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Osteoblast Response to Rest Periods During Bioreactor Culture of Collagen–Glycosaminoglycan Scaffolds

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Flow perfusion bioreactors have been shown to enhance fluid transport and improve cell viability throughout tissue-engineered bone constructs. Furthermore, osteoblasts have been shown to be stimulated by flow during bioreactor culture, although the optimum flow regime to promote an osteogenic response has yet to be found. One problem is that bone cells lose their ability to respond to stimulation; however, mechanosensitivity can be restored by introducing resting periods between bouts of loading. The aim of this study was to analyze the effect of rest-insertion on the response of osteoblasts seeded on collagen–glycosaminoglycan scaffolds in a flow perfusion bioreactor over culture periods up to 14 days. Short-term rests of 5, 10, or 15 s and long-term rests of 7 h were incorporated into stimulation patterns. Cell distribution was enhanced in all flow groups, whereas static culture controls exhibited encapsulation. Cyclooxygenase-2 expression and prostaglandin E_2 levels increased significantly because of bioreactor culture over static controls. Osteopontin expression was significantly higher for the rest-inserted groups than the static control group or steady-flow group. These results indicate that the insertion of resting periods during flow enhances cellular distribution and osteogenic responses on collagen–glycosaminoglycan constructs cultured in a flow perfusion bioreactor.

Introduction

In tissue engineering applications, bioreactors are used to influence biological processes by the application of a mechanical stimulus.[1] Their purpose is to aid in the *in vitro* development of new tissue by providing stimuli to cells and encouraging them to produce extracellular matrix prior to *in vivo* implantation. Bioreactor culture also overcomes problems associated with static culture conditions, such as poor nutrient and waste exchange, limited cell viability, and extracellular matrix formation in the center of the construct leading to core degradation.[2–4] The aforementioned problems are caused as the cells on the periphery of the construct grow and secrete extracellular matrix, and consequently, diffusion to the center becomes increasingly difficult as movement of fluid into the core is impeded (leading to an encapsulation effect). Cell necrosis occurs in the center and is a major obstacle in the formation of viable tissue *in vitro*. Bioreactors can induce fluid flow throughout scaffolds and thus enable nutrients to be delivered to the cells at the center of the scaffold and waste to be removed from that area. This can increase cell viability throughout scaffolds and thus deliver a more homogeneous construct.[2,3,5–7]

Numerous types of bioreactor have been used in tissue engineering, however, for flow-dependent cellular stimulation such as with osteoblasts, the most commonly used is the flow perfusion bioreactor. Flow perfusion bioreactors generally consist of a pump and a scaffold chamber joined by tubing. The scaffold is held in position across the flow path of the device and the medium is perfused through the scaffold, thus enhancing fluid transport. In our laboratory, such a device has been designed and validated.[8] It has been shown that osteoblasts respond to mechanical signals which play a key role in the formation of bone[9–11]; mechanical stimulation leads to an increase in proliferation and matrix synthesis.[12,13] In particular, *in vitro* experiments have shown fluid flow to have a number of effects on bone cells. Osteoblasts respond to flow with increases in early, mid, and late stage bone formation marker levels. After short-term culture periods, increases in intracellular calcium oscillations have been noted,[14] whereas longer culture periods can increase alkaline phosphatase (ALP) levels,[2] and ultimately, mineralization can be enhanced by bioreactor culture.[7]

A problem with many attempts to stimulate bone cells is the phenomenon that they become accustomed to their mechanical environment and stop responding to it.[15,16]

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However, the mechanosensitivity of bone cells can be restored by inserting recovery periods into the stimulation pattern. This enables their sensitivity to be restored, allowing them to be further stimulated by the same magnitude of stimulus, once the rest period has elapsed. *In vivo*, full mechanosensitivity has been shown to be restored with 4–8-h long-term rest periods between bouts of stimulation and higher bone formation rate results.[17] *In vitro*, in a previous study in our laboratory using the flow perfusion bioreactor, which will be used in this study, instead of a rest period, the use of low-magnitude steady flow for 7 h between bouts of high flow loading for 1 h was shown to allow cells to regain their mechanosensitivity while still providing nutrients to them.[18] In this study, osteoblasts showed increased expression of the early-stage bone formation marker cyclooxygenase-2 (COX-2) due to the use of the long-term rest period in conjunction with the 1-h stimulation periods.[18] The use of short-term rest periods (7–14 s) between cycles of stimulation has also proved to have an osteogenic effect *in vivo*.[17] In the case of short-term recovery periods, rests of 5, 10, and 15 s have been used between bouts of 10 s of oscillatory flow in two-dimensional (2D) *in vitro* study. Significant increases in Ca^{2+} levels resulted from the use of 10- and 15-s rest periods, and osteopontin (OPN) levels increased with rest-inserted flow when compared with continuous flow.[19] These data suggest that the combination of the two recovery periods (short- and long-term) might provide the best regime for bone cell stimulation *in vitro*.

To date, work on rest-inserted loading has been done primarily *in vivo* or in 2D *in vitro*. In one 3D study, Vance *et al.*[20] found that prostaglandin E_2 (PGE_2) release increased with the use of a short bout of oscillatory flow in combination with steady flow. *In vivo*, rest-inserted loading remains to be optimized,[21] and *in vitro*, further experiments are needed to examine the role of rest period duration.[19] The goal of this study was, using the flow perfusion bioreactor developed previously in our laboratory,[8,18] to investigate the effects of introducing rest periods during flow on osteogenic stimulation of cells seeded on collagen–glycosaminoglycan (CG) scaffolds for periods of up to 14 days to establish the optimal culture conditions for bone tissue engineering.

