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Evaluation of different culture techniques of osteoblasts on 3D scaffolds

Research Article

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Abstract: Bones adjust their structure to withstand the mechanical demands they experience. It is suggested that flow-derived shear stress may be the most significant and primary mediator of mechanical stimulation. In this study, we designed and fabricated a fluid flow cell culture system that can load shear stress onto cells cultured on 3D scaffolds. We evaluated the effect of different culture techniques, namely, (1) continuous perfusion fluid flow, (2) intermittent perfusion fluid flow, and (3) static condition, on the proliferation of osteoblasts seeded on partially deproteinized bones. The flow rate was set at 1 ml/min for all the cells cultured using flow perfusion and the experiment was conducted for 12 days. Scanning electron microscopy analysis indicated an increase in cell proliferation for scaffolds subjected to fluid shear stress. In addition, the long axes of these cells lengthened along the flowing fluid direction. Continuous perfusion significantly enhanced cell proliferation compared to either intermittent perfusion or static condition. All the results demonstrated that fluid shear stress is able to enhance the proliferation of cells and change the form of cells.

Keywords: *Osteoblast • 3D scaffold • Fluid shear stress*

1. Introduction

Osseointegrated dental implants are undoubtedly one of the most significant scientific breakthroughs in dentistry over the past 40 years. Brånemark *et al*. recommended a stress-free unloaded healing period in order to minimize the risk of implant failure [1,2]. However, to achieve the targeted success rate, a strict protocol needs to be followed even if patients encounter pain with prolonged use of uncomfortable interim prostheses [2-4]. Recently, researchers have begun to focus attention on intermediate loading at the post-surgery

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stage, as well as the parameters for gauging successful loading. It is well known that bones adjust their structure to withstand the mechanical demands they experience. Physical loading and routine activities have been shown to inhibit bone resorption, which would otherwise occur upon disuse [5]. The bone around the implant consists of a structured extracellular matrix (ECM). The ECM is composed of inorganic and organic elements containing a conglomeration of cell types responsible for its metabolism and upkeep in response to a variety of signals [2]. We speculate that osteoblasts react upon the immediate loading placed on the dental implants after insertion. Bancroft *et al*. demonstrated that bone cells cultured *in vitro* respond to a variety of different mechanical signals such as fluid flow, hydrostatic pressure, and substrate deformation [6]. However, current studies indicate that fluid flow is a potentially stronger stimulus for bone cell behavior compared to either hydrostatic compression or substrate deformation [6,7]. Klei-Nulend *et al*. stimulated cells by fluid shear stress and found that osteocytes are activated by the fluid flow in the lacuna-tubule during osseointegration progression [8]. Knote further confirmed the existence of fluid flow in the bone mineralization matrix through molecule marking [9].

In order to stimulate bone cells *via* fluid shear stress, traditional methods utilizing spinner flasks and rotating wall bioreactors have been suggested. However, for the purpose of our study, these methods cannot fully satisfy our objectives. The use of spinner flasks subjects cells on the outer periphery of scaffolds to turbulent flow while the interior of the cells exhibit static molecular diffusion. Similar drawbacks exist in the use of a rotating wall bioreactor [6,10-13]. As a result, scholars have focused on the study of fluid flow systems and have suggested that flow-derived shear stress may be the most significant and primary mediator in the mechanical stimulation [14]. In this study, we designed and fabricated a fluid flow cell culture system that can load shear stress on cells that are cultured on 3D scaffolds. This method mimics the mechanical stress experienced in bone, as well as the processes by which the bone shapes itself in a 3D manner in response to such stress.

In previous studies, some authors have focused on the effect of long-time shear stress on cells [6,10-12, 15-17]. Meanwhile, others have investigated fluid shear stress loading on cells for short time periods, believing that over-loading would inhibit cell growth [18,19]. Incidentally, the effects of various fluid flow culturing methods on experimental results have not been clearly elucidated.

Thus, the objective of this study is to evaluate the effect of different culture techniques, namely, (1) continuous perfusion fluid flow, (2) intermittent perfusion fluid flow, and (3) static condition on the proliferation of osteoblasts cultured on partially deproteinized bones (PDPB) for a period of 12 days.

2. Experimental Procedures

2.1 Cell isolation and culture

Ten 2- to 3-day old Sprague-Dawley (SD) rats were sacrificed and their craniums were removed. Excised tissue was first rinsed with penicillin and streptomycin solution, and then rinsed three times with phosphate buffered saline (PBS) buffer solution before being cut into 1 mm^3 pieces. The pieces were placed in cultured flasks containing 5 ml F-12 media supplemented with 10% fetal bovine serum (FBS) and antibiotics (GIBCO, USA). The flasks were placed in a 37°C incubator and turned over after about 4 h. The animal experiment of this study was authorized by West China Hospital Ethics Committees.

