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In vitro engineering of bone using a rotational oxygen-permeable bioreactor system

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Abstract

Tissue engineering of bone may supersede the need in the future of autograft procedures to treat bone defects resulting from trauma or developmental diseases. A Rotational Oxygen-Permeable Bioreactor System (ROBS) has recently been developed in our laboratory to reproduce dynamic and gas-permeable culture conditions that would supply optimal oxygen and continuous loading to cell/polymer constructs in culture. The cell culture media in ROBS were examined at 1, 24 and 48 h to evaluate the kinetics of pO_2, pCO_2 and pH without culturing cells. The results were compared to the kinetics in 100 mm diameter cell culture dishes (Control I: static, gas permeable) and 50 ml centrifuge tubes (Control II: dynamic, non-gas permeable). The results showed the same kinetics in ROBS and Control I, whereas Control II failed to maintain the gas conditions of the media. Next, osteoblasts derived from mesenchymal stromal cells (MSCs) of neonatal rats were cultured in three-dimensional poly(DL-lactide-co-glycolide) (PLGA) foams using ROBS to study the effectiveness of this bioreactor system to support cell growth and differentiation. Mineralization was observed within 2 weeks of culture and was shown throughout the polymer at 7 weeks with embedded osteocytic cells. This study demonstrates the usefulness of ROBS for in vitro bone tissue engineering. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: Bioreactor; Tissue engineering; Bone; In vitro; Partial gas pressure

1. Introduction

The repair of bone defects, resulting from tumors, infection or trauma remains a challenge for orthopedic surgeons. Currently, autologous bone grafting continues to be the gold standard, but this technique has several disadvantages, including a limited amount of bone supply and donor site morbidity. For more than 30 years, researchers have tried to develop bone graft substitutes that would supersede the need for an autograft harvest. Osteoconductive biomaterials, including bone cement, hydroxyapatite and tricalcium phosphate were first proposed, but many complications occurred, such as infection, high extrusion rates and stress shielding [1]. Recent advances in the fields of cell biology and materials engineering have allowed the development of engineered organs or tissues in the laboratory [2]. These techniques are based on the combination of osteocompetent cells and resorbable scaffolds, and have been already explored both in vivo and in vitro. Using the in vivo approach, Isogai et al. showed excellent bone formation using biodegradable polymers seeded with cell resources and implanted subcutaneously in nude mice. After 40 weeks, both cortical and cancellous bone formation were observed in the tissue-engineered construct [3]. In vitro tissue engineering of bone is presumed to be more useful for mass production and may allow more precise control of osteogenesis during the process. Ishaug et al. [4] first reported the in vitro formation of a three-dimensional osseous structure after 56 days of culture, using poly(α-hydroxy ester) biodegradable foams seeded with osteoblasts. The in vitro engineered bone showed a good distribution of the cells throughout the polymer, synthesis of an extra-cellular matrix, but partial mineralization. The amount of bone formation in both the in vivo and in vitro approaches may be influenced by many factors, including growth factors, hormones, sufficient and continuous level of oxygen and nutrients supplies and appropriate mechanical stress on cells.

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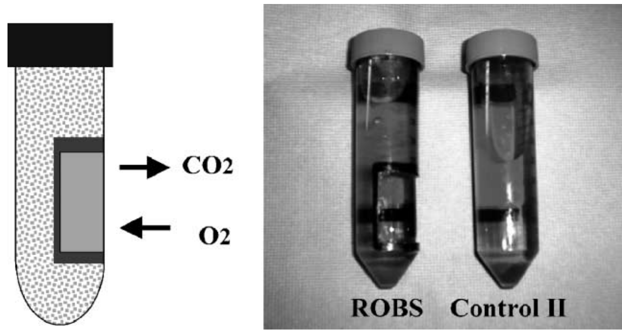

Fig. 1. Polystyrene vessel with 30 × 40 mm window, coated with silicone membrane inside the vessel that allows gas exchange (left). A 50-ml centrifuge tube (right) was used as a dynamic but non-gas permeable model (Control II).

