Hydrodynamic 3D Culture for Bone Tissue Engineering

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1. Introduction

Bone tissue engineering provided a promising approach for treatment of large bone defects resulting from maladies such as birth defects, trauma, or tumor resection. In vitro culture of a porous scaffold seeded with osteoprogenitor cells may enhance its bone regeneration potential. In this chapter, we describe the design of a novel perfusion bioreactor system with oscillatory flow for cultivating multiple 3D cellular constructs with clinical applicable size. It can perfuse culture media in a small volume repeatedly throughout the scaffolds. The mixing effect of the oscillatory flow may enhance the uniform growth of cells in the scaffold in prolonged culture in vitro. The system was characterized. Mouse osteoblast-like cells, MC 3T3-E1, were dynamically seeded and cultured in large porous ceramic β-tricalcium phosphate (β-TCP) scaffolds by the oscillatory perfusion system, and then the cell growth within the scaffolds was evaluated by fluorescent study. The oscillatory perfusion system could be a simple and effective bioreactor for bone tissue engineering.

2. Bone regeneration

Although bone tissue is able to regenerate after injuries and can remodel in relation to local stresses, treatment of large bone defects resulting from maladies such as birth defects, trauma, or tumor resection has produced an extremely large clinical demand for bone graft well distributed across the population. Autologous bone grafts have been considered the gold standard for augmenting bone regeneration. However, there are limited sites where bone may be harvested without loss of function (Brown and Cruess, 1982; Enneking et al 1980). Autografts are less effective in irregularly shaped defects and may be resorbed prior to complete healing. Furthermore, autografts harvested from the iliac crest of the hip are associated with a 10% complication rate including infection, fracture, pain, paresthesia, nerve injury, and donor-site morbidity (Gitelise and Saiz, 2002; Younger and Chapman, 1989). Allografts derived from cadavers are another commonly used bone graft material. However, disease transmission and immunologic rejection are serious concerns with unprocessed allografts, and processed allografts, such as demineralized bone matrix, lack bone growth inducing factors necessary for efficacy. Xenografts, or bone grafts obtained from different species, are also a poor option due to the danger of disease transmission or immunological rejection (Erbe et al, 2001).
2.1 In vitro bone tissue engineering

Tissue engineering may provide functional substitutes of native tissues, to serve as grafts for implantation (Langer and Vacanti, 1993) and physiologically relevant models for controlled studies of cell function and tissue development. In one of the most typical approaches, 3D structures are generated by the association of osteoprogenitor cells (autologous or allogeneic) with porous scaffolds. This may enhance the bone regeneration potential of the graft, because the seeded cells are not only a cell source for regeneration, but can also coat the scaffold surface with their osteoinductive extracellular matrix even if they did not survive after implantation of the biomaterial (Holtorf et al, 2005; Goldstein et al, 2001; Sikavitsas et al, 2003).

But in conventional static culture, nutrients and oxygen transported through a diffusion process could favor cells to live within 1 millimeter from the outer and upper surface of the scaffold, with only dead cells in the center of the scaffolds after an extended period of time (Figure 1A).

![Figure 1](calcein-am-pi-double-staining-of-mouse-osteoblast-like-cells-after-6-days-of-culture-on-the-porous-ceramic-scaffolds-under-fluorescent-stereomicroscope-middle-section-view-static-culture-living-shell-of-cells-with-a-dead-center-perfusion-culture-by-oscillatory-fluid-flow-living-cells-distribute-uniformly-throughout-the-scaffold-dead-cells-were-stained-red-and-living-cells-green-scale-bar-2mm)

Fig. 1. Calcein-AM/PI double staining of mouse osteoblast-like cells after 6 days of culture on the porous ceramic scaffolds under fluorescent stereomicroscope (middle section view). A) Static culture: living shell of cells with a dead center; B) Perfusion culture by oscillatory fluid flow: living cells distribute uniformly throughout the scaffold. Dead cells were stained red and living cells green. Scale bar=2mm

