times a week for four weeks, with 10.5 ml of medium made available to each scaffold per week (Fig. 2A). No major change of pH (Phenol Red) occurred in the culture medium during the experiments to suggest the depletion of nutrients from either group.

This biaxial rotation bioreactor system consists of a spherical vessel for culture (volume 500 ml), where the cellular-scaffold constructs are anchored to the lip of bioreactor by pins, and a medium reservoir (500 ml), which allows the continuously replenishing medium and the real time monitoring and control of oxygen, pH, and temperature. The spherical vessel and reservoir are connected by tubing to form a perfusion system with medium flow circulating between each other (as indicated in red arrow) (Fig. 2B). The entire bioreactor was placed in an incubator with humidified atmosphere at 37 °C and 5% CO₂. Gaseous exchange was enabled through a special membrane incorporated into the spherical vessel. The spherical vessel was programmed to rotate in two perpendicular axes (X and Z), with both axial rotation set to 5 rpm. This setting achieves a perfusion flow rate of 3.8 ml/min (Fig. 2B), allowing a complete change of medium every 132 min. A rotational speed of 5 rpm was chosen as it resulted in the greatest cellular proliferation in preliminary experiments investigating rotational speeds between 3 and 15 rpm.

2.7. Cellular adhesion, viability and proliferation of hfMSC cellular scaffolds

The morphology of the cell in 3D culture, cellular adhesion and extracellular matrix (ECM) production were examined daily by phase contrast light microscope (PCLM) over 28 days. Scaffolds were examined in two perpendicular planes, in planar (top) and side view points (Fig. 3B).

The qualitative analysis of cell viability in 3D was performed via fluorescent diacetate/propidium iodide (FDA/PI) staining, where FDA stains viable cells green, and PI stains necrotic and apoptotic cell nuclei red. Scaffolds were biected in half to expose the centre of the scaffold to achieve a core view of the scaffold (Fig. 4A), stained with FDA/PI as previously described [16], and viewed under a confocal laser
microscope (Olympus, FV300 Fluoview, Japan). Cellular scaffolds were examined in both planar view and side view on days 14 and 28 (Fig. 4A).

The total cell number in the 3D cellular scaffold on days 0 (day of transfer to the bioreactor or static culture conditions, as illustrated by Fig. 1A) 14, 28 (n = 3) was estimated by quantifying the dsDNA content of each scaffold using a PicoGreen dsDNA Quantification Kit (Molecular Probes, USA). The total DNA was extracted from each cellular scaffold by incubating the cellular scaffolds in 0.4 ml enzymatic cocktail (consisting of 0.1% collagenase A (Roche) with 0.1% Trypsin mixed in PBS) at 37°C for 2 h, with vortex every 30 min then followed by three cycles of freeze and thaw; and assayed by following the manufacturer’s instruction. The proliferation of the hfMSC inside 3D scaffold was interpreted by the changes of dsDNA amount.

2.8. Comparison of osteogenic differentiation and mineralization in 3D scaffold culture

2.8.1. ALP activity assay

The intracellular ALP activities of hfMSC cellular scaffolds under two culture conditions were compared on days 0, 7, 14, 28 (n = 3, triplicates). Cell lysates were tested for ALP activity using SensoLyte™ pHNPP Alkaline Phosphatase Assay Kit (Anaspec USA) and the ALP activities were normalized to the total protein content determined using the Bradford assay (Bio-Rad Laboratories, US) as previously described [16].

2.8.2. von Kossa staining

hfMSC cellular scaffolds on days 14 and 28 were stained with von Kossa staining. Briefly samples were gently rinsed twice with PBS then fixed in 10% formalin for 1 h, and washed in dH2O. Finally, they were stained with freshly made 2% silver nitrate in dH2O (w/v) for 10 min in the dark and exposed to an incandescent lamp (100 W) for 30 min.

2.8.3. Calcium content assay

The calcium content of the hfMSC cellular scaffolds on days 7, 14 and 28 (n = 3) was assayed as previously described [16]. Briefly, the calcium deposition is dissolved in 0.4 ml 0.5 N acetic acid and determined by a colorimetric assay using calcium assay kit (BioAssay Systems, USA). And control cell-free empty scaffolds cultured as above (n = 3 per time point) were used as a negative control to offset the elution of calcium from the tricalcium phosphate component in the scaffold (27.5 ± 4.3 mg/acellular scaffold).

2.8.4. Scanning electron microscope (SEM) and energy dispersive X-ray spectrometer (EDX) analysis

After micro-CT scanning, the same 3 scaffolds were dehydrated, gold sputtered, viewed under the SEM and element component of crystal-like structure inside the samples was analyzed by EDX as previously described [16].