While many studies have reported the positive effects of biological factors, such as the bone morphogenetic proteins (BMPs) [5,6], few have focused on optimal oxygen supply and physical stresses in order to enhance osteogenesis in vitro [7].

We recently developed a new Rotational Oxygen-Permeable Bioreactor System (ROBS) that aims at supplying optimal oxygen levels and continuous hydrostatic pressure to a tissue-engineered structure consisting of a biodegradable polymer scaffold seeded with osteoblasts. The following studies are the subjects of this report: (1) we examined oxygen and carbon dioxide partial pressures (pO_2 and pCO_2) and pH of the cell culture media in ROBS and compared them to other culture conditions such as 100 mm cell culture dishes (static and gas permeable culture model) or 50 ml centrifuge tubes (dynamic and non-gas permeable model) to prove the gas permeability of ROBS; (2) biodegradable polymer foams seeded with osteoblastic cells, harvested and differentiated from rat bone marrow mesenchymal stromal cells (MSCs), were cultured in ROBS and examined histologically to prove the efficiency of this device in producing tissue engineered bone.

Fig. 2. Original designed vessel holder placed on roller device (6 RPM) in an incubator (5% CO_2 in air, 37 °C, fully humidified).

2. Experimental procedure for bioreactor system

2.1. Rotational Oxygen-Permeable Bioreactor System (ROBS)

This system was designed to provide sufficient oxygen tension to cell culture media under dynamic cell culture conditions. A thin coating of silicone elastomer (SYL-

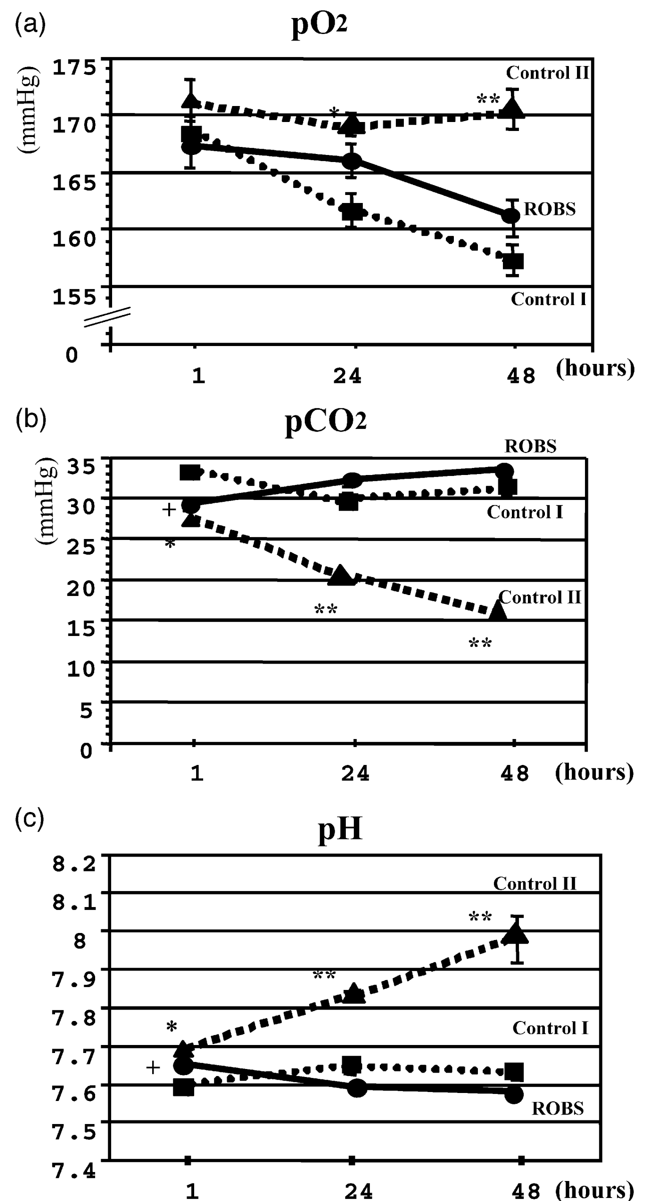

Fig. 3. (a) Graph of pO_2 measurements. (b) Graph of pCO_2 measurements. (c) Graph of pH measurements. Standard error bar is shown at each point. All measurements are statistically analyzed (*t*-test) and decided as significantly different at $p < 0.01$ (● = ROBS, ■ = Control I, ▲ = Control II).