Materials and Methods

Scaffold fabrication and crosslinking

CG slurry was fabricated by blending fibrillar collagen (Integra Life Sciences, Plainsboro, NJ) with 0.05 M acetic acid and adding chondroitin-6-sulphate sodium salt (Sigma–Aldrich, Dublin, Ireland). The slurry was lyophilized using a final freezing temperature of −40°C.[22] Scaffolds were crosslinked using dehydrothermal crosslinking for 24 h at a temperature of 105°C to sterilize and strengthen the scaffolds. CG scaffold samples of 12.7 mm diameter were further crosslinked using EDC/NHS (14 mM *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride and 5.5 mM *N*-hydroxysuccinimide in distilled water; Sigma–Aldrich) for 2 h[23] to decrease the percentage shrinkage of the scaffolds due to cell-mediated contraction over time.

Construct preculture

Scaffold samples were seeded with 2 million MC3T3-E1 preosteoblast cells. For experiments carried out up to 49 h (study 1), constructs were cultured in α-MEM supplemented with 2% penicillin/streptomycin, 1% L-glutamine, 10% fetal bovine serum, and 0.1% amphotericin (Sigma–Aldrich) for 6 days of preculture before insertion into the bioreactor to allow for cell attachment, infiltration, and proliferation.[18] For constructs cultured up to 14 days (study 2), the medium was replaced with osteogenic medium (supplemented with 50 μg/mL ascorbic acid and 10 mM β-glycerolphosphate) on the third day of the preculture period and was used thereafter until the end of the experiment. The scaffolds were kept in an incubator with 5% CO_2 at 37°C and the medium was changed every 2–3 days during preculture. In total, the scaffolds were precultured in static conditions for 6 days after which point the constructs were cultured in the flow perfusion bioreactor or static culture conditions for 1, 25, or 49 h (study 1) and 4, 7, or 14 days (study 2).

Bioreactor culture

Four scaffold chambers were used for each experiment and each experiment was repeated at least twice, providing a minimum sample size of eight per group (study 1), whereas six scaffold chambers were used for study 2 (providing a sample size of six). The scaffolds were stimulated with a flow pattern incorporating both short- and long-term rest periods for 1, 25, or 49 h (study 1) and 4, 7, or 14 days (study 2). Short-term periods of no flow were incorporated into 1 h bouts of stimulation. They were of durations 0 (steady-flow group), 5, 10, and 15 s and were inserted between bouts of 10 s of 1 mL/min flow. This hour of stimulation was followed by either 7 h of 0.05 mL/min flow for study 1 (1–49 h) or no flow for study 2 (4–14 days) acting as a long-term rest period (Fig. 1). This 8-h cycle was repeated for the duration of the culture period. For cultures longer than 4 days the medium in the reservoir was replaced with fresh osteogenic medium every 2–3 days during the 7-h resting period.

Two different control groups were used to assess the effect of bioreactor culture on cellular activity. The first was a "static control" group, in which cell-seeded constructs were maintained in static culture in an incubator. The second was a "bioreactor control," in which constructs were placed into the bioreactor and immediately removed. This group was used to examine the effect of the process of setting up the bioreactor on cellular activity. After the culture period, constructs were removed from static or bioreactor culture and either flash frozen or fixed in formalin. Samples of medium were also flash frozen.

DNA quantification

To analyze DNA content, constructs were digested, homogenized, and mixed with Hoechst 33258 dye solution (Sigma–Aldrich, Dublin, Ireland). The fluorescence of the samples was measured at 460 nm after excitation at 355 nm in a Wallac Victor^{2}™ 1420 multilabel counter (Perkin Elmer Life Sciences, Turku, Finland). Fluorescence readings were compared with a standard curve to calculate the cell number.

Gene expression

Real-time reverse transcriptase–polymerase chain reaction (RT-PCR) was used to analyze gene expression. RNA was extracted using an Rneasy Mini Kit (Qiagen, Crawley, United Kingdom) and quantified by absorbance at 260 nm

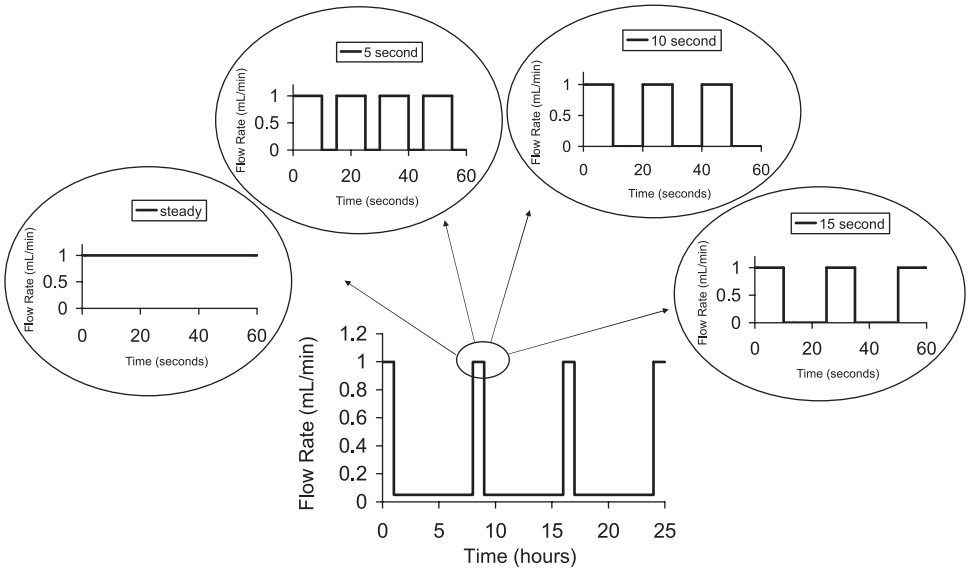

FIG. 1. Stimulation patterns used both short- and long-term rest periods. Short-term rests were of durations 0, 5, 10, or 15 s. Long-term rests were 7 h in duration. For study 1 (1–49 h), flow was administered at 0.05 mL/min, whereas for study 2 (4–14 days) there was a no-flow phase. This 8-h cycle was repeated for the duration of bioreactor culture.