2.1.1 Development and evaluation of an oscillatory perfusion bioreactor for bone regeneration in vitro

Thus various bioreactors have been designed and developed for in vitro culture of 3D cell-scaffold constructs under conditions that support better nutrition of cells, possibly combined with the application of mechanical forces to direct cellular activity and phenotype. Spinner flasks and rotating wall vessel (RWV) bioreactors are two kinds of most basic bioreactors applied for bone tissue engineering, as shown in Figure 2 A-B. In the spinner flasks, scaffolds are attached to the needles hanging from the lid of the flask, and convective forces generated by a magnetic stirrer bar allow continuous mixing of the media surrounding the scaffolds\[10]. Medium stirring enhances external mass-transfer but also generates turbulent eddies, which could be detrimental for the development of the tissue. In the rotating wall vessel reactors, the vessel walls are rotated at a rate that enables the drag force (Fd), centrifugal force (Fc) and net gravitational force (G) on the construct to be balanced; the construct thus
remains in a state of free-fall through the culture medium (Sikavitsas et al., 2002; Botchwey et al., 2001; Unsworth et al., 1998) also presenting a low fluid shear stress. The microgravity state may present some advantages because it avoids cell deposition, and at the same time promotes cellular interactions (Botchwey et al., 2001). However, it is also known that microgravity is deleterious for bone, leading often to losses in total bone mass (Sinha et al., 2002). A common aspect of both systems was that the cells were not homogeneously distributed throughout the scaffolds structure in the two systems (Skavitsas et al., 2002) probably due to the inefficient internal mass-transfer while the external limitation reduced.

Direct perfusion bioreactors have been applied to fabricate uniform artificial bone grafts in vitro (Figure 2C).

To reduce mass transfer limitations both at the construct periphery and within its internal pores, the media is directly conveyed throughout the interconnected pores to continuously introduce nutrients and remove wastes. Direct perfusion bioreactors have been shown to enhance early cell proliferation, osteogenic differentiation, and mineralized matrix production of bone marrow stromal osteoblasts seeded in three-dimensional scaffolds (Goldstein et al., 2001; Cartmell et al., 2003; Wang et al., 2003; Meinel et al., 2004; Dolder et al., 2003; Bancroft et al., 2002; Sikavitsas et al., 2003; Porter et al., 2005; Holtorf et al., 2005; Botchwey et al., 2003). In addition to enhancing chemotransport by shear flow over bone cells attached to the scaffold surface, a perfusion system may also simulate the fluidic mechanical environment bone cells experience in vivo and elicit a flow-induced osteogenic response of the bone cells, as evidenced by increased activity of alkaline phosphatase (ALP), an early osteogenic differentiation marker (Pavalko et al., 1998; Kapir et al., 2003; Jessop et al., 2002; McAllister et al., 1999), and the expression of osteocalcin (Lan et al., 2003), osteopontin (Kreke et al., 2004; Kreke et al., 2005), and bone sialoprotein (Kreke et al., 2005). So perfusion bioreactors are very important tools for research on bone tissue engineering.
Most existing perfusion bioreactors have still some problems to be addressed: 1) the culture media circuit, the typical structure of them, includes multiple tubes and junctions, making them complicated, difficult to handle. The bubbles are also easy trapped and alter the flow field. 2) Cell seeding of scaffolds – that is, the dissemination of isolated cells within a scaffold – is the first step in establishing a 3D culture, and might play a crucial role in determining the progression of tissue formation (Vunjak-Novakovic et al., 1998). But the large volume of medium necessary for circulation does not allow for a small seeding volume required for efficient inoculation; therefore, to improve seeding efficiency, researchers prefer to seed cells into scaffolds outside the perfusion system and then transfer them to the system, significantly increasing the risk of contamination. 3) Growth factors are essential for tissue formation and play an important role in tissue engineering. Bone morphogenetic proteins (BMPs), transforming growth factor beta (TGFβ), fibroblast growth factors (FGFs), insulin growth factor I and II (IGF I/II), and platelet derived growth factor (PDGF) have been proposed for bone tissue engineering applications (Jadlowiec et al., 2003; Lind and Bunger, 2001; Yoon and Boden, 2002). Unfortunately, they are still very expensive now. So the large volume of medium required also made traditional perfusion cultures very costly when such kinds of expensive conditioning agents as well as valuable transfected cells or vectors are used. It also prevented the retention of newly synthesized extracellular matrix components or transfected gene products within the construct. In addition, it has been suggested that oscillatory fluid flow instead of unidirectional flow mimics the physiologic fluid flow profile observed in bone during mechanical loading and demonstrated to have beneficial effects on osteogenesis, such as increasing osteopontin and osteocalcin expression (Vance et al., 2002; Ponik et al., 2006; Wu et al., 2006; Li et al., 2004; You et al., 2000; You et al., 2001; Donahue et al., 2003; Batra et al., 2005; Qin et al., 2003).