GARD® 184, Dow Corning, MI, USA) was applied inside a 50-ml polypropylene centrifuge tube (Fisher, NJ, USA). After curing, a 30 × 40 mm window was cut out from the polypropylene tube to allow for gas exchange (Fig. 1). This gas-permeable vessel was placed in the originally designed vessel holder and placed on a roller culture apparatus (Wheaton, NJ, USA) in an incubator maintained at 37 °C in a fully humidified atmosphere of 5% CO_2 in air (Fig. 2). The rotating speed was set to 6 RPM to allow for motion of the cell/polymer constructs inside the vessel during rotation.

2.2. Analysis of oxygen and carbon dioxide partial pressures and pH of medium

In order to compare the ability of gas exchange of ROBS, a 100-mm diameter cell culture dish and a 50-ml centrifuge tube were used as controls. A 100-mm cell culture dish was used as a static and gas permeable culture condition model (Control I). A 50-ml centrifuge tube was placed in the same holder with the ROBS and used as a rotational but non-gas permeable culture model (Control II). Each model was filled with 35 ml of primary cell culture media (Dulbecco's Modified Eagle Medium (GIBCO, NY, USA) with 10% Fetal Bovine Serum (Hyclone, UT, USA) and 1% Penicillin–Streptomycin (Sigma, MO, USA) and placed in the same incubator. Before use, the cell culture media was stored overnight in the same incubator in a gas exchangeable flask to standardize the initial gas and the pH of the media. After 1, 24 and 48 h, 1 ml of cell culture media was taken from each model with a heparinized syringe simultaneously to analyze pO_2, pCO_2 and pH by using an arterial blood gas analyzer (RapidLab™ 840, Bayer). Twelve samples from each group were examined ($n = 12$). Statistical analysis (Student's *t*-test) was used to evaluate the results, and *p*-values of less than 0.01 were considered significant.

3. Experimental procedure for in vitro tissue engineered bone

3.1. Polymer foam fabrication

Polymer foams were fabricated by a solvent-casting, particulate-leaching technique using D-(+)-glucose (Sigma) as the leachable component. A similar technique has been reported by Ishaug et al. [4]. Briefly, a 5% solution of poly (DL-lactide-co-glycolide) (PLGA 85:15) (Sigma) in chloroform was prepared. A total of 3 ml of the PLGA solution were cast in a 43-mm diameter aluminum dish containing 3 g of glucose (particle size < 300 μm). After drying and leaching of the sacrificial phase in distilled water overnight, the samples were cut into 10 × 10 mm squares (thickness 0.8–1.0 mm). The samples were stored under vacuum for at least 48 h after leaching to minimize the chloroform content. For sterilization, samples were placed in 70% ethanol for 1 h [8]. The polymer foams were soaked in a purified collagen solution (Cohension, CA, USA) overnight prior to cell seeding to facilitate cell attachment.

3.2. Cell resource

Osteoblastic cells were obtained from the marrow of 3–7 days old neonatal Lewis rats. Femora were aseptically excised, cleaned of soft tissue and cut at both metaphyseal