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Three acellular scaffold implants were utilized as negative controls to cancel out the scaffold and known bone tissue was used for delineating ectopic bone from the scaffold and bone tissue. The threshold value between un-transplanted empty imaged together with transplanted acellular scaffolds, un-transplanted empty software, VGstudio (Volume Graphics GmbH, Germany) to stack the 2D image into Skyscan (Belgium). The reconstructed data were loaded onto the 3D modeling reconstructed at a step size of 1 using a modified Feldkamp algorithm as provided by a 1 mm aluminum filter were used during the scanning. The scan files were 3.1. Characterization of hfMSC

2.9.1. Cellular-scaffold constructs' preparation

hfMSC were seeded onto PCL–TCP scaffolds pre-cultured in D10 medium for one week and pre-differentiated in osteogenic differentiation medium under either bioreactor culture or static culture condition for two weeks before implantation (as illustrated in Fig. 1A).

2.9.2. Surgical procedure

After inducing general anesthesia, a midline longitudinal skin incision was made on the dorsal surface of each mouse, and subcutaneous pockets created, into which the scaffolds were inserted. The skin was closed with interrupted 6-0 vicryl sutures. After three months, animals were euthanized, and the implants retrieved for histological and micro-CT analysis (Fig. 1A).

2.9.3. Histology

Cellular-scaffold constructs (n = 3) from each group were embedded in OCT medium (Tissue-Tek, USA), and sectioned at 10 μm thickness and laid onto poly-L-lysine slides. Sections were stained with Masson Trichrome and von Kossa (counterstained with nuclear fast red) to ascertain evidence of bone formation and mineralization respectively.

2.9.4. Human to mouse chimerism

Human-specific lamin A/C immunostaining was used to investigate chimerism of human cells in murine tissue as previously described [25,28]. Briefly, sections from each sample were blocked with 5% normal goat serum for 2 h, and left to react with monoclonal mouse anti-human Lamin A/C antibody (1:100, Vectorlabs, UK) overnight at 4 °C; sections were then incubated with biotin anti-immunoglobulin antibodies (1:100 Alexaflour 488, Invitrogen, UK) for 1 h, and counterstained with propidium iodide (PI). Images were visualized through confocal microscopy as described above. The number of human and murine cells within scaffolds (n = 3) was enumerated manually on six low powered fields (LPFs) to calculate the rate of chimerism of human cells. A mean of 217 cells was counted for each LPF (range 157–313) of each specimen.

2.9.5. Micro-CT analysis

After cellular scaffolds (n = 3) have been harvested and fixed in 10% neutral buffered formalin, they were placed in the sample holder and scanned through 180° with a rotation step of 1° at a spatial resolution of 35 μm. An averaging of 5 and a 1 mm aluminum filter was used during the scanning. The scan files were reconstructed at a step size of 1 using a modified Feldkamp algorithm as provided by Skyscan (Belgium). The reconstructed data were loaded onto the 3D modeling software, VGstudio (Volume Graphics GmbH, Germany) to stack the 2D image into a 3D model for quantitative histomorphometric analysis. Cellular scaffolds were imaged together with transplanted acellular scaffolds, un-transplanted empty scaffold and bone tissue. The threshold value between un-transplanted empty scaffold and known bone tissue was used for delineating ectopic bone from the scaffolds through visual and histogram analysis, and a value of 195 was chosen. Three acellular scaffold implants were utilized as negative controls to cancel out the influence of the TCP component within the scaffold on mineralization.

2.10. Statistical analyses

Parametric data have been represented as mean ± SD, and compared using either two-way ANOVA or Student t-test. A value of p < 0.05 was taken as significant.

3. Results

3.1. Characterization of hfMSC

Culture expanded hfMSC grew as plastic-adherent spindle-shaped cells (Fig. 2A), with 45.1% of hfMSC being positive for the osteoprogenitor marker Stron-1. (Fig. 2B). hfMSC revealed an immunophenotype which was negative for haemopoietic and endothelial markers CD14, CD34, CD45, CD31, VWF and HLA II and positive for mesenchymal markers CD105 (SH2), CD73 (SH3, SH4); intracellular marker vimentin and laminin; cell adhesion molecules CD29, CD44, CD 106, CD 90; and HLA I as previously reported [25,28]. Furthermore, a small proportion of hfMSC was positive for the embryonic stem cell markers Oct-4 (25.6 ± 1.4% positive) and Nanog (34.7 ± 2.1% positive) (Fig. 2C). Under permissive culture conditions, they underwent osteogenic, adipogenic and chondrogenic differentiation (Fig. 2D), confirming their bona-fide MSC phenotype.