We have designed and developed a compact oscillatory perfusion bioreactor recently. As shown in Figure 2D, the base of flow well was sealed with a 0.3-mm silicon film. The movement of the syringe back and forth was controlled by a continuous cycle syringe pump, consequently forcing the silicon film to move up and down, thus driving the media in the flow wells to perfuse up and down through the scaffolds in an oscillatory manner. Glass cloning rings were attached to the caps to enable the system to hold a limited volume (<500μm) of cell suspension during seeding for high seeding efficiency as well as enable conditioned culture in small volume. Because the syringe could be released from the pump easily, we were able to move only the syringe-connected chamber (without pump) onto a clean bench and change the medium just as one would do with a conventional 6-well plate. We have successfully fabricated a uniform tissue engineering bone with a clinical relevant size (10 mm in diameter, 8 mm in height) in only 1.5ml culture osteogenic media by this oscillatory perfusion bioreactor for prolonged period.

At first, the cellularity of the scaffold was evaluated by determining the double-standed DNA (dsDNA) content using the PicoGreen assay kit (Molecular Probes). The oscillatory perfusion group scaffold had significant higher number of cells than the static group 24 postseeding (p<0.05), as determined from measuring the DNA content (Fig. 3A). The scaffold DAN content after 5 days of differentiating culture was also higher in the oscillatory perfusion culture than in the static culture (p<0.1). During the 5 days of differentiation culture by oscillatory perfusion, the doubling time of the cells was significantly longer than that by the static culture method(Fig. 3B), indicating that the proliferation rate in the perfusion culture during this period (p<0.05).
Fig. 3. A: DNA content of MC 3T3-E1 cells per scaffold after 24 h of seeding and 5 days of differentiation (day6). B: Doubling time of static culture and oscillatory perfusion culture during 5 days of differentiation culture, calculated from dsDNA content. All the experiments were conducted independently (static group: n=4, oscillatory perfusion group: n=3). Error bars represent SD. The asterisk (*) indicates a statistically significant difference between the static group and the perfusion group (p<0.05). (Du et al., 2007)

Fig. 4. ALP activity of seeded MC3T3-E1 cells after 5 days of differentiation culture. A: Total ALP activity per scaffold. B: ALP activity per dsDNA content per scaffold, as an estimate of cellular ALP activity. All the experiments were conducted independently (static group: n=4, oscillatory perfusion group: n=3). Error bars represent SD. The asterisk (*) indicates a statistically significant difference between the static group and the perfusion group (p<0.05). (Du et al., 2007)

To evaluate the osteogenic function of the attached MC 3T3-E1 cells, activity of ALP, an early osteogenic differentiation marker, was assayed. The activity of ALP was measured by a clolorimetric endpoint assay. The same supernatant samples as those prepared for the DNA content assay were used. After 5 days of differentiating culture, the total ALP activity...
was significantly higher in the oscillatory perfusion culture than in the static culture (p<0.05) (Fig. 4A). As shown in Figure 4B, perfusion culture showed a higher average ALP activity per dsDNA content than that in the static culture (p<0.1), suggesting higher levels of ALP per cell in the oscillatory perfusion culture.

Stereomicroscopic observation of living cells darkly stained by MTT indicated that after 24 h of static seeding by top dropping, cells indeed penetrated into the center of the scaffolds (Fig. 5C) but remained at a much higher density on the top surface (Fig. 5A) and with cells seeded very sparsely in the lower regions (Fig. 5 D, E). After 5 days of differentiation culture, the density of cells increased significantly, and the cells appeared as a uniform layer on the top surface of the scaffolds (Fig. 5); far fewer living cells were found in the deeper pores (Fig. 5L-O). The cell density also increased along the side surfaces of the scaffolds with a top-to-bottom gradient of cells (data not shown), but MTT staining was pale in the center of the scaffold (Fig. 5M). On the contrary, the MTT staining of the oscillatory perfusion culture scaffolds showed a uniform seeding density (Fig. 5F-J) and subsequent homogeneous cell proliferation throughout the scaffold (Fig. 5P-T).

