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A comparison of bioreactors for culture of fetal mesenchymal stem cells for bone tissue engineering

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ABSTRACT

Bioreactors provide a dynamic culture system for efficient exchange of nutrients and mechanical stimulus necessary for the generation of effective tissue engineered bone grafts (TEBG). We have shown that biaxial rotating (BXR) bioreactor-matured human fetal mesenchymal stem cell (hfMSC) mediated-TEBG can heal a rat critical sized femoral defect. However, it is not known whether optimal bioreactors exist for bone TE (BTE) applications. We systematically compared this BXR bioreactor with three most commonly used systems: Spinner Flask (SF), Perfusion and Rotating Wall Vessel (RWV) bioreactors, for their application in BTE. The BXR bioreactor achieved higher levels of cellularity and confluence (1.4–2.5x, $p < 0.05$) in large 785 mm^3 macroporous scaffolds not achieved in the other bioreactors operating in optimal settings. BXR bioreactor-treated scaffolds experienced earlier and more robust osteogenic differentiation on von Kossa staining, ALP induction (1.2–1.6×, $p < 0.01$) and calcium deposition (1.3–2.3×, $p < 0.01$). We developed a Micro CT quantification method which demonstrated homogenous distribution of hfMSC in BXR bioreactor-treated grafts, but not with the other three. BXR bioreactor enabled superior cellular proliferation, spatial distribution and osteogenic induction of hfMSC over other commonly used bioreactors. In addition, we developed and validated a non-invasive quantitative micro CT-based technique for analyzing neo-tissue formation and its spatial distribution within scaffolds.

1. Introduction

Bone graft is the second most transplanted tissue in the world, with more than 1.5 million transplantation performed in United States annually [1,2]. However, this demanding need for effective bone grafts to treat non-union fractures cannot be fulfilled by existing bone grafts. This is due to the limited availability and donor-site morbidity associated with the use of autografts [3,4], the significant risk of disease transmission and immune reaction with the use of allografts [5]and vulnerability of fatigue in synthetic grafts secondary to their inability to remodel [6]. Bone tissue engineering (BTE) has been proposed as a promising strategy to develop tissue engineered bone grafts (TEBG), which are not only available off-the-shelf like allo- and synthetic grafts, but also have potent bone repair capacity like autografts. The success of BTE strategy requires the integrated technological advances from research fields of scaffold, stem cell and bioreactor culture system [7].

Mesenchymal Stem Cells (MSC) are readily isolated, non-immunogenic and have well defined osteogenic differentiation pathways, and have been investigated as osteogenic cell sources for BTE [8–11]. However, the clinical use of human adult MSC has been hindered by their low frequencies in vivo, limited proliferation capacity and early senescence in culture, and hence has limited capacity for generating adequate cell numbers for clinical applications, especially in the older age groups [12]. More recently, human

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fetal MSC (hfMSC) have been identified and characterized from ontologically primitive sources [13,14]. In a head-to-head comparative study, hfMSC have significantly higher proliferative and osteogenic potential and lower immunogenicity than MSC types isolated from perinatal or postnatal origins, suggesting their greater potential as allogeneic osteogenic cellular source for BTE [15].

Dynamic bioreactor culture systems are essential for the in vitro cultivation and maturation of TE bone grafts, especially for larger grafts where the core of the scaffold is more than 200 μm from the surface. Bioreactors improve the mass transport of nutrients and allow the diffusion limitation of traditional static culture, which is generally taken to be around 200 μm, to be overcome [16,17]. In addition, the dynamic media flow applies a mechanical stimulus to the cells, enhancing cellular osteogenesis and mineralization through triggering of mechano-transduction signaling pathways [18,19]. Currently, several types of bioreactors have been developed for BTE applications. This includes the Spinner Flask (SF) bioreactor, Perfusion bioreactor and Rotating Wall Vessel (RWV) bioreactor, all of which have been shown to promote cellular proliferative and osteogenic differentiation [18,20–22]. Notably, most of these bioreactors are uni-axial in design, which may place a constraint on the homogenous flow pattern of media. In order to overcome this, we have developed a Biaxial Rotating (BXR) bioreactor which integrates a biaxial rotating wall vessel design with a media perfusion system, evidenced with improved flow dynamics over uni-axial rotation under in-silico simulation [23]. The dynamic culture and osteogenic priming of hfMSC-mediated macroporous polycaprolactone/tri-calcium phosphate (PCL-TCP) scaffolds in this BXR bioreactor generated an effective TEBG which resulted in the healing of a critical sized defect [24,25].

While the use of bioreactors for the culture of MSC for BTE has been reported by several groups, there is a paucity of studies which have compared different bioreactors in an optimised manner with the use of relevant osteogenic cell sources loaded onto macroporous scaffolds suitable for BTE. In this study, we performed a head-to-head comparison of this BXR bioreactor with SF, Perfusion and RWV bioreactors for BTE. After optimization of each individual parameter for BXR, SF and Perfusion bioreactors, we conducted a systematic comparison of the bioreactors and their effect on the proliferation and osteogenic differentiation of hfMSC seeded within macroporous PCL-TCP scaffolds.

2. Materials and methods

2.1. Samples and ethics

Fetal tissue collection was approved by the Domain Specific Review Board of National University Hospital (D06/154), Singapore in compliance with international guidelines regarding the use of fetal tissue for research [26]. 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. In this study, a fetal sample at 14^{+2} (weeks + days) gestations was utilized for both preliminary and comparative experiments.