(GeneQuant Pro RNA/DNA calculator; Biochrom, Cambridge, United Kingdom). Four hundred nanograms of RNA per sample was used for reverse transcription (Quantitect RT Kit; Qiagen) using a Peltier Thermal Cycler 200 (MJ Research, Waltham, MA). Real-time PCR was then carried out (7500 Real-Time PCR System; Applied Biosystems, CA). The QuantiTect SYBR Green PCR Kit (Qiagen) was used, according to the manufacturer's instructions, with QuantiTect Primers (designed by Qiagen). Results were quantified for COX-2, collagen-1 (COL-1, α1), OPN, and ALP via relative quantification (ΔΔCt method) using 18-S rRNA as the endogenous reference. For each gene, results are expressed relative to the bioreactor control group. All PCR reactions were conducted in triplicate for each sample.

PGE_2 concentration

A PGE_2 enzyme immunoassay was used (Cayman Chemicals, Ann Arbor, MI) to analyze PGE_2 concentration in the culture medium. A standard curve was run with each plate using PGE_2 standards. After incubation and development, plates were read at 405 nm absorbance using a Wallac Victor[2] 1420 multilabel counter (Perkin Elmer Life Sciences). A sample size of six was used.

Histology

Paraffin-embedded constructs were sliced at 10 μm and stained using hematoxylin and eosin to examine cell distribution. Digital images of all stained sections were obtained using an imaging system (AnalySIS; Olympus, Dublin, Ireland) in conjunction with a microscope (Olympus IX51; Olympus).

Statistics

Results are expressed as mean ± standard deviations. Statistics were done in SigmaStat 3.0 (SPSS, Chicago, IL) using a general linear model analysis of variance with the Holm-Sidak *post hoc* multiple comparison test. Nonnormal data (RT-PCR data for COX-2, COL-1, ALP, OPN, and PGE_2) was normalized using logarithmic or square root transforms so that the conditions of the statistical test were met.[24] A p value of < 0.05 was considered statistically significant.

Results

Figures 2–4 show the results for study 1 for culture up to 49 h. There were significantly more cells on the static group

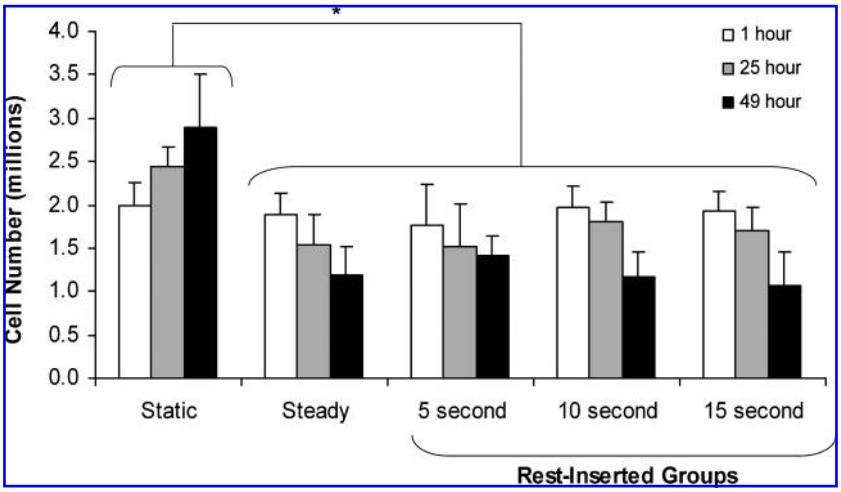

FIG. 2. Cell number of groups cultured for up to 49 h (study 1); $n = 6$ for all groups and asterisk represents $p \leq 0.0002$. While there was a significant decrease in cell number due to bioreactor culture, a large number of cells still resided on the constructs after the culture period.