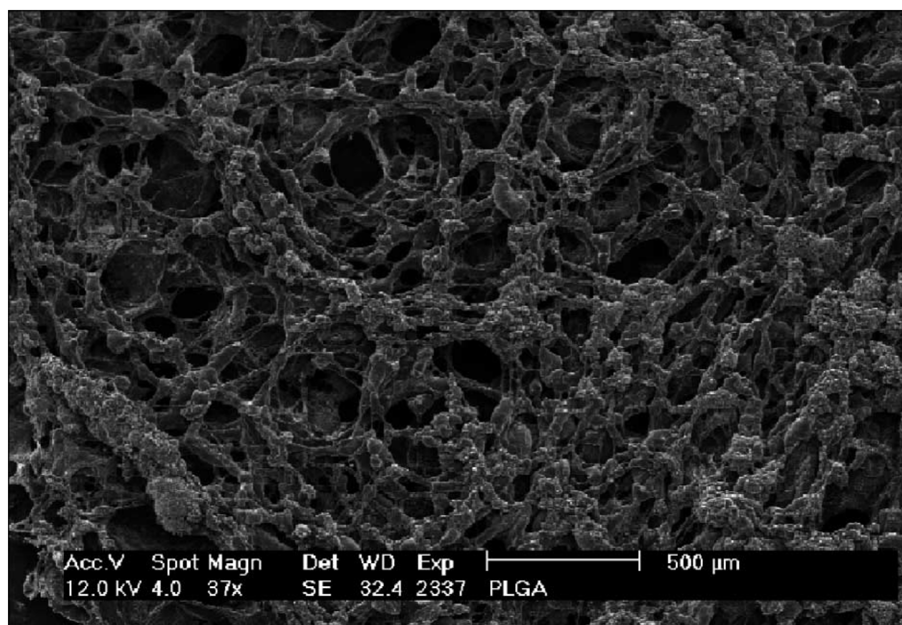

Fig. 4. Microstructure of PLGA foam is shown by SEM. PLGA has a three-dimensional interconnected network with pores of various sizes. Glucose (particle size < 300 μm) was used as the porogen.

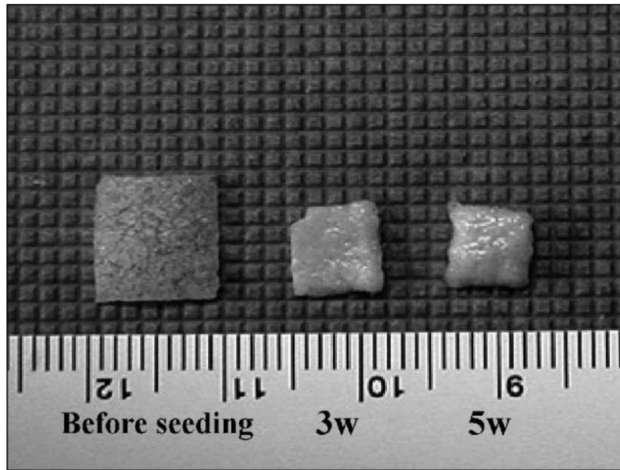

Fig. 5. A gross view of PLGA foam cultured in ROBS with MSCs derived osteoblasts. From the left: PLGA foam at day 0, after 3 weeks of culture, and after 5 weeks of culture.

ends. Bone marrow was flushed from the midshaft with primary cell culture media using a syringe equipped with a 22-gauge needle and filtered with a 70-μm nylon mesh. Filtered cell clumps were collected and broken up by repeated pipetting. Subsequently, the cells were centrifuged at $400 \times g$ for 10 min. The resulting cell pellets were resuspended in primary cell culture media and divided approximately 2×10^8 cells per T-75 flask. Unattached hematopoietic cells were removed after 24 h by changing the media. Cell culture media was replaced every 3 days. Osteogenic supplements (1 mM Na β-glycerol phosphate (Sigma), 0.2 mM L-ascorbic acid (Sigma) and 0.1 μM dexamethasone (Sigma)) were added to promote osteogenic differentiation when cells reached 70% confluence. These primary cultured cells were enzymatically detached with 0.05% trypsin and used for cell seeding.

PLGA sheets, together with 2×10^6 cells/cm^2 PLGA, were suspended in 35 ml primary cell culture media and cultured in the ROBS. The cell culture media was replaced after the first 24 h. The cell/polymer constructs were initially cultured in the primary cell culture media. After the first week, the primary media were replaced by media containing osteogenic supplements.