![Fig. 5. MTT staining observed by fluorescence stereoscopy. A-J, 24 h after seeding; KT, 5 days of differentiation culture. A-E, and K-O are images from a static culture sample at the indicated region; F-J, and P-T are images from an oscillatory perfusion culture sample at the indicated regions of the scaffold. The dark crystals layering the pores represent the locations of living cells. Scale bar = 250 µm. (Du et al., 2007)](image)
Fig. 6. A: Calcein-AM/PI double staining of the middle section of the scaffolds after 5 days of differentiation culture. a-e images from fluorescence stereomicroscopy of the indicated regions from a scaffold in static culture; f-j, images of the same regions from a scaffold in an oscillatory perfusion culture. Living cells are stained green and dead cells red. Scale bar = 100 mm. B: Number of living and dead cells per view at the same magnification of A. C: Cell viability in the indicated regions. The results are derived from B. Error bars represent SD, n=6. Asterisks (*) indicate a statistically significant difference between the static group and the oscillatory perfusion group (p<0.05). (Du et al., 2007)
To elucidate the fate of cells seeded in the center of the scaffold in the static culture and whether the oscillatory shear flow led to severe damage to cells, Calcein-AM,PI double staining to visualize living and dead cells, respectively, and quantitative study of cell distribution and viability were conducted. The double staining showed that after 6 days of static culture, green (calcein) and red (PI) fluorescence were both strong on the top surface (Fig. 6Aa). However, only a few PI-stained cells-apparently undergoing apoptosis or necrosis-were found in the center of the scaffold (Fig. 6Ac), and only a sparse presence of shrunken red-stained nuclei of dead cells was noted in the lower region (Fig. 6Ad). On the other hand, green-stained living cells were distributed evenly throughout the scaffold cultured in the oscillatory perfusion system with a similar ratio of sparse dead cells to more abundant living cells throughout the scaffold (Fig. 6Af-j). The cell distribution was quantified (Fig 6B). Living cells were distributed homogeneously throughout the section views in the oscillatory perfusion culture, whereas mainly dead cells were distributed heterogeneously in the static culture scaffolds. Analysis of the viability of the cells by scaffold section revealed homogeneous cell viability in the oscillatory higher than the viability of the static cultured cells (p<0.05, n=6; Fig. 6C).

In summary, the present study demonstrates that a relatively large engineering bone construct (10 mm diameter x 8 mm height) could be developed in vitro by culturing composites of osteoblast-like cells and porous ceramic blocks with an oscillatory perfusion system. The oscillatory flow condition not only allow a better seeding efficiency and homogeneity, but also facilitates uniform culture and early osteogenic differentiation, which suggests that the oscillatory perfusion culture system may be a valuable and convenient tool for bone tissue engineering. Another significant advantage of the perfusion system is that only a small volume of medium is needed to load oscillatory flow in this bioreactor, which makes it an especially efficient tool for conditioned culture with expensive growth factors or gene vectors with more economical feasibility. The produced cellular matrix might also be concentrated in such a small volume. In addition, similar studies by other kinds of cell types and scaffold materials would be helpful to investigate furtherly about the biological effects of oscillatory flow on 3D culture for tissue engineering.

### 2.1.2 Oscillatory perfusion culture of CaP-based tissue engineering bone with and without dexamethasone (Helvetica, 9 pt, bold)

It is important to develop not only a feasible 3D culture system to maintain the tissue, but also an effective osteogenic system enhance and maintain the osteogenic potential of the graft. Dexamethasone is a key component in the classic osteogenic supplements (ascorbic acid, sodium b-glycerophosphate, and dexamethasone). It exerts a powerful effect on the osteogenic differentiation. Continual exposure to dexamethasone, beginning shortly after cell harvest, is required to drive and maintain the osteoblastic phenotype of marrow-derived progenitor cells. Although maintenance of the osteoblastic phenotype is desirable for long-term cultures, dexamethasone has been suggested to drive osteoprogenitors down the osteogenic pathway so quickly that they do not proliferate well, and even to induce apoptosis. Because previous studies have suggested synergistic osteogenic effects when both dexamethasone and unidirectional flow perfusion culture were used, (Holtorf et al., 2005) we supposed that beneficial synergistic effects could also be induced by dexamethasone and oscillatory fluid flow. Because the flow rates would have different biological effects on bone cells not only in two-dimensional (2D) culture on parallel plates (Bacabac et al., 2005; Bacabac et al., 2004; Bakker et al., 2001; Kreke et al., 2004; Thi et al., 2003), but also in 3D
bone construction by a perfusion system (Cartmell et al., 2003), it would also be important to study the influence of different flow rates of the oscillatory flow on the administration of dexamethasone.