2.2. Isolation and characterization of hfMSCs

hfMSC were isolated through plastic adherence, and culture expansion, and characterized through immunophenotyping, colony-forming capacity and tri-lineage differentiation into osteoblasts, adipocytes and chondrocytes as previously described [15,24]. Briefly single-cell suspensions of fetal bone marrow were prepared by flushing the marrow cells out of 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) (referred as D10 medium), and then plated onto 100 mm dishes at 10^6 mononuclear cells/ml in D10 medium. Media were changed every 2–3 days and non adherent cells were removed, and sub-cultured at $10^4/cm^2$ to sub-confluence. hfMSCs at passage 3 were used for characterization and at passage 4 were used for both preliminary and comparative experiments.

2.3. Experimental design

2.3.1. Generation of macroporous PCL-TCP scaffolds

A bioactive PCL-TCP composite scaffold with high porosity was fabricated using the fused deposition modeling technique, which results in a honey-comb-like pattern of triangular pores with a porosity of 70%, average pore size of 0.523 mm, and a lay-down pattern of 0/60/120° as previously described (Fig. 1A) [15].

2.3.2. Seeding hfMSC to PCL-TCP scaffold

hfMSC were seeded onto the porous scaffolds by adding cell suspension media to scaffolds (seeding density 2500 cells/mm^3 scaffold), placed in 24-well culture plates, and incubated for 3 h in an incubator to allow for the initial cellular attachment to the scaffolds. Thereafter, 3 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% CO_2 for 1 week with D10 medium changes 3 times a week to acclimatize the cellular scaffolds before dynamic culture (Fig. 1B).

2.3.3. Optimisation of bioreactor parameters

First, a "Preliminary Experiment" was designed to optimize the culture conditions and setting of the bioreactor parameters for BXR, Perfusion and SF bioreactors (Fig. 1B). These were a biaxial rotation speed of 2, 5, 10 rpm with a fixed perfusion flow rate of 3.5 ml/min for BXR bioreactor; a perfusion flow rate of 0.2, 2.0 and 5.0 ml/min for Perfusion bioreactor; and a stirring speed of 10, 20 and 30 rpm for SF bioreactor. After seeding hfMSC to PCL-TCP scaffolds ($4 \times 4 \times 4$ mm, Fig. 1A) in a dropwise manner and pre-cultured for one week as above, hfMSC cellular scaffolds were then transferred to BXR, Perfusion and SF bioreactors operated in different culture conditions for 1 week and assessed for cellular adhesion, proliferation and viability ($n = 4$).

2.3.4. Comparative experiment

After the optimal culture condition has been determined, a comparative experiment was conducted to compare BXR, Perfusion, SF, and RWV bioreactors for BTE application (Fig. 1B). During the experiment, BXR, Perfusion and SF bioreactors were operated at the optimal conditions derived from the initial experiment, while RWV bioreactor was operated at the rotation speed of 47 rpm and perfusion flow rate of 3.5 ml/min, which achieved free suspension of the scaffolds in the media. We use large cylindrical shaped scaffolds (10 mm in diameter and height, Fig. 1A) for this comparative study. After the loading of the cells and pre-culture in static condition for 1 week as above, hfMSC cellular scaffolds were randomly divided into four groups and cultured in osteogenic induction media (D10 medium supplemented with 10 mM β-glycerophosphate, 10^{-8} M dexamethasone and 0.2 mM ascorbic acid) within the four different bioreactors over 4 weeks. Each group was compared for cellular adhesion, proliferation, viability, distribution within the scaffold, osteogenic differentiation and mineralization. In all bioreactors, media were changed once a week over four weeks, with 28 ml of medium change per scaffold per week being kept as a constant between groups to ensure identical access to nutrients. The bottom end of each cylindrical scaffold was defined as the side of the scaffold sitting on the culture plate during the initial static loading of the scaffold.

2.3.5. Bioreactor setups

The BXR bioreactor consists of a spherical culture chamber, where the cellular scaffolds are anchored to the cap of bioreactor by pins, a medium reservoir and a perfusion system, which connects culture chamber and medium reservoir, as previously described [23,24]. The spherical culture chamber is designed to rotate in two perpendicular axes (Y and Z, as indicated in blue block arrows, Fig. 1C) simultaneously and perfused with media flow circulating between culture chamber and medium reservoir (as indicated by red arrows, Fig. 1C). The perfusion bioreactor consists of a number of perfusion culture chambers, where cellular scaffolds are placed, a medium reservoir and a peristaltic pump (Masterflex, USA). Culture chambers, reservoir and pump are connected to each other in series, with medium circulating among each other (as indicated by red arrows, Fig. 1C). The SF bioreactor (Bellco Biotechnology, USA) consists of a culture chamber, where the cellular scaffolds are anchored to the cap of culture chamber by pins, and a magnetic stir bar, which can rotate in the *Z*-axis (as indicated by blue block arrows, Fig. 1C). Finally, an RWV bioreactor (Synthecon, USA), consisting of a horizontally rotated culture chamber, where the cellular scaffolds are suspended, and a perfusion system with media continuously flowing through the culture chamber (as indicated in red arrows, Fig. 1C) was used. The culture chamber can rotate in the *X*-axis (as indicated in blue block arrows, Fig. 1C) at certain speed to suspend the cellular scaffolds in a free floating culture condition. All four bioreactor systems were placed within an incubator during the culture.

2.4. Cellular adhesion and proliferation of hfMSC cellular scaffolds

The morphology of hfMSC in 3D culture, cellular adhesion and extracellular matrix (ECM) production were examined weekly by Phase Contrast Light Microscope (PCLM) over 4 weeks. The qualitative analysis of cell viability in 3D was performed by fluorescein di-acetate/propidium iodide (FDA/PI) staining, where FDA stains viable cells green, and PI stains necrotic and apoptotic cell nuclei red. Cellular