3.3. Histological examination

Cell/polymer constructs were prepared for histology after specific culture time periods. The samples were fixed and stored in 10% neutral buffered formalin, then embedded in

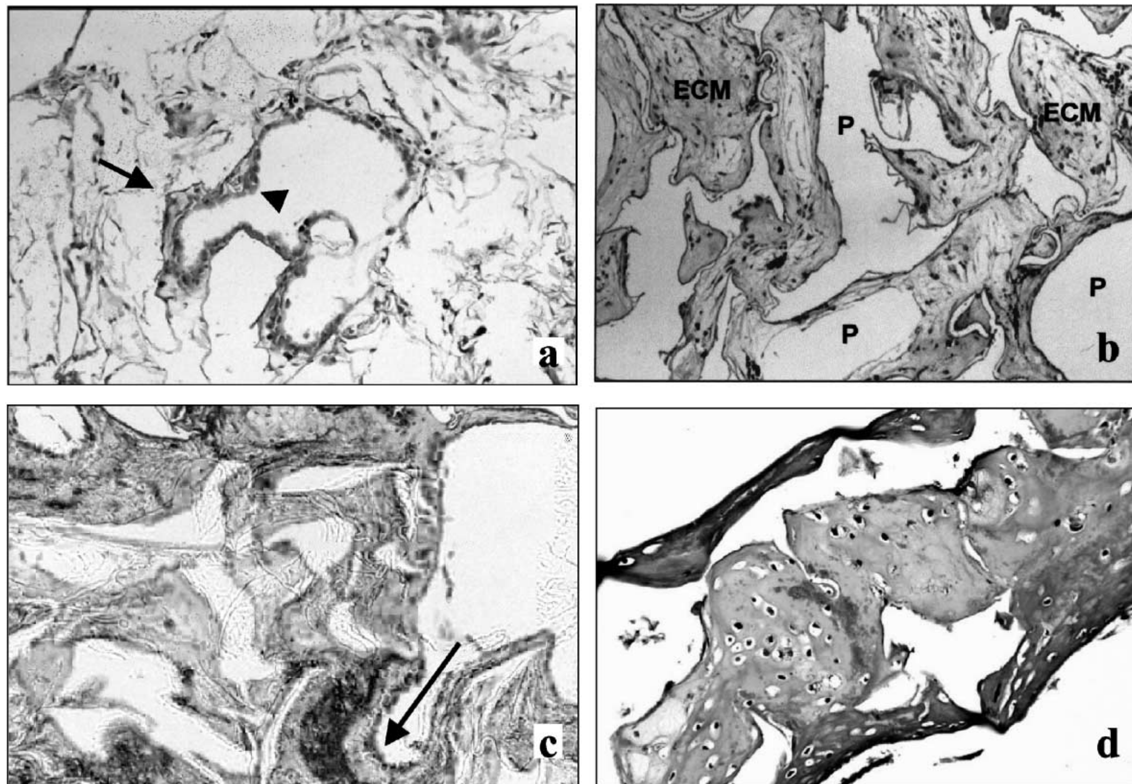

Fig. 6. (a) A confluent layer of osteoblasts lining a lumenal space (arrowhead) in a 1-week specimen (HE, 10 ×). Homogenous extracellular matrix is adjacent to osteoblasts (arrow). (b) Abundant extracellular matrix (ECM) located between the polymers (P) with scant foci of mineralization in a 2-week specimen. Viable cells are seen within the matrix. PLGA dissolved during the histological process. (HE, 10 ×). (c) Adjacent to the osteoblasts is an osteoid-like substance which is centrally mineralized in a 4-week specimen (arrow) (HE, 10 ×). (d) Scattered lacunae with osteocyte-like cells are seen within the calcified matrix in a 7-week specimen (HE, cross-section, 10 ×).

paraffin. Tissue blocks were sectioned at 4 μm and stained with hematoxylin and eosin for cell visualization. Decalcification for 1 h was applied to the specimens that had been cultured more than 2 weeks.

4. Results

4.1. Analysis of oxygen and carbon dioxide partial pressures and pH of medium

The results of pO_2, pCO_2 and pH measurements of the media in 100 mm diameter dishes (Control I), 50 ml centrifuge tubes (Control II) and ROBS at 1, 24 and 48 h. after seeding are shown in Fig. 3a, b and c, respectively. ROBS showed no difference in pH or pCO_2 compared to Control I at 24 and 48 h, with a stable shift (pH: 7.59–7.64, pCO_2: 29.7–33.2 mm Hg). The values of Control II at 24 and 48 h were significantly higher in pH (7.82–7.98) and lower in pCO_2 (20.2–16.0 mm Hg) compared to both ROBS and Control I. No difference was detected in pO_2 between ROBS and Control I at any measured time points. The pO_2 in ROBS and Control I decreased sequentially (ROBS: 167 → 160 mm Hg, Control I; 168 → 157 mm Hg), whereas Control II maintained a pO_2 level around 170 mm Hg.