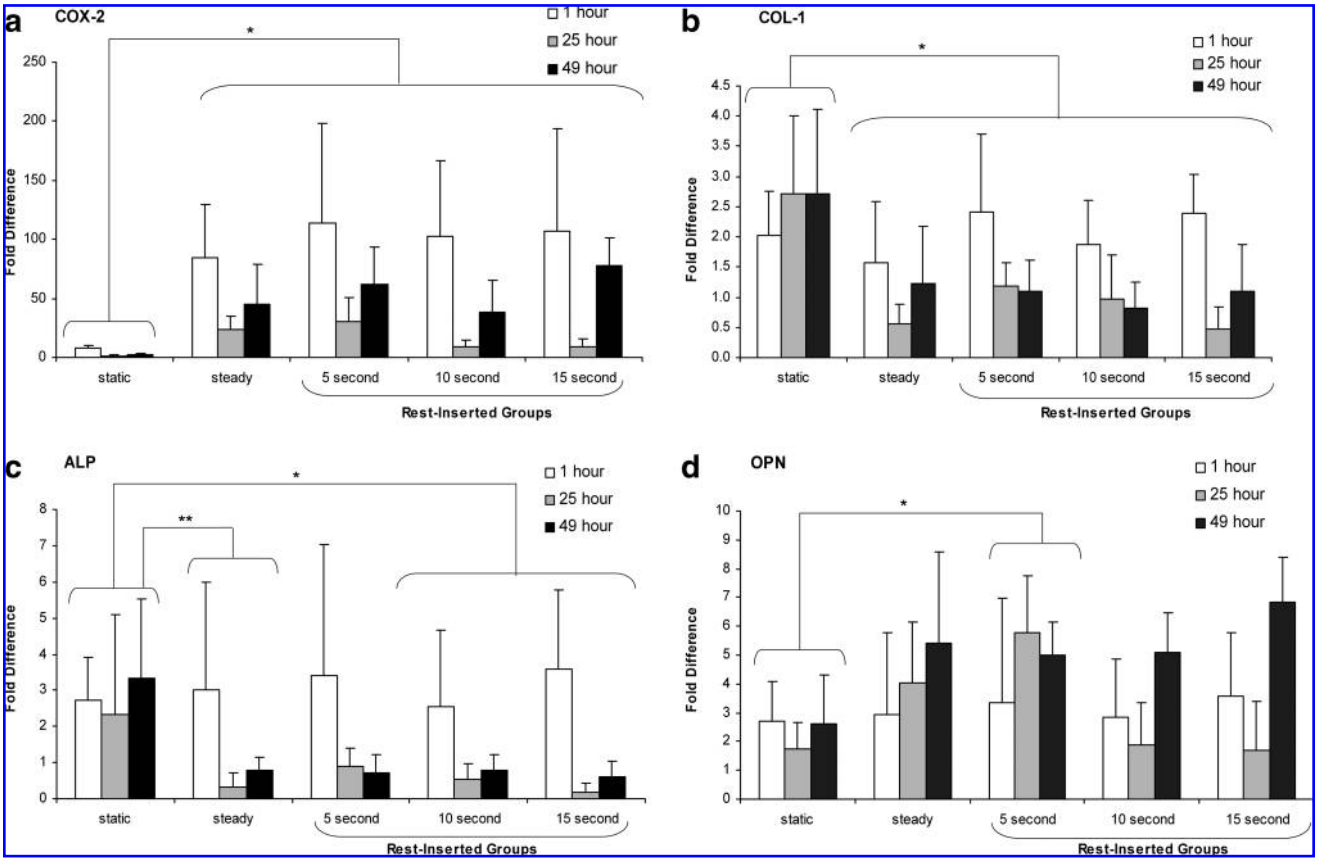

FIG. 3. (**a**) Gene expression of cyclooxygenase-2 (COX-2) ($*p < 0.00005$). All groups cultured in the bioreactor showed significant increases in cyclooxygenase-2 expression compared with the static group. (**b**) Gene expression of collagen-1 (COL-1) ($*p \leq 0.0262$). All groups cultured in the bioreactor showed significant decreases in COL-1 expression. (**c**) Gene expression of alkaline phosphatase (ALP) ($*p \leq 0.0156$, $**p < 0.0001$). While ALP expression did not change after 1 h of flow in the bioreactor, expression decreased for all bioreactor-cultured groups at 25 and 49 h compared with the static group. (**d**) Gene expression of osteopontin (OPN) ($*p = 0.0074$). A significant increase in expression of OPN was noted for the 5-s rest-inserted group. All groups are scaled to the bioreactor control group.

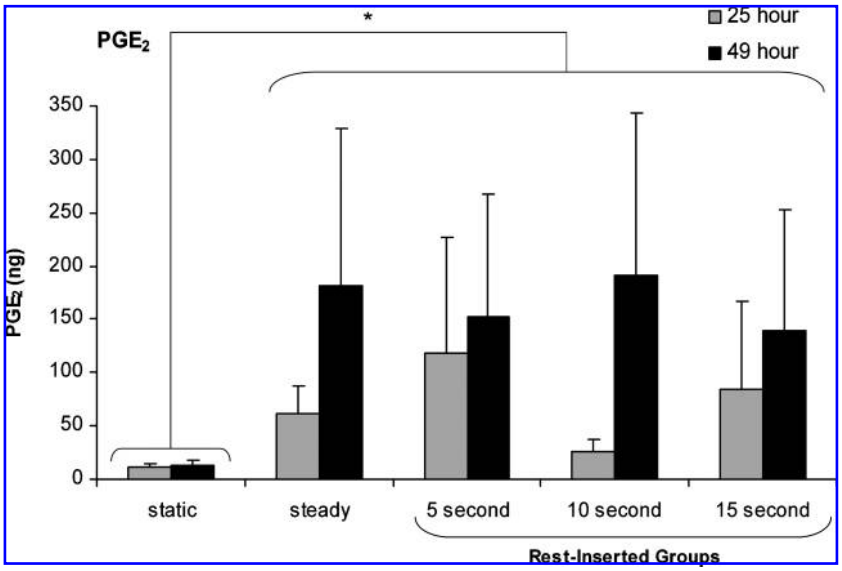

FIG. 4. Prostaglandin E_2 (PGE_2) production ($*p \leq 0.0017$). Increased PGE_2 production was seen for all flow groups. It was also noted that PGE_2 production increased over time for the flow groups (25 vs. 49 h, $p = 0.0138$).

than on any flow group ($p \leq 0.0002$; Fig. 2). Cell number decreased over time because of flow, with a significant reduction in cell number on flow group constructs after 49 h (1 h vs. 49 h, $p = 0.0026$). Despite the reduction in cell number due to flow, there were still substantial numbers of cells (over 50% of the amount initially seeded onto them) on scaffolds in all flow groups after 49 h of bioreactor culture. There was no effect of short-term rest-insertion on cell number over the culture period (steady vs. rest-inserted groups, $p > 0.9536$).