Moreover, CaP ceramic scaffolds were most widely used in clinical practice, but CaP materials had potential absorbing ability which might influence the performance of dexamethasone for osteogenesis. Therefore, it would be important for bone tissue engineering to confirm whether it would work the same way as 2D culture or not when CaP-based scaffolds were used for 3D culture. In addition, the 3D hydrodynamic environments in bioreactor would also make dexamethasone work differently from that of 2D static culture condition.

The mouse osteoblast-like cell line MC 3T3-E1, which has the potential to be further differentiated, was used in this work. The cells were seeded onto porous ceramic scaffolds using the oscillatory perfusion method to achieve a similar cellularity before culture, and eliminate any confounding effects arising from the use of different cell-seeding techniques. The seeded constructs were then cultured by either the static method or the oscillatory perfusion method continuously for 6 days either with or without dexamethasone. The flow rate of oscillatory perfusion was set at 0.05, 0.5, 1 mL/min per scaffold. The cell proliferation, early osteogenic effects and viability were evaluated.

From an MC3T3 sample of a known cell number, which was mixed with an equal volume of knifeminced scaffold, the DNA content of MC3T3 per cell was determined to be 6.02 pg/cell, so the cell number could be evaluated by DNA content measurement. After seeding all cells by the oscillatory method, there were no significant differences among the groups, as shown in Fig. 7.

Fig. 7. The cell number per scaffold after 6 days of culture. P0.05,P0.50, 1.00 referred to the perfusion groups with the flow rate of, respectively, 0.05m 0.50, and 1.00 mL/min. Error bars represent the means = SD. N=6. (Du et al., 2008)

ALP activity is an important early osteogenic marker. As shown in Fig. 8, there were no significant differences in ALP activity between the cultures with and those without dexamethasone in either the perfusion or static culture groups. Total ALP activity per
scaffold was higher in all the perfusion culture groups than the static culture groups (p<0.05), except for the group perfused at 0.05 mL/min without dexamethasone. The total ALP activity was highest in the 0.5 mL/min perfusion groups, which were significantly higher than static culture and all perfusion groups at all other flow rates (p<0.05). The average ALP activity per cell in the 0.5 min/mL perfusion groups was significantly higher than that in the static culture group (p<0.05). The 1.0 mL/min perfusion group without dexamethasone had the lowest average ALP activity, and the activity in this group was significantly lower than that by other flow rates in the same medium.

Fig. 8. ALP activity analysis. (a) The total ALP activity per scaffold after 6 days of culture and (b) the average ALP activity per scaffold after 6 days of culture. P0.05,P0.50,P1.00, referred to the perfusion groups with the flow rate of, respectively, 0.05, 0.50, and 1.00 mL/min. Error bars represent the means = SE. N=6. The asterisk (*) undicated a statistically significant difference between the static group and the perfusion group (p<0.05); (#) indicates a statistically significant difference from groups of other flow rates in the same medium (p<0.05). (Du et al., 2008)

The status of the cells in the center of the scaffolds under different culture conditions was observed under SEM, as shown in Fig. 9. No living cells were found in the center of the
scaffolds subjected to static culture; higher magnification revealed dead cells that were shrunken and slack, with a preserved porous membrane and no surrounding extracellular matrix (Fig. 9a). However, numerous living cells were found in the center of the scaffold formed by perfusion culture; these cells were smooth, stretched out, and surrounded with a cellular matrix (Fig. 9b).

![Fig. 9. The cells in the center of scaffolds after 3 days of culture observed under a scanning electron microscope. (a) Static culture: no stretched cells were found except the porous and shrunken dead cells marked with an arrow and (b) perfusion culture (0.5 mL/min): living cells covered the surface of the pores in a stretched and intact shape. Scale bar =25 μm. (Du et al., 2008)](image)

A gross view of the cell viability distribution throughout the various sections is shown in Fig. 10a. We can see that there is only a thin shell of living cells covering the surface of the scaffolds cultured by the static method, with few living cells inside; in the 1 mL/min perfusion group; however, the living cells were distributed uniformly throughout the scaffold. The uniformity of distribution in the perfusion groups was dependent on the flow rate with the necrotic area decreasing as the flow rate increased. There was no clear increase in necrotic area by the administration of dexamethasone. As shown in Fig. 10b, 10x objective of the cells in the center of the scaffolds revealed that there were almost no green-stained living cells in the scaffolds subjected to static culture, but the cellular viability in the center of the scaffolds in the perfusion culture increased as the perfusion rates increase, especially in the groups perfused at 0.5 and 1.0 mL/min, in which the green-stained living cells covered the pore surfaces uniformly with a stretched shape. The groups treated with dexamethasone did not show any clear difference compared to those without dexamethasone.