2.2 Osteoblast identification *via* Alizarin red stain

Cell-climbing slices were placed into a six-well plate. For the nodule formation assay, 50 μg/mL ascorbic acid, and 10 mmol/L sodium β-glycerophosphate (Sigma, USA) was added into the F-12 culture media with 10% FBS and antibiotics. Until Day 18 of culturing, cells were washed twice with PBS and then fixed with 100% ethanol. Fixed osteoblasts were stained with 1% Alizarin red S, pH 6.3, for five minutes until the nodules were stained red. Fixed cells were then washed with distilled water and air-dried [20,21].

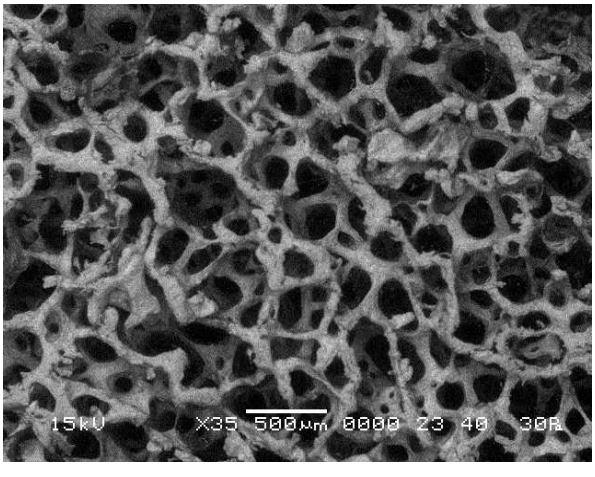

Figure 1. SEM images of the surface of the PDPB. Partial deproteinized bone with a volumetric porosity of 79±1.2% and the average pore size is 300-500 μm (×35).

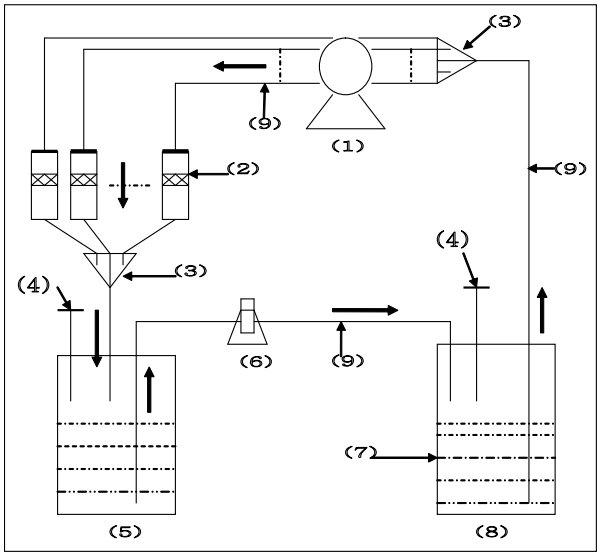

Figure 2. Schematic of the fluid flow cell culture system. (1) pump; (2) chamber; (3) transfer board; (4) gas filter; (5) waste vessel; (6) through connection; (7) complete culture media; (8) media reservoir; (9) platinum-cured silicon tubing.

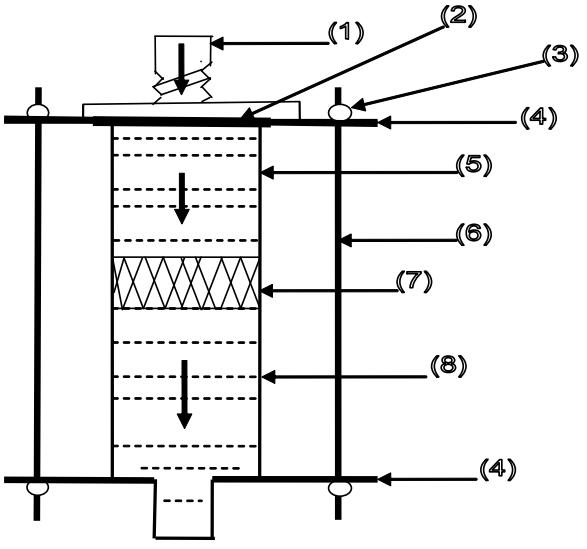

Figure 3. Schematic of the chamber. (1) inlet of media; (2) O-ring; (3) screw cap; (4) titanium board; (5) syringe; (6) titanium stand; (7) scaffold seeded with cells; (8) culture media.

2.3 Scaffolds

Scaffold material consisted of partially deproteinized bones (Yantai Zhenghai Biology Techonology Co. Ltd., China) with a volumetric porosity of 79±1.2%. The average pore size of the mesh was about 300-500 μm. The prepared scaffolds were round with a diameter of 10 mm and a thickness of 2 mm (Figure 1).

2.4 Fluid flow cell culture system

A fluid flow cell culture system was developed consisting of eight flow chambers, platinum-cured silicon tubing, reservoirs, gas filter, and a pump (Tianjin Xieda Weiye Electron Co. Ltd., China) (Figure 2).