4.2. Polymer foam fabrication

Polymer foams were fabricated using 85:15 PLGA by the solvent-casting particulate-leaching technique using D-(+)-glucose particles (particle size < 300 μm) as the leachable porogen. The resulting polymer foams had a thickness between 800 and 1000 μm. The microstructure of the PLGA foam was characterized using scanning electron microscopy (SEM). A typical micrograph is shown in Fig. 4, which reveals a three-dimensional interconnected network with pores of various sizes.

4.3. Gross and histological examination of tissue engineered constructs

PLGA foams without cells (before seeding), cultured for 3 and 5 weeks are shown in Fig. 5. We observed that the cell/polymer constructs shrunk sequentially (decrease in length of up to 40%), whereas their thickness and hardness increased with calcified matrices. Histological examination revealed extracellular matrix formation and active osteoblastic cell rimming in HE stained specimens at 1 week (Fig. 6a). The density of the extracellular matrix increased in the polymer foams and became partially calcified after 2 weeks (Fig. 6b). At 3 weeks, the gap was occupied with a dense extra cellular matrix and extensive calcification was observed (Fig. 6c). A single osteocytic cell embedded with dense calcified tissues can be seen in the cross-section of a 7-week specimen (Fig. 6d).

5. Discussion

Since the first description of in vitro bone formation using three-dimensional biodegradable scaffolds [4], several types of bioreactor systems have been developed to enhance bone formation. However, these studies did not show a sufficient level of bone formation compared to in vivo studies. Our ROBS was designed to facilitate cell activities in vitro by maintaining simultaneous oxygen supply and physiological load to cells during culturing. Based on the data for pO_2, pCO_2 and pH of media in ROBS, we have shown that the ability of gas exchange is greatly enhanced compared to closed tubes cultured under dynamic conditions, e.g., stirred flasks or Rotating Microgravity Bioreactors (RMB).

When effects of cell activities are avoided, the shift in pH of the media depends mainly on pCO_2 of the media because of the higher solubility of CO_2 in water compared to O_2 (solubility in water at 308.15 K, O_2: 4.80×10^{-4}, CO_2: 1.982×10^{-5}). In an incubator maintained at 37 °C with a fully humidified atmosphere of 5% CO_2 in air, the calculated theoretical values of pCO_2 and pO_2 are 35.6and 142 mm Hg, respectively (P_{air} = 760 mm Hg, $P_{H_2}O$ (37 °C) = 47.1 mm Hg, P_{CO_2}=[760–47.1] × 0.05, P_{O_2}=[760–47.1] × 0.95 × 0.21; based on the standard value of oxygen in the atmosphere of 21%). Ideally, pCO_2 of the media should be closer to 35.6 mm Hg if gas exchange is not restricted. Our data showed that the pCO_2 levels in ROBS and Control I at 48 h were close to the theoretical value (31.6 and 33.2 mm Hg, respectively), compared to Control II (16.0 mm Hg). These results support the fact that ROBS has the same gas permeability as Control I. The difference between ROBS and Control I in pCO_2 and pH at 1 h is explained by the accelerated diffusion of CO_2 from the liquid phase caused by rotational agitation of the media. In contrast, within the closed vessel, CO_2 diffuses from the media into the gas phase until it is saturated. This may cause a lower pCO_2 and higher pH of the media in Control II. The theoretical value of pO_2 in the same incubator is calculated to be approximately 142 mm Hg. Thus, pO_2 values of the media in a gas-permeable device in the incubator should also exhibit the shift to the theoretical value sequentially, mimicking the shift observed in pCO_2. In fact, the results in ROBS and Control I show similar shifts but a slower gradient to 142 mm Hg. This is attributed to the lower solubility of oxygen. The pO_2 results suggest that the cell culture medium stored in a 4 °C refrigerator maintained a pO_2 of approximately 170 mm Hg that decreased approximately 10 mm Hg after 48 h by diffusion without cells. In our experiment, the cell culture media used did not contain HEPES (a strong buffer) but contained Na+ and K+ (weak buffers) that may play a role in slowing down gas diffusion. The use of other medium containing various buffers could have a potential effect on the capacity of gas diffusion. We conclude that gas exchange ability of ROBS is at least as good as Control I.