The cell viability was further studied quantitatively under 10x objective. The uniformity of the viability as the total viability decreased as the flow rate was decreased to 0.05 mL/min (Fig. 11). The perfusion culture had higher viability than static culture from the section view.
In conclusion, the biological effects of dexamethasone on oscillatory perfusion culture of tissue engineering bone on a CaP scaffold was investigated. The results showed that the oscillatory flow could enhance early osteogenesis of osteoblast-like cells in 3D culture on ceramic scaffolds, with a peak function at the flow rate of 0.5 mL/min. The cell viability was significantly higher and more uniform in the perfusion groups than in the static culture groups. The uniformity decreased as the perfusion rates decreased. However, dexamethasone was suggested to have no effect when CaP ceramics were used as scaffold, and thus other osteogenic growth factors should be used under this condition. Because most osteogenic growth factors are very expensive, the oscillatory perfusion culture using small medium-to-volume ratio perfusion culture using CaP scaffolds. Such a dynamic conditioned culture could also produce bioactive engineered bone tissue that would function as a DDS for bone regeneration.

Fig. 10. Calcein-AM/PI double staining of the midline section of the scaffolds culture with MC 3T3-E1 cells for 6 days by the static method or the perfusion method observed under a fluorescent stereomicroscope. The living cells were stained green and the dead cells red. (a) The gross views of the whole sections, scale bar=2 mm and (b) the center of scaffolds, scale bar = 100 μm. (Du et al., 2008)
Fig. 11. Quantitative study of the cell viability distribution according to Calcein-AM/PI double staining of the middle section after 6 days of culture observed under a fluorescent stereomicroscope under 10x objective; (b) the viability of the corresponding regions in (a); and (c) the total cell viability of each culture method. Error bars represent the means = SE, n=6. The asterisk (*) indicates a statistically significant difference between the static group and the perfusion group (p<0.01). (Du et al., 2008)

2.1.3 3D culture by unidirectional or oscillatory flow for bone tissue engineering

As we mentioned in 2.1.2, the compact perfusion system with oscillatory flow appeared to enhance early osteogenesis and the uniformity of cultured bone constructs. Here, we compared the biological effects of a perfusion system with unidirectional flow on the 3D
construction of cell-seeded bone grafts against those of a perfusion system with oscillatory flow. Mouse osteoblast-like cells, MC 3T3-E1 were cultured in porous ceramic scaffolds by either static, unifirectional perfusion, or oscillatory perfusion culture for 6 days. Cell proliferation, early osteogenic effects, and viability were then evaluated.

A unifirectional perfusion system was designed as shown in Figure 12A. A media tank was set on each end of the perfusion camber as both media reservoirs and gas bubble trappers.

![Diagram of perfusion system](https://www.intechopen.com)

Fig. 12. Illustrations of the 3D perfusion culture systems for tissue engineering bone by either unifirectional flow or oscillatory flow. A: The unidirectional perfusion system; (b) the oscillatory perfusion system. (Du et al., 2009)

A gas-permeable bag was set in the circuit loop as a gas exchanger. The unifirectional media flow was driven by a syringe pump. A couple of silicon tubes led from Tank 2 to the clean bench, so that the medium could be exchanged in the sterile environment without moving the whole complicated system. The perfusion system with the scaffolds set inside was sterilized by ethylene oxide gas.

As shown in Figure 13, the calcein-AM/PI double staining of the midline section of the samples after 6 days of culture demonstrated that the living cells grew only on the surfaces of the scaffolds cultured statically (Fig. 13A; the cells grew extremely inhomogeneously in unidirectional perfusion culture, where there was a clear inverted arch-shaped interface between living cells and the non-living area (Fig. 13B).
Fig. 13. Calcein-AM (green; living cells)/propidium iodide (red; dead cells) double fluorescence staining of the scaffolds cultured with MC3T3-E1 cells for 6 days by static and perfusional methods with either unidirectional flow (1mL/min) or oscillatory flow (0.5 mL/min): (A) static culture; (B) unidirectional perfusion culture at 1 mL/min; (C) oscillatory perfusion culture at 0.5 mL/min; (D) oscillatory perfusion culture at 1 mL/min. (Du et al., 2009)