The chambers were made of 2 ml glass syringes. The body of the chamber formed a hollow 10 mm in diameter and 60 mm in height. The top of the syringe was enclosed by a Polytetrafluoroethene O-ring to ensure that no media leakage would occur around the edge of the chamber (Figure 3 and 4). The scaffold was placed and fixed by a cut radial plunger. The vertical flow of media through the scaffold was generated by gravity. Fluid flow through each chamber was driven by a multichannel roller pump, and all flow chambers drew from a common reservoir containing 200 ml of media. After each cycle, the media would return to the waste vessel, which was connected to the media reservoir. The fluid head pressure would then bring back the media to the reservoir, and the flow cycle would be repeated. Components of the flow circuit were connected with platinum-cured silicon tubing.

Prior to use, all the components (tubing, reservoirs, connectors, and chambers) were sterilized *via* autoclaving, and the system was then assembled using a sterile technique in a laminar flow hood. The cell-seeded scaffolds were sealed in individual flow chambers, and the entire flow system was placed into a standard cell culture incubator (37°C and 5% CO_2).

Figure 4. Image of the chamber.

2.5 Cell seeding and culture

After cells had grown into their third passage, they were detached and seeded on the PDPB scaffolds. Scaffolds were placed in a 24-well plate using a cell suspension culture. After 4 h of attachment, complete media was added to each well to cover the scaffolds. The cell/scaffold constructs were then incubated overnight for further attachment. Scaffolds were then divided into three groups. Group A and group B were placed into the fluid flow cell culture system with an initial flow rate of 0.3 ml/min on the first day to ensure good cell attachment; the flow rate was then subsequently increased to 1 ml/min for a total of 12 days [13]. The constructs in group B were subjected to 1 h fluid flow per day. The velocity of the fluid was kept constant for constructs subjected to either continuous or intermittent fluid flow. For the control group, the seeded scaffolds were placed in a standard 24-well plate with 2 ml complete media, and then cultured under static conditions. Media was changed every other day, and samples were removed for analysis on Days 4, 8, and 12 for all three groups.

2.6 Scanning electron microscopy (SEM) analysis

On Day 4, 8 and 12, samples seeded with cell suspension at a density of 2×10^6 cells/piece were rinsed in calcium-free PBS and fixed in 2.5% glutaraldehyde at 4°C overnight. The scaffolds/cell constructs were then dehydrated in a gradient series of ethanol, dried with tetramethylsilane, sputter-coated with gold, and examined using a JEOL 6310 SEM at an acceleration voltage of 15 kV.

2.7 Cell proliferation assay

Cell proliferation was monitored by counting viable cells with a Cell Counting Kit-8 (CCK-8) (Dojindo, Japan). At the time points indicated previously, samples seeded with the cell suspension at a density of 1×10^4 cells/piece were removed and placed into 24-well plates, 50 µl of Cell Counting Assay Kit-8 solution was added to each well, the cells were incubated for another 2 h. Optical density (OD) was measured at a wavelength of 450 nm using a microplate reader (VARI OSKAN FLASH 3001, Thermo, USA).

3. Results

3.1 Inverted phase contrast microscope analysis

After three days of culture, several polygonal or shuttle-shaped cells with multiple umbos, as well as round cells, were observed attached to the wall (Figure 5a). The speed of cell growth increased during the passage culture.

3.2 Calcium tubercles assay

Using the Alizarin method, the dense, round, and light-tight intercellular red staining mass can be observed under an inverted phase contrast microscope. The large staining mass had a wide color range (Figure 5b). The number of calcium tubercles in culture increased with time.