For RT-PCR analysis, in all cases, the bioreactor control group was taken as the base level and scaled to 1. The increase or decrease in gene expression was then calculated as a fold change compared with the bioreactor control group. Gene expression of COX-2 was found to increase significantly in all flow groups over the static control ($p < 0.00005$; Fig. 3a). Although there was a significant decrease in expression from 1 to 25 h ($p < 0.00005$) for all groups, there was a significant increase in expression from 25 to 49 h ($p < 0.00005$) for the flow groups only. The static control showed significantly higher expression of COL-1 than every other group ($p \leq 0.0262$; Fig. 3b). Expression decreased over time on the flow groups (1 h vs. 49 h, $p < 0.00005$) but appeared to level off at later time points, as there was no change in expression from 25 to 49 h ($p = 0.5919$). ALP levels were also highest for the static group. Expression was not changed because of 1 h of flow, but 25 and 49 h of bioreactor culture decreased the expression of ALP ($p < 0.0001$; Fig. 3c). All groups, with the exception of the 5-s group, had significantly lower expression of ALP than the static group ($p \leq 0.0156$). No significant differences in OPN levels were found between any groups, the exception being the 5-s rest-inserted group which showed significantly higher levels of OPN expression than the static control ($p = 0.0074$; Fig. 3d). Expression did not increase from 1 to 25 h but a significant increase in expression was seen from 1 to 49 h ($p = 0.0013$). By 49 h, expression for all markers was the same for all flow groups.

PGE_2 expression was examined at 25 and 49 h. It was found that production of PGE_2 was increased on all flow groups compared with the static control ($p \leq 0.0017$; Fig. 4). Flow groups showed a 9- to 14-fold increase in PGE_2 expression at 49 h compared with the static group. PGE_2 expression also increased significantly over time (25 vs. 49 h, $p = 0.0138$).

In study 2 we examined the long-term effect (up to 14 days) of flow on gene expression (COL-1, ALP, and OPN), cell number, and distribution. As there was no significant difference in any of the markers by 49 h for all of the flow groups, two characteristic flow groups (steady and 10 s rest-inserted) were chosen and were compared with the static group. As with the 49 h experiments, we observed significantly higher cell numbers on statically cultured constructs compared with the flow groups at all time points ($p \leq 0.025$). For static culture, all scaffolds retained more than 2×10^6 cells, whereas constructs cultured in the bioreactor maintained approximately $0.5–1 \times 10^6$ cells. There was no significant difference in cell number between the flow groups (Fig. 5).

COL-1 and ALP expression decreased from 1 h to day 14 for all groups ($p \leq 0.07$; Fig. 6a, b). However, the static control showed significantly higher expression of COL-1 than either of the flow groups ($p \leq 0.025$; Fig. 6a). OPN expression (Fig. 6c) remained fairly constant up to day 7 and then peaked at day 14 for the static group (1 h vs. 14 days, $p \leq 0.004$); for the steady group it peaked at day 4, whereas the rest-inserted group showed a steady continuous increase up to day 14 (1 h vs. 14 days, $p \leq 0.004$). Overall, OPN levels were higher on the rest-inserted group than the steady group and static control ($p \leq 0.025$).

Histological analysis (Fig. 7) revealed that there was a difference in the cellular distribution of scaffolds that were cultured under static conditions compared with the bioreactor. Hematoxylin and eosin staining of the scaffolds revealed that there were more cells on the periphery of static constructs, with few or no cells in the center of these constructs (encapsulation effect; Fig. 7a), compared with constructs exposed to flow where the cells appeared to have been detached from the edges and surfaces (Fig. 7b). By day 14 the encapsulation effect became more pronounced for the static constructs (Fig. 7c).

Discussion

The goal of this study was to use a flow perfusion bioreactor to stimulate an osteogenic response from osteoblastic cells on CG scaffolds using rest periods during flow for culture periods up to 14 days. Following the promising work in our laboratory, a long-term rest period of 7 h and short-term rest periods of 5, 10, or 15 s between every 10 s of 1 mL/min steady flow were used. The results show that

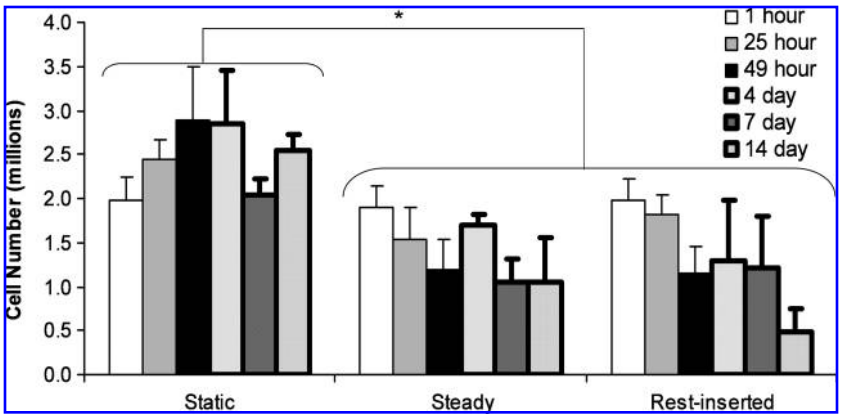

FIG. 5. Cell number of all groups (*$p \leq 0.025$). A decrease in cell number was observed in constructs cultured in the bioreactor. Data for 1–49 h are from study 1 and data for 4–14 days are from study 2.