Fig. 14. Calcein-AM (green; living cells)/propidium iodide (red; dead cells) double fluorescence staining of the scaffolds cultured with MC3T3-E1 cells for 6 days by static and perfusional methods with either unidirectional flow (1mL/min) or oscillatory flow (0.5 mL/min). Images of scaffolds observed at 10x objective of a fluorescence stereomicroscope are shown according to the regions indicated by the broad views on the left. (Du et al., 2009)
In contrast, the scaffolds cultured by the oscillatory flow had a relatively uniform distribution of living cells throughout the scaffolds. The inhomogeneous living cells distribution of the unidirectional perfusion culture was further verified by higher magnification, as shown in Figure 14. In the unidirectional perfusion culture, the top surfaces of the scaffolds were covered by a thick layer of cells, and above the arch-shaped interface, there were dense living cells over the pore surface. However, very few cells were living below the interface or at the bottom of the scaffolds.

As shown in Figure 15, the quantitative study of cell viability in the section further confirmed the findings presented above. In static culture, there were few living cells in the section view, and these were accumulated only near the top surface. In the unidirectional perfusion culture, the living cells and dead cells were distributed extremely inhomogeneously, with living cells favoring the upper positions (Fig. 15A).

![Graph A](image)

**Fig. 15.** Quantitative study of cell viability distribution according to the calcein-AM/PI double staining of the middle section after 6 days of culture observed under fluorescent stereomicroscope under 10x objective. The numbers 0.5 and 1.0 refer to the flow rates of these groups in mL/min. A: Living and dead cell numbers per view of respective regions were counted at the same magnification as in Figure 3. U: upper; M: middle; L: lower, (B) viability of respective region derived from (A); Error bars represent means ± SD. N=6. (Du et al., 2009)

However, although there were fewer cells in the lower part of the scaffold in the unidirectional perfusion culture, their viability was not low (Fig. 16B); in the oscillatory perfusion culture, the cells proliferated uniformly throughout the scaffolds.
Fig. 16. The cell number per scaffold after 6 days of culture. The numbers 0.5 and 1.0 refer to the flow rates of the groups in mL/min. Error bars represent means = SD, n=6. The asterisk (*) indicates a statistically significant difference between the static group and all other groups. (Du et al., 2009)

Fig. 17. The average ALP activity per cell after 6 days of culture. The numbers 0.5 and 1.0 refer to the flow rates of the groups in mL/min. Error bars represent means = SD, n=6. The asterisk (*) indicates a statistically significant difference between the static group and the perfusion group (P<0.05). # indicates a statistically significant difference between unidirectional perfusion group and the oscillatory perfusion group (P<0.05). (Du et al., 2009)
The total cellularity of the scaffolds after 6 days of culture was evaluated by DNA content analysis, as shown in Figure 17. The total cell number in unidirectional flow perfusion was significantly higher than in any of the other groups (p<0.05). The average ALP activity did not differ significantly between the unidirectional perfusion culture and the static culture (P>0.05), although that of the oscillatory perfusion at 1 mL/min was also not significantly higher than that of the static group (Fig. 17).

Osteoblast-like cells were cultured with porous ceramic scaffold three-dimensionally in vitro for 6 days under static and hydrodynamic conditions with either unidirectional or oscillatory flow. Although the unidirectional flow increased cell proliferation, the proliferation was extremely inhomogeneous, which rendered the engineered bone unsuitable for transplantation. On the other hand, the oscillatory flow enabled uniform proliferation of osteogenic cells and increased early osteogenesis. This suggested that the oscillatory fluid flow might be better than unidirectional flow for 3D culture of engineered bone in vitro. The oscillatory perfusion system could be a compact, safe, and efficient bioreactor for bone tissue engineering.

3. Conclusion

As bone is a loading-dependent remodeling tissue, design of bioreactors for bone tissue engineering could not only culture a uniform “living” tissue, with suitable type and appropriate amount of physical stimuli, we may also “exercise” the bone graft in vitro and achieve a functional active engineering bone. As well as an efficient culture device for chemotransport and physical environment simulation, the design of future bioreactors would also be preferred to be safe, economy acceptable, customized-scaffold and nondestructive evaluation supported culture system.

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