In ROBS, the cell/polymer constructs were observed to be moving along the vessel wall and not merely floating in

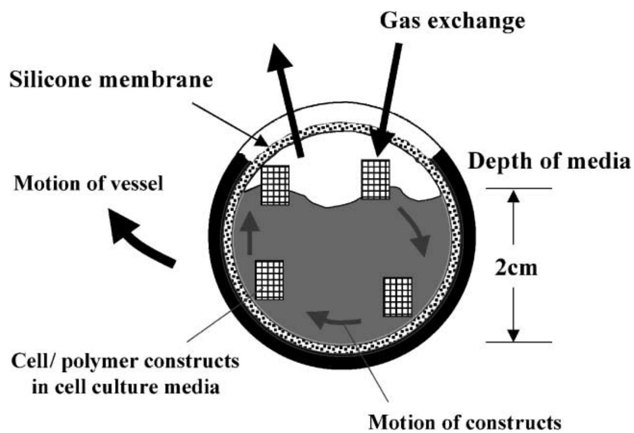

Fig. 7. Schematic view of fluid flow inside ROBS. Cell/polymer constructs are moving along the vessel wall at 6 RPM. This applies a continuous shift of hydrostatic pressure from 0 to 2 cm H_2O.

the media. This movement supposedly induces a continuous hydrostatic pressure shift from 0 to 2 cm H_2O corresponding to the level of medium in the vessel when placed in the rotating holder on the cell/polymer constructs (Fig. 7). It is likely that ROBS provides sufficient mechanical stress to accelerate cell differentiation [9], although quantitative data of hydrostatic pressure shifts have not been shown in this experiment. The key feature of ROBS, which distinguishes it from other currently used bioreactors, is its ability to produce a cyclic shear stress (6 RPM) due to the motion of the cell/polymer construct in the vessel. In contrast, RMB does not produce a shear stress but a microgravity environment by collecting cell/polymer constructs in the center of the rotating medium in the vessel, which is supposed to be beneficial to cell proliferation. A stirred flask imposes a shear stress on the constructs which is of constant magnitude rather than cyclic [10].

In this study, we have shown that bone formation in three-dimensional PLGA foams cultured in ROBS could be achieved. The seeding cell density used in this study (2×10^6/cm^2 of PLGA) was based on Ishaug et al. [4] and Holy et al. [11]. The seeded cells were observed in the center of the polymer with an equal distribution at 1 week, even though no special technique for cell seeding was used in this study. This result suggests the use of ROBS as a cell seeding and proliferation device. On gross examination, the cell/polymer constructs had shrunk the incubation period. This shrinkage was primary caused by the contraction of the extracellular matrix produced by osteoblasts. A higher polymer concentration in the foam might provide more resistance the shrinkage. Microscopic observation showed active osteoblasts (lining large cells with abundant basophilic cytoplasm) in a series of the cell/polymer constructs. Small granules of calcification were first seen within 2 weeks throughout the extracellular matrix. When taking into account the period for differentiation of osteoblasts from MSCs, these cells started calcification approximately 3 weeks after seeding, comparable to the time necessary for MSCs to induce calcification under two-dimensional culture conditions [12]. At 7 weeks, massive mineralization was observed throughout the constructs, with embedded single osteocytic cells. However, the degree of mineralization was intensified on the surface compared to the center portion of the specimen (Fig. 6d). This observation suggests that the diffusion of media into the center portion is obstructed after completion of mineralization on the surface. To overcome this problem, fabrication of capillary-like networks for oxygen and nutrient transport into the polymer may provide a route towards large three-dimensional tissues [13,14].

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