3.2. Bioreactor culture resulted in homogenous cellular distribution, rapid proliferation and maintenance of cellular viability at the scaffold core

Under bioreactor culture, hfMSC proliferated rapidly, saturating all the spaces within the scaffolds and reached confluence in 7 days, as seen by light microscopy (Fig. 3C–F), which was confirmed through measurement of total double-stranded DNA content (Picogreen dsDNA assay, Fig. 3S). In contrast, static-cultured scaffolds reached confluence within the scaffolds only after 28 days of culture (Fig. 3G–J, S), and achieved a lower final cell number at 28 days compared to the bioreactor treated scaffolds (0.6×, p < 0.001, Fig. 3S).

Bioreactor-cultured scaffolds demonstrated homogenous cellular distribution at the surface (planar view, Fig. 4A) as well as the internal core (core view, Fig. 4A) of the scaffolds at days 7 and 14, which was evident with both light microscopy and FDA/PI staining (Figs. 3E and F and 4B and C). While bioreactor-cultured scaffolds maintained good cellular viability at the core of the scaffold after 28 days of culture (Fig. 4D and E), we observed massive cellular death in core of the scaffold in static cultures under static culture conditions (Fig. 4I), despite good viabilities earlier in culture (day 14, Fig. 4F and G).

3.3. Bioreactor culture enhanced osteogenic differentiation and mineralization

We observed the deposition of extracellular calcium crystals appearing in bioreactor treated scaffolds by day 14 (Fig. 3L), which was only noticeable at day 28 in static-cultured scaffolds (Fig. 3R). This observation was confirmed through the darker von Kossa staining of the bioreactor cultured over static-cultured scaffolds at both days 14 and 28 time points (Fig. 5A).

Scanning electron microscopy (SEM) revealed higher levels of crystal-like extracellular matrix (ECM) deposition in bioreactor cultured vs. static-cultured scaffolds (Fig. 5B). These deposits were calcium phosphate salts as shown through element component analysis (EDX) to consist mainly of P, Ca and O elements (Fig. 5C).

Supporting this observation, we found that bioreactor treated scaffolds expressed significantly higher levels of ALP activity than the static culture scaffolds from day 7 through day 28 (1.5×, p < 0.001, Fig. 5D), and higher calcium deposition (4.9× at day 14, p < 0.001, and 5.7× at day 28, p < 0.001, Fig. 5E).

hfMSC assumed an osteoblastic morphology after bioreactor culture (day 28, Fig. 4D and E), appearing ovoid in shape as observed on FDA/PI staining, in contrast to static-cultured scaffolds, where hfMSC maintained a typical spindle-shaped morphology (Fig. 4H and I).

3.4. Bioreactor treatment promoted higher ectopic bone formation in vivo

Three months after subcutaneous implantation in immunodeficient mice, scaffolds from both bioreactor and static culture were found to be well integrated into the surrounding host tissues, with no evidence of any tumour formation (n = 18) (Fig. 6A and B). Bioreactor-cultured scaffolds demonstrated higher human:mouse chimerism rates than static-cultured scaffolds (78.5 ± 14.6% vs. 57.6 ± 8.3%, p = 0.02) (Fig. 6C–G). In addition, bioreactor-cultured scaffolds generated more woven (Masson’s Trichrome staining, Fig. 6H and I), and calcified bone (von Kossa, Fig. 6J and K). In line with these observations, micro-CT analyses demonstrated more ectopic bone formation in bioreactor over static-cultured scaffolds (3.2×, p < 0.001) (Fig. 6L and M).
4. Discussion

Bioreactors have been developed in order to address the need to circumvent the limitations of mass transfer in thick scaffold cultures, and in the case of bone tissue engineering, to apply suitable mechano-transduction forces which in turn facilitate osteogenic differentiation through specific signaling pathways [19,22]. In this study, we demonstrated that biaxial bioreactor matured hfMSC/PCL–TCP scaffolds resulted in significantly higher cellular proliferation, homogenous cellular distribution and in-vitro and in-vivo osteogenic differentiation than those cultured in static condition. In addition, biaxial bioreactor-cultured scaffolds retained high cellular viabilities in the core of the scaffold not achieved with static cultures.