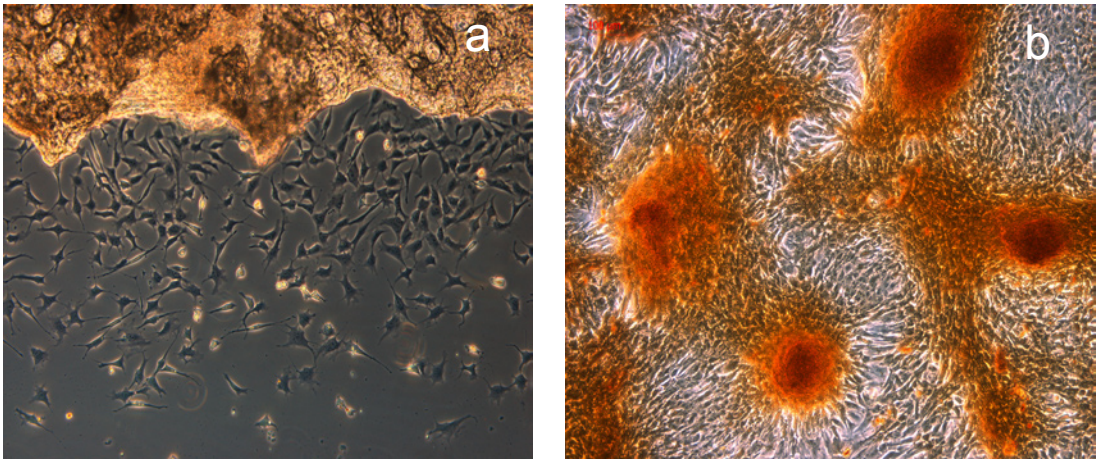

Figure 5. Image of primary culture of osteoblasts and positive staining of the calcified nodule. a: A few osteoblasts were found in the primary culture by inverted phase contrast microscope (×100); b: The dense and round intercellular red staining mass with wide coloring range (Alizarin method ×100).

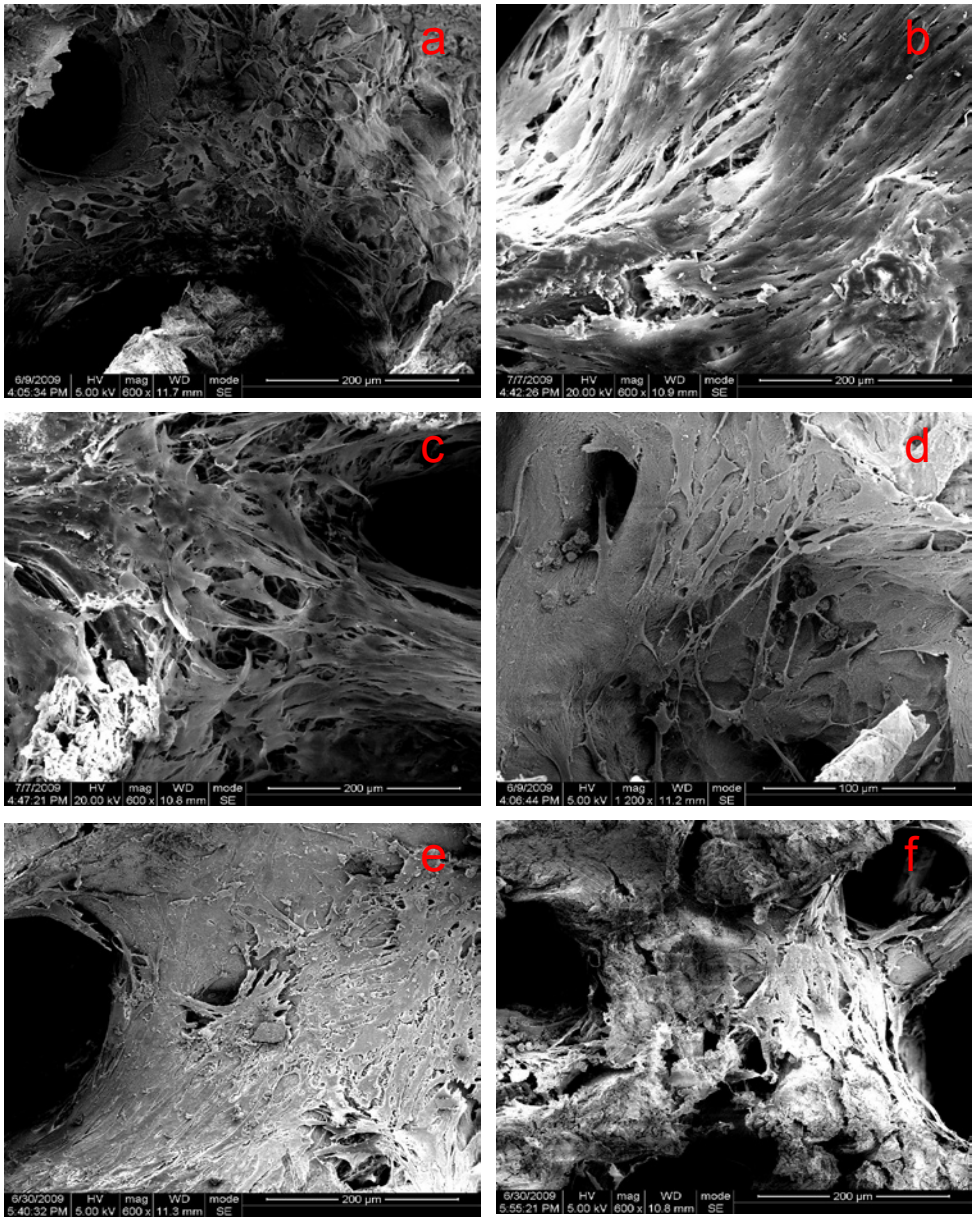

Figure 6. SEM images of the seeded scaffolds. The surface of the seeded scaffold cultured in static condition for 4 days (a, ×600); subjected to the continuous fluid flow for 4 days (b, ×600); subjected to intermittent fluid flow for 4 days (c, ×600). The interior of the seeded scaffold cultured in static condition for 4 days (d, ×600); subjected to the continuous fluid flow for 4 days (e, ×600); subjected to intermittent fluid flow for 4 days (f, ×600).