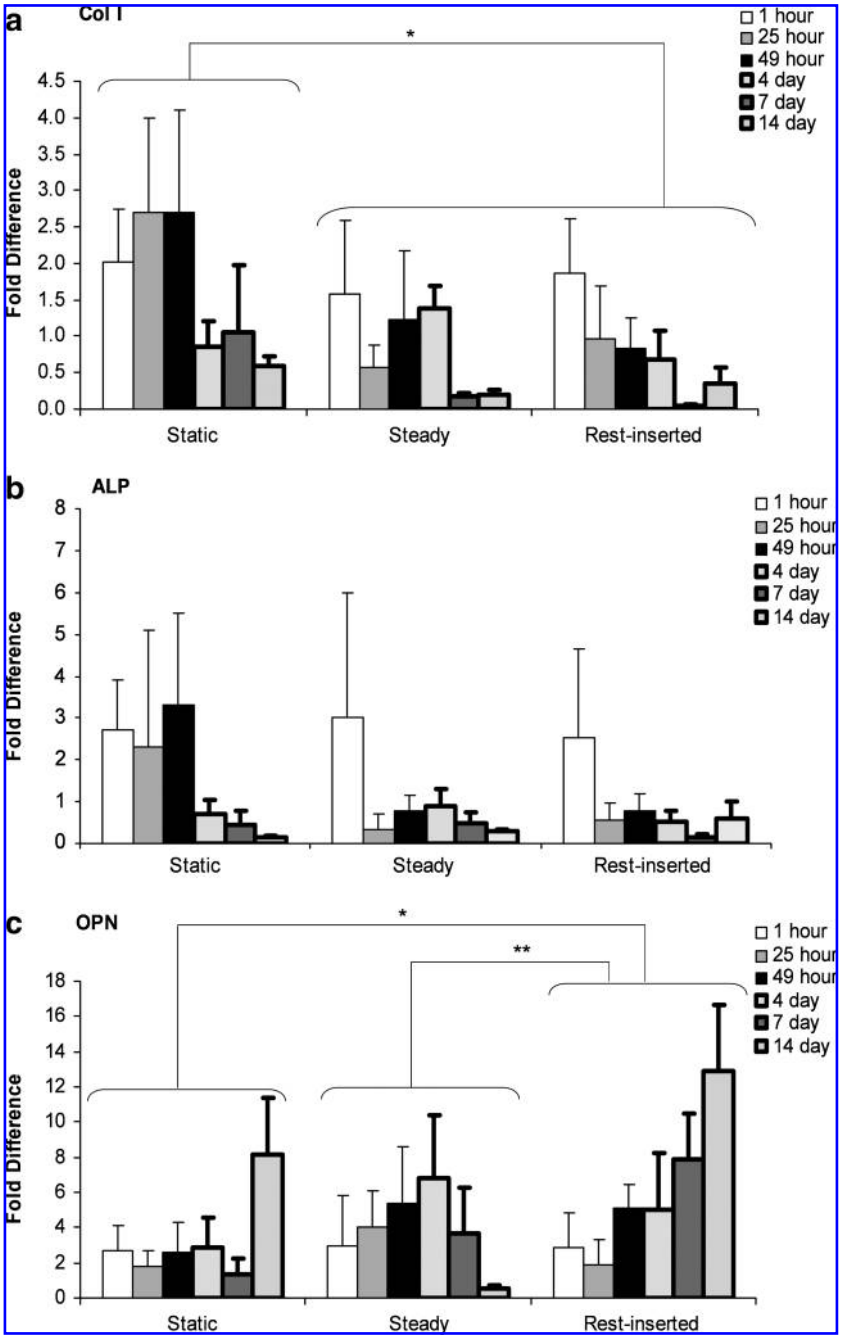

FIG. 6. (**a**) Gene expression of COL-1 ($*p \leq 0.025$). All groups cultured in the bioreactor showed significant decreases in COL-1 expression compared with the static group. (**b**) Gene expression of ALP. ALP expression decreased from 1 h to day 14 for all groups whilst there are no differences in its expression because of culture conditions. (**c**) Gene expression of OPN ($*p \leq 0.017$, $**p \leq 0.025$). A significant increase in OPN expression was observed for the rest-inserted group in comparison to the static and steady groups. All groups are scaled to the bioreactor control group. Data for 1–49 h are from study 1 and data for 4–14 days are from study 2.

bioreactor culture enhanced cell distribution compared with constructs cultured statically and led to upregulation of a number of bone formation markers analyzed, with the rest-inserted group showing the most promising results for bone tissue engineering.