Currently, several bioreactors such as spinner flasks, perfusion bioreactors and rotating wall vessel (RWV) bioreactors have been investigated for bone tissue engineering applications, but have been beset by various limitations. The use of spinner flasks resulted...
Fig. 3. Cell adhesion and proliferation of hfMSC cultured in the PCL–TCP scaffolds. A) High-porosity PCL–TCP scaffolds measuring 6 mm × 6 mm × 4 mm were seeded with hfMSC for these experiments; B) Scaffolds were viewed from the planar (top) and side profile under phase contrast light microscopy through the culture. C–R) Scaffolds which had been cultured for one week were transferred to either bioreactor or static culture conditions over 28 days. Biaxial bioreactor-cultured scaffolds achieved confluence of all the available space within the scaffolds by day 7 (D–F), with the appearance of extracellular crystals from day 14 of culture (red arrow, L), which increased in amount by day 28, limiting the passage of light through the scaffold (K–N). In contrast, static-cultured scaffolds reached confluence only after 28 days of culture, with evidence of mineralization (red arrow, R) appearing only at day 28 (G–J & O–R). S) Quantification of dsDNA content in cellular scaffolds by Picogreen assay supported the significantly higher cellular proliferation rates (*p* < 0.001), achieving a confluence by day 7, and achieving a higher final cell content within the scaffolds at all time points.

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in improved fluid flow through a simple design [29–31], although the turbulence generated can be detrimental for seeded cells and newly laid down ECM [21,32]. Perfusion bioreactors, which have been shown to enhance MSC proliferation and osteogenic differentiation in scaffold constructs [33–35], have been limited by non-homogenous cellular distributions, with cells at the frontal zones being washed away by the oncoming perfusion flow with higher flow rates [36]. Rotating wall vessel (RWV) bioreactors [37–39], which generate low shear forces and 3D high mass transfer capacity, are prone to similar problems of non-homogenous cellular growth and ECM deposition [21,33,40]. In addition, during the free floating culture, collision between the scaffolds and the bioreactor walls can induce cellular damage and disrupt cellular attachment and matrix deposition on the scaffolds [21,33,40].

Consequently, the biaxial rotating bioreactor used here has been designed to address the deficiencies found in current bioreactor designs: firstly, a perfusion system was included to allow the exchange between the vessel and reservoir, allowing maximal mass transfer with consequential low shear stress, without the washout associated with RWV bioreactors; secondly, biaxial rotation of the culture vessel improved upon uniaxial rotating designs, leading to more homogenous cellular and ECM distribution of the scaffold, as previously predicted from in silico [23], and; lastly, cellular scaffolds were secured by pins and were not kept in free suspension, avoiding the risk of scaffold collisions with the vessel walls.

By day 28 of static culture, we found cellular necrosis within the core of scaffolds, which is 2000 μm (2 mm) away from the surface. This coincided with the achievement of cellular confluence within the scaffold in static culture, and hence the need for nutrients and oxygen to reach the core through diffusion alone. The cellular necrosis observed here is largely predictable as the limits of mass transfer of nutrients are generally held to be around 200 μm [19]. In contrast, biaxial bioreactor-cultured scaffolds, which achieved cellular confluence by day 7 of culture, resulted in high degrees of cellular viability at the core of the scaffolds from 7 through 28 days despite the high cellularity and mineralization, with consequential higher final cellular numbers and osteogenic differentiation and mineralization. This performance was better than the experience with spinner flasks, where Sutherland et al. found a central necrotic core in 1 mm spheroid, which is 500 μm from the surface [41].

Culturing scaffolds in bioreactor resulted in higher cellularity which was obvious through both light microscopy and dsDNA content measurements, indicating the beneficial effects of improved mass transport. In addition, shear stresses found within the biaxial bioreactor can provide key mechanical stimulation which has been implicated in the promotion of cellular proliferation of osteoprogenitor cell types [22,42].

In the bioreactor culture, shear forces would have been applied to both the surface and the interior of the scaffold during the first week of culture, in line with in-silico fluid dynamic modeling simulations [23]. At the end of one week, however, cellular confluence of the scaffold would prevent any further convection from taking place within the scaffold itself. The maintenance of cellular viability at the scaffold core, which is 2000 μm from the surface, would have been impaired. This would prevent any further convection from taking place within the scaffold itself. The maintenance of cellular viability at the scaffold core, which is 2000 μm from the surface, would have been impaired.