3.3 SEM analysis

On Day 4, examination of the control group construct revealed osteoblasts attached to the surface of the scaffold, with most cells 10-30 μm in size and polygonal- or shuttle-shaped. Among connected cells, some cell growth extended into the pores and only a small quantity of the matrix was secreted by osteoblasts (Figure 6a). In group A, osteoblasts covering most of the surface of the scaffold demonstrated confluence to the point of flaking; their long axis lengthened clearly along the flowing fluid direction. Several of the osteoblasts spanned pores and several small pores were completely covered. The extracellular matrix was larger among cells in the experimental groups than that of the cells in control group (Figure 6b). In group B, a scan of the surface of the constructs revealed that the osteoblasts proliferation and growth of the extracellular matrix was more significant than the control. However, less cells were observed compared to the continuous fluid flow-loading scaffolds. Part of the scaffold was covered

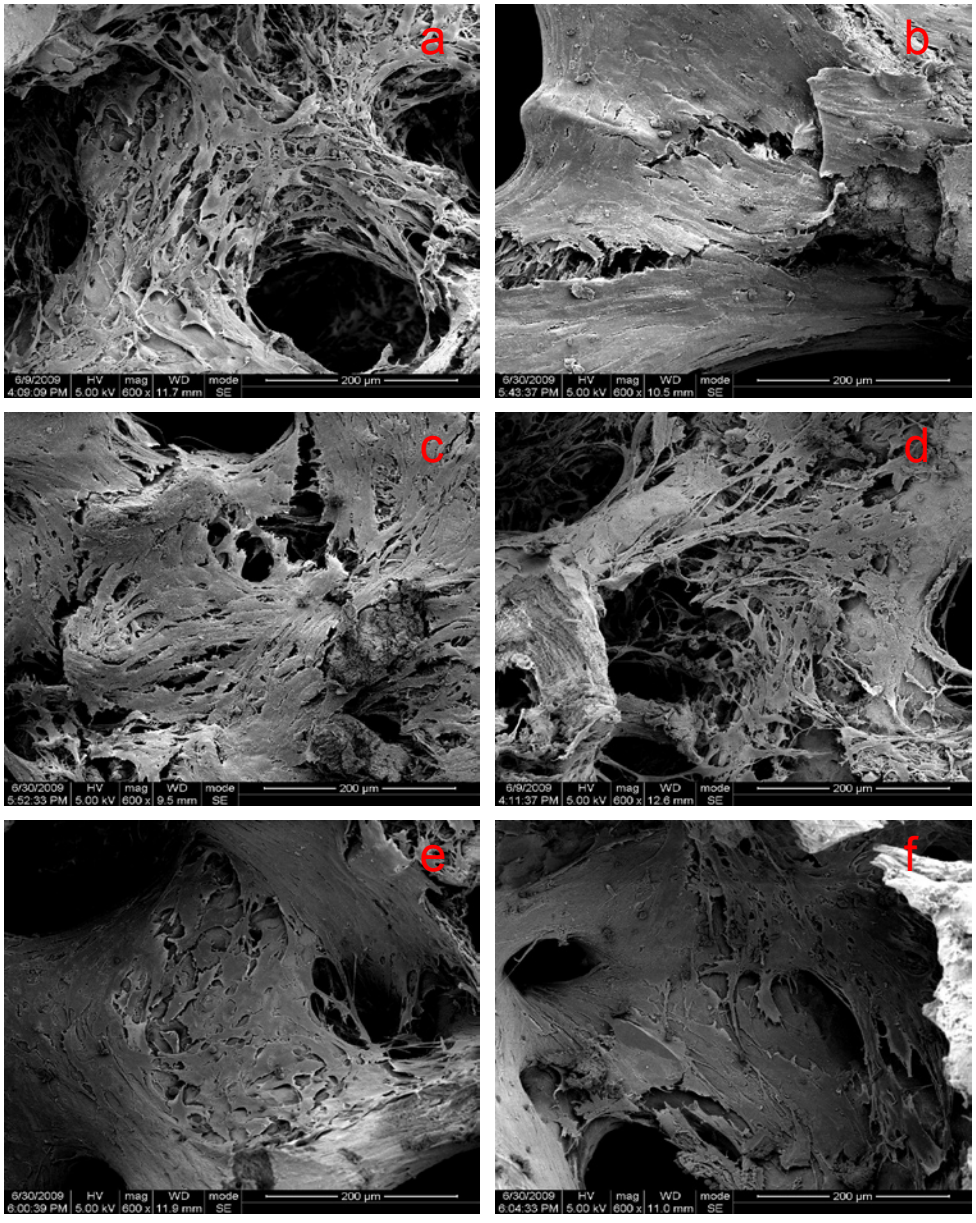

Figure 7. SEM images of the seeded scaffolds. The surface of the seeded scaffold cultured in static condition for 8 days (a, ×600); subjected to the continuous fluid flow for 8 days (b, ×600); subjected to intermittent fluid flow for 8 days (c, ×600). The interior of the seeded scaffold cultured in static condition for 8 days (d, ×600); subjected to the continuous fluid flow for 8 days (e, ×600); subjected to intermittent fluid flow for 8 days (f, ×600).