Significant changes in gene expression of genes associated with bone formation due to the different flow types were noted. COX-2 levels were higher for all bioreactor groups compared with the static control, particularly after just 1 h of bioreactor culture. COX-2 is vital for the production of PGE_2[25] and the increase in COX-2 expression resulted in higher PGE_2 production in all flow groups over the static control at both 25 and 49 h. PGE_2 is involved in osteoblast differentiation[26] and may be linked to osteocalcin expression.[27,28] In 2D studies, it has been found that PGE_2 release is similar for continuous and short-term rest-inserted loading, a finding which is similar in 3D in this case.[19]

COL-1 expression decreased for all bioreactor groups compared with the static control. After 1 h of bioreactor culture, expression of COL-1 remained constant across all groups, but by 49 h, all bioreactor groups had significantly lower expression of COL-1 than the static group. With the exception of the 5-s rest-inserted group, ALP expression was lower for all bioreactor groups than for the static control. After 1 h of bioreactor culture, no effect on ALP expression was seen because of flow. Twenty-five and 49-h culture periods result in ALP downregulation in flow groups. Notably, OPN expression was higher for the 5-s rest-inserted group

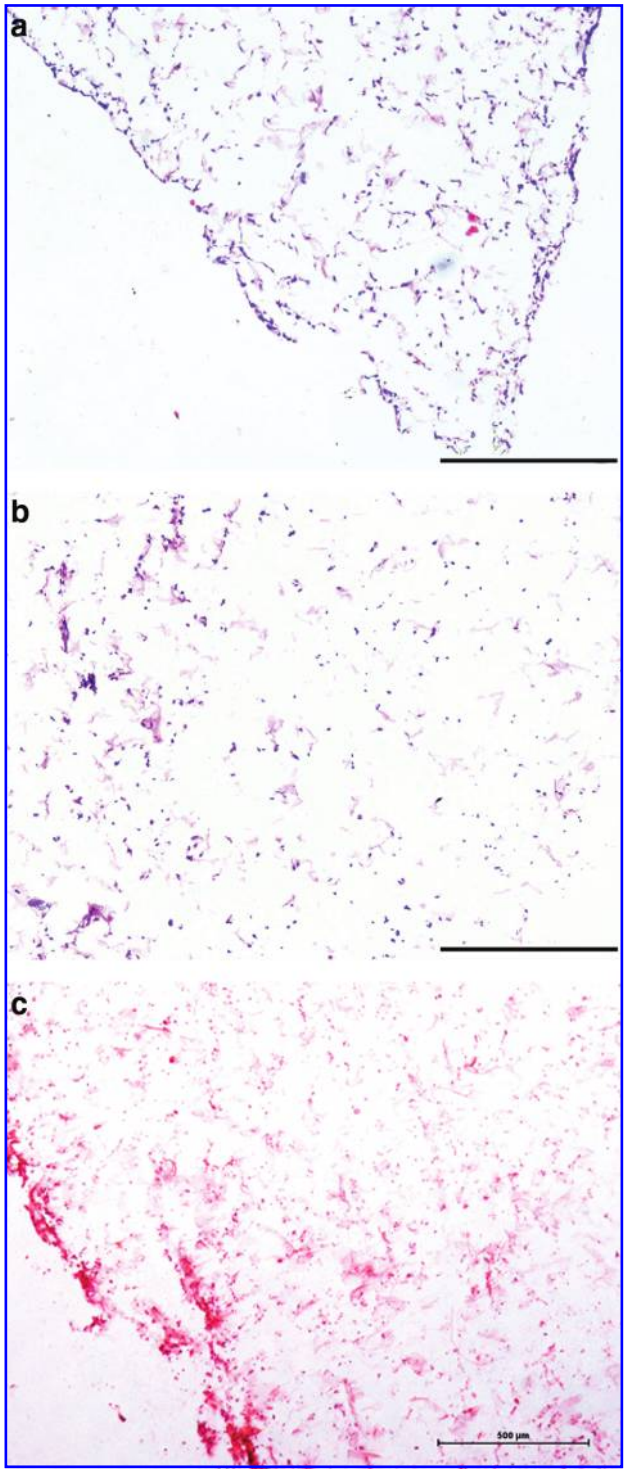

FIG. 7. Images of hematoxylin and eosin-stained construct slices after 25 h of culture. (**a**) The static group after 49 h of static culture (excluding preculture); (**b**) the 10-s rest-inserted group, which is representative of all flow groups after 49 h of bioreactor culture; (**c**) image of construct after 14 days of static culture. The scale is 500 µm in length in all cases. The static group exhibits a line of cells along the periphery, which is not evident in the flow groups. Cell distribution is greatly enhanced in this flow group and this is representative of all flow groups. By day 14, the encapsulation effect became more pronounced for the statically cultured constructs. Color images available online at www.liebertonline.com/ten.

than on the static group. This was the only significant change noted for this gene, although a trend for higher OPN values was seen on all bioreactor cultured groups by 49 h.

Because there was no significant difference between any of the markers by 49 h for all of the flow groups, two characteristic flow groups (steady and 10-s rest-inserted) were cultured up to 14 days and compared with the static control. It is important to note that there was a difference in the 7-h resting phase; for the 1–49-h experiments a flow rate of 0.05 mL/min was used; however, it was found that if this flow rate was used at 4 days and beyond, the cells did not survive (data not shown). We attributed this effect to the cells becoming detached during the 7-h low-flow phase. It has been reported that extended periods of continuous steady flow may hinder the development of an osteoblastic extracellular matrix because of cell detachment; as fluid flow reorganizes the actin cytoskeleton it may affect cell retention.[5] Therefore, this flow rate (0.05 mL/min) was eliminated for bioreactor culture longer than 49 h and it was found that the medium in the chamber (about 1 mL) was sufficient for the cells to survive during the 7 h without flow.