**Fig. 4.** Cellular viability studies (FDA/PI staining): A) FDA/PI was used to stain for live and dead cells respectively, through confocal microscopy imaging of the planar (top) view, and after bisecting the scaffold into two in the middle, achieving a view of the scaffold’s centre (core view). B–I) Biaxial bioreactor-cultured scaffolds demonstrated confluence of the scaffold surface and interior, with homogenous cellular distribution at an earlier time point (B and C) then static-cultured scaffolds (F and G) (D14 shown here). By day 28, hMSC in bioreactor-cultured scaffolds assumed an osteoblast morphology, appearing ovoid in shape (D and E), while retaining high cellular viabilities in the core of the scaffolds (E). In contrast, hMSC in static-cultured scaffolds remained spindle shaped (H), with poor cellular viabilities in the core of the scaffolds (I). (All images here are confocal z-stack images, constructed from 44 horizontal image sections with 300 μm in depth. Mag. 100×.)

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surface, would depend on the improvement of mass transfer of nutrients afforded by convection forces at the surface of the scaffold [19].

Shear stress generated by the biaxial bioreactor can trigger mechano-transduction signaling pathways, which in turn upregulates production of cyclic adenosine monophosphate (cAMP) [43], transforming growth factor beta1 (TGF-β1) [44] and nitric oxide [45], all of which have been implicated in bone tissue repair mechanisms. The improved osteogenic differentiation and mineralization of hfMSC cellular scaffolds in a dynamic bioreactor environment over a static culture system are in keeping with the general observation that shear forces stimulate osteogenic programming in MSC [4,33,46–49]. In addition, we have observed that biaxial bioreactor-cultured hfMSC assumed an ovoid

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**Fig. 5.** Osteogenic differentiation and mineralization of cellular scaffold. A) von Kossa staining of scaffolds in bioreactor culture demonstrated more robust osteogenic differentiation and mineralization than static culture, as shown in much darker of von kossa staining at days 14 and 28. B) Scanning electron microscopy (SEM) images of cellular scaffolds at day 28 of culture demonstrating higher mineralization in the scaffolds grown in bioreactor cultures. C) EDX analysis of the element components revealed the mineralised nodules as calcium phosphate salts, consisting of P, Ca and O elements. D) Cellular scaffolds cultured in biaxial bioreactor expressed higher level of ALP activity than those in static culture from day 7 (**p < 0.01, ***p < 0.001), and finally, E) analysis of calcium deposition in the scaffolds revealed significantly higher calcium deposition in bioreactor-cultured scaffolds than static-cultured scaffolds from days 14 to 28. **p < 0.01.

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Fig. 6. In-vivo ectopic bone formation A and B) Implanted scaffolds from both groups integrated into the surrounding tissues with no evidence of tumour formation. C and G) Immunostaining for human-specific lamins A/C (green nuclei, counterstained with propidium iodide) showed a higher human:mouse chimerism rate in bioreactor-cultured vs. static-cultured scaffolds (78.5 ± 14.6% vs. 57.6 ± 8.3%, \( p < 0.05 \)). (S indicates scaffold). H–K) Bioreactor-cultured scaffold showed more woven and mature bone formation and mineralization by Masson’s Trichrome and von Kossa staining respectively (scale bar 100 μm). L and M) This was confirmed by micro-CT analysis of the new ectopic bone formation around the cellular scaffolds, with 3.2-fold more ectopic bone formed in bioreactor-cultured scaffolds compared with static-cultured scaffolds (*\( p < 0.05 \), **\( p < 0.001 \)).
morphology similar to those of mature osteoblasts, suggesting a higher degree of differentiation being achieved compared to static-cultured hfMSC.

The improved in-vitro performance of biaxial bioreactor-cultured scaffolds over static-cultured scaffolds was accompanied by higher cell proliferation and differentiation of bone tissue engineered grafts compared to standard static culture systems. More importantly, the use of our biaxial bioreactor resulted in a ten-fold improvement in mass transfer in thicker grafts, which will have important implication for their eventual clinical application in bone tissue engineering.

5. Conclusion

The use of this biaxial rotating bioreactor should allow shorter in-vitro maturation culture time to be achieved, along with improved cellular and ECM distribution, and more efficient osteogenic induction and mineralization of bone tissue engineered grafts compared to standard static culture systems. More importantly, the use of our biaxial bioreactor resulted in a ten-fold improvement in mass transfer in thicker grafts, which will have important implication for their eventual clinical application in bone tissue engineering.

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Appendix

Figures with essential colour discrimination. Certain parts of the majority of the figures in this article are difficult to interpret in black and white. The full colour images can be found in the on-line version, at doi:10.1016/j.biomaterials.2009.01.028.

Appendix. Supplementary data

Supplementary data associated with this article can be found in the online version, at doi:10.1016/j.biomaterials.2009.01.028.

References


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