by flaky osteoblasts, meanwhile, the long axes of the osteoblasts also showed a lengthened trend along the flowing fluid direction (Figure 6c). Upon examination of the interior of the static-cultured scaffold, we found that only a few osteoblasts were observed (Figure 6d). In comparison, more cells grew into the pores in group A, and certain small pores were covered by osteoblasts (Figure 6e). For the group B, the cell number was slightly higher than the control group, although cell contact was increased (Figure 6f).

Increased culture time (Day 8) revealed that osteoblasts in polygonal or shuttle shapes proliferated greatly and ranked closer compared to Day 4 (Figure 7a and 7d). For all the constructs subjected to perfusion, the cells on the scaffolds indicated significantly enhanced proliferation capability of the osteoblasts. In group A, the cells on the surface of the scaffold had converged and overlapped in growth. The majority of the pores were covered and matrix secretion was greatly increased. A scan of the interior of the scaffold revealed cell growth in

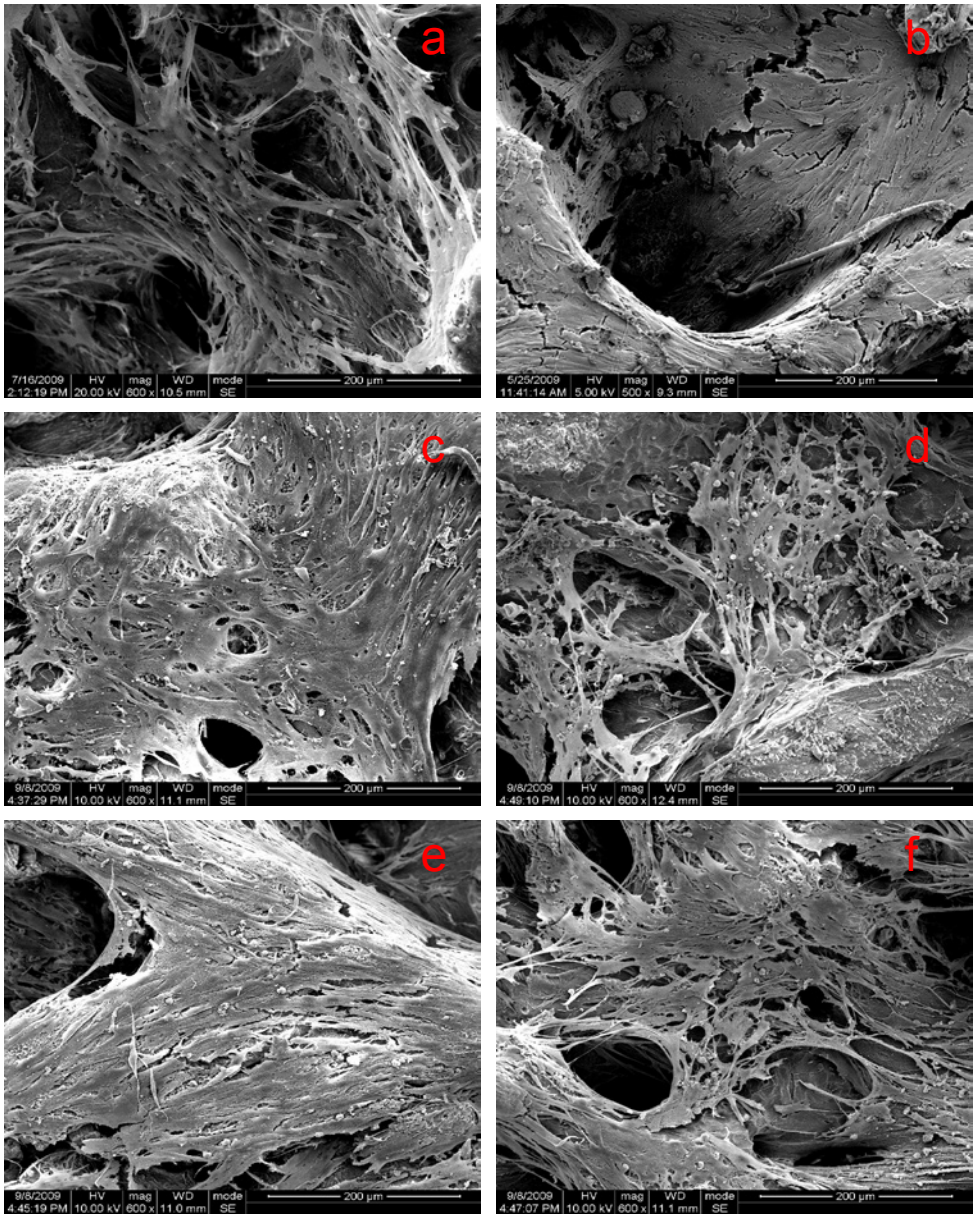

Figure 8. SEM images of the seeded scaffolds. The surface of the seeded scaffold cultured in static condition for 12 days (a, ×600); subjected to the continuous fluid flow for 12 days (b, ×600); subjected to intermittent fluid flow for 12 days (c, ×600). The interior of the seeded scaffold cultured in static condition for 12 days (d, ×600); subjected to the continuous fluid flow for 12 days (e, ×600); subjected to intermittent fluid flow for 12 days (f, ×600).

the pores. Cells had converted into brittleness and grew deeper into the pores and several cells reached the other side of the scaffold (Figure 7b and 7e). For group B, fewer cells were observed and cell contact was not as compact as seeded cells of group A. However, cells did confluence, growing into the pores in the direction of fluid flow (Figure 7c and 7f).

On Day 12, upon examination of all the scaffolds of the three groups, more cells and extracellular matrix can be found compared to Day 8, however, the proliferation of osteoblasts was not so notable in comparison to the early culture periods. In addition, the form of the cells changed similar to the cells that were cultured in the same condition in Day 8 (Figure 8a-f).

3.4 Cell proliferation assay

In order to assess the effect of fluid shear stress on cell proliferation in 3D scaffolds, a CCK-8 assay was performed. The amount of the formazan dye, generated by the activity of dehydrogenases in cells, is directly proportional to the number of living cells (Figure 9a).