COL-1 expression was lower for all groups by day 14 compared with 1 h. By day 14, both the steady and rest-inserted groups showed a significant decrease in COL-1 expression compared with the static group. COL-1 is expressed during proliferation in 2D culture, which is then gradually downregulated but is expressed at low levels throughout osteoblast differentiation and maturation.[29] This suggests that culture in the bioreactor may either be downregulating this gene or accelerating osteoblast maturation. A very similar trend for ALP expression was observed as it also decreased by day 14 for all groups. ALP expression peaked at 49 h for the static group. Including the 6-day preculture, constructs were actually in culture for a period of 8 days; however, for constructs cultured in the bioreactor, ALP expression decreased after 1 h (or 6 days including the preculture period). In 2D culture, ALP expression generally increases after the proliferation phase has ended, meaning that expression begins to increase after 7 days and peaks at 21 days.[29] It may be the case that proliferation has been stopped because of bioreactor culture (which is supported by the cell number quantification) and ALP expression decreases because the proliferative phase is now over. Although enhanced ALP activity suggests enhanced osteoblastic function,[2] ALP expression was downregulated just before mineralization occurs,[30] and so a decrease in expression may indicate the onset of mineralization. Expression of OPN increased from 1 h to day 14 for all groups, except for the steady group which peaked at day 4 and then decreased by day 14. OPN levels were fairly constant for the static group and then peaked at day 14, whereas for the rest-inserted group there was a gradual and steady increase up until day 14. This increase in OPN levels has also been found using rest-inserted flow in 2D culture.[19] OPN is a late-stage marker in the mechanotransduction cascade. It regulates bone cell attachment and mineralization[31] and is important in bone remodeling.[32] Taken together, increased OPN expression coupled with decreased COL-1 expression may indicate that bioreactor culture has enhanced expression of postproliferative genes at the expense of those found during proliferation.

Upon examining cell number, it was found that there were significantly more cells in the static group than in the flow

groups following bioreactor culture. This decrease in cell number due to bioreactor culture has been noted in other studies.[5,20] An explanation may be that the shear stress exerted on the cells by the flow has caused some of them to become detached from the scaffolds. It is worth noting that despite the significant decrease in cell number due to flow, there are still approximately 0.5 million cells on the constructs after the 14-day culture period. Despite the reduction in cell numbers seen for the constructs cultured in the bioreactor, cell distribution appeared to be more homogeneous on flow groups than on the static control group, as has been seen in numerous other studies.[2,3,5–7,18] The static control group exhibited a line of cells on the periphery of the scaffolds. As cells proliferate in static culture, they block off the pores of the scaffold and decrease diffusion of nutrients to and waste removal from the center of the scaffold. This leads to an inhomogeneous tissue-engineered construct, with cells residing predominantly on the periphery. In the flow groups, the cells remaining on the scaffold following culture are well distributed, with those attached to the periphery of the scaffold predominantly being washed off. Therefore, the decrease in cell number seen under bioreactor culture may potentially be of benefit to tissue development. By removing cells clustered on the periphery and increasing cell viability in the center of the scaffold, it is envisaged that a more homogeneous scaffold may develop preventing core degradation occurring *in vitro*.

For a number of tissue types, the move toward clinical trials has been slow, and the progress to date in engineering significant quantities of functional tissue *in vitro* for implantation in humans *in vivo* has been somewhat disappointing; one reason for this lack of success has been the issue of core degradation. Core degradation is the necrosis of cells at the center of tissue-engineered constructs that results due to insufficient fluid transport throughout the grafts.[4] This problem becomes increasingly manifested as cells on the periphery of the construct grow and secrete extracellular matrix; consequently, diffusion of nutrients to the center of the construct becomes increasingly more difficult as movement of fluid into the core is impeded, resulting in an encapsulation effect and eventually acellular necrosis occurs in the center which is a major obstacle in the formation of a viable tissue *in vitro*.

One caveat to the study that is worth noting is that the bioreactor system is not a looped system but a linear one. This means that once the syringe has been emptied, the flow direction must be changed in order for flow to continue. During the 1-h stimulation periods, the flow direction was changed every 15 min and during the low-flow long-term rest period, flow direction changed every 3.5 h. These changes in flow direction may have an effect on cellular activity and cell distribution. Although either effect cannot be isolated, effects on distribution may be beneficial as scaffolds are perfused in both directions, so there is no net stimulus encouraging movement of cells in one direction. Another consideration to be taken into account is the cell type used; although an immortalized cell type (MC3T3s) was used as they provide reproducible results, human mesenchymal stem cells may be a better cell source to use to determine the physiological role of mechanical stimuli in inducing osteogenesis.

To summarize, a flow perfusion bioreactor has been used to examine the effect of flow patterns with short-term rest periods on cellular activity. The results show that cell number is decreased by flow but scaffolds still retain over 50% of the amount initially seeded onto them after 14 days of bioreactor culture; of great significance is that flow perfusion of scaffolds in the bioreactor provides a better distribution of cells throughout the scaffold with no encapsulation effect occurring. From an osteogenesis perspective, COX-2 and PGE_2 expression increase, whereas COL-1 expression decreases because of bioreactor culture. The gene expression for ALP and OPN are more complicated, with ALP being downregulated in all groups, and OPN being upregulated significantly in the rest-inserted groups compared with the static control group or steady-flow group. These results indicate that the insertion of resting periods during flow enhances cellular distribution and osteogenic responses on CG constructs cultured in a flow perfusion bioreactor which is promising for the development of homogeneous bone graft substitutes using tissue engineering.

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Disclosure Statement

No competing financial interests exist.

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