Growth curves show a steady increase in the number of living cells on the scaffolds from Day 4 to Day 12 in all the three groups. Additionally, the cells in group A showed a significant increase in proliferation relative to that of group B and control group cells (Figure 9b).

4. Discussion

In this study, we designed a fluid flow cell culture system that can be easily and inexpensively fabricated. We checked the effect of the fluid flow on osteoblasts that were seeded on 3D scaffolds. The chambers of the cell culture system were made from syringes for the following reasons: (1) the plunger and the body of the syringe formed negative pressure, which would reduce non-perfusion bypassing fluid; (2) syringes would not influence the growth of cells; (3) we could observe the condition of the scaffolds because the syringes were transparent; and (4) various syringes could be chosen according to the size of the scaffolds, making the experiment more practical and convenient. The whole cell culture system was easy to assemble, and its volume was so small that the whole system could be placed into the incubator, which is beneficial for long-termed experiments. Moreover, each flow chamber had its own independent pumping circuit, which ensured a consistent and controllable flow rate. As a result, the number of chambers could be easily altered based on the needs of the study and thus, allowing flexibility in the design of the experiment.

In order to mimic bone mechanical stress and the process by which it shapes itself in response to fluid shear stress in a 3D manner, we loaded such stress on cells that were cultured on 3D scaffolds. The 3D culture could improve the supply of nutrients and mass transport of O_2 inside the scaffolds. Apart from these, the fluid flow could enhance the removal of cell metabolic products from the interior of the scaffolds, which is beneficial for interior cell growth [14]. In addition, we used PDPB as scaffolds. The material was selected because they originate from the cortical bone of cattle and, as such, its porosity size and porosity rate is similar to that of the human jaw. Moreover, the wide range of pore sizes and the rough surface provide for a good adhesion base for the cells [13].

Although the stimulation of osteoblastic cells by fluid shear stress for a short period or a long period have all been well documented, the different results induced from the various fluid flow's culturing methods have not been clearly elucidated. This study compared the proliferation and morphologic diversity of osteoblasts that were seeded on 3D PDPB scaffolds cultured in three different techniques: as subjected to continuous perfusion fluid flow, as subjected to intermittent perfusion fluid flow, and in static condition.

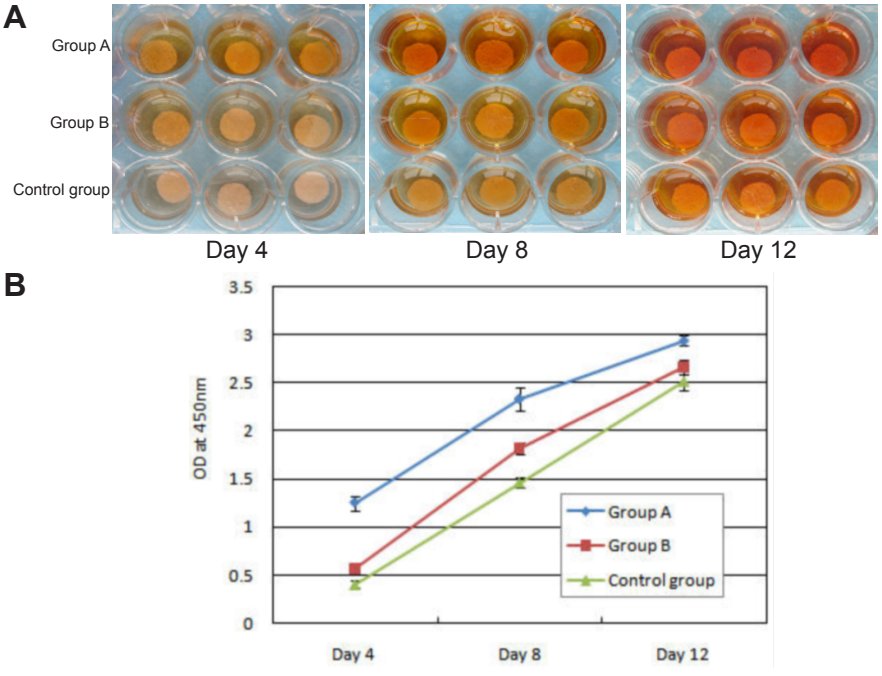

Figure 9. Effect of perfusion fluid flow on cell proliferation. a: The formazan dye is generated by the activities of dehydrogenases in cells, and the amount of the dye is directly proportional to the number of living cells. b: OD values obtained by using a microplate reader. Briefly, the significant enhancement of cell proliferation was observed for continuous perfusion fluid flow (group A) compared to either intermittent perfusion fluid flow (group B) or static condition (control group) at all the time points. Error bars represent means ±SD.

In our study, the shear forces experienced by the cell cultured on scaffolds under fluid flow was about 1 dyne/cm^2(1 N/m^2=10 dyne/cm^2=1 Pa), as calculated by a cylindrical-pore model in the approximation of the geometry of the scaffold porosity [22]. The stress gradients in bone resulted in pressure gradients within the tissue. Since bones are porous materials, these gradients could induce fluid movement from high to low pressure regions. The shears experienced by the resident bone cells as a consequence of this fluid transfer were initially argued as the most significant mechanical stress at the cellular level. In fact, modeling studies predict that the magnitude of the shear stress is in the range 0.8-3.0 Pa [23], albeit this is at the lower limit of what previous studies have indicated as sufficient to induce stimulatory effects in bone cells [6].

Our SEM analysis showed that the use of a dynamic cell culture approach leads to enhanced cell proliferation. In the early culture periods, the high number of cells at post-flow culture agrees with results from other research groups, which also demonstrated that the use of a dynamic cell culture approach leads to an enhanced cell proliferation. Also, the continuous flow of media that is applied by the culture system was found to be superior in the intermittent perfusion flow fluid culture condition in relation to the construct cellularity and distribution of cells. This phenomenon can also be seen from the results of the cell proliferation assay, which indicated the significant enhancement of cell proliferation for continuous perfusion compared to either intermittent perfusion or static condition. Hence, this could be considered a product of enhanced availability of nutrients, removal of inhibitory waste products, and the application of fluid flow exerted by the media to the cells [14,23]. Moreover, the long axes of the osteoblasts significantly lengthened along the fluid flow direction for all cells that were cultured in the perfusion fluid flow condition, which may imply the adaptive change of the cells for the purpose of promoting bone formation and reducing relative bone resorption. In addition, the stress and resistance on cells would decrease when the long axes of the cells are the same with the fluid flow direction; this may be somewhat related to the osteogenous direction [24].

Additionally, the results of the cell proliferation assay also showed that the rate of cell proliferation during later culture period was not so notable in comparison to early culture period. This was found to have the same trend seen by SEM analysis. However, this phenomenon can only be observed in long-term perfusion fluid flow since the actual experienced shear rates dynamically increase with culture time as the cells and matrix occupy an increasingly larger amount of the scaffold [6].

All of the results elucidate the impact of fluid shear stress over greater periods of time (*i.e.*, days as opposed to hours), which is significant even at lower rates of fluid flow that do not damage cells. At low flow rates over longer periods of time, fluid shear stress enhances the adhesion and proliferation of cells. Thus, this supplies a basis for future research in terms of identifying immediate loading on implants after insertion.

In conclusion, the study performed a newly developed fluid flow cell culture system that was designed to demonstrate how fluid shear stress could enhance the proliferation of osteoblasts cultured on scaffolds, increase the extracellular matrix secreted by cells, and change the form of osteoblasts. In addition, we discovered that the effect of the continuous perfusion fluid flow is more effective compared to the intermittent perfusion fluid flow. Our results thus supply a basis for future study on the availability of immediate loading of implant at post-surgery. However, the relationship between fluid flow forces and the actual forces that are loaded on the bone around the implant after restoration needs further investigation.

Acknowledgments

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