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Title: 3 hours of perfusion culture prior to 28 days of static culture, enhances osteogenesis by human cells in a collagen GAG scaffold

Running title: Bioreactor culture improves osteogenesis on a collagen GAG scaffold

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Abstract

In tissue engineering bioreactors can be used to aid in the *in vitro* development of new tissue by providing biochemical and physical regulatory signals to cells and encouraging them to undergo differentiation and/or to produce extracellular matrix prior to *in vivo* implantation. This study examined the effect of short term flow perfusion bioreactor culture, prior to long term static culture, on human osteoblast cell distribution and osteogenesis within a collagen glycosaminoglycan (CG) scaffold for bone tissue engineering. Human Foetal Osteoblasts (hFOB 1.19) were seeded onto CG scaffolds and pre-cultured for 6 days. Constructs were then placed into the bioreactor and exposed to 3x1hr bouts of steady flow (1ml/min) separated by 7hrs of no flow over a 24hr period. The constructs were then cultured under static osteogenic conditions for up to 28 days. Results show that the bioreactor and static culture control groups displayed similar cell numbers and metabolic activity. Histologically however, peripheral cell-encapsulation was observed in the static controls, whereas, improved migration and homogenous cell distribution was seen in the bioreactor groups. Gene expression analysis showed that all osteogenic markers investigated displayed greater levels of expression in the bioreactor groups compared to static controls. While static groups showed increased mineral deposition; mechanical testing revealed that there was no difference in the compressive modulus between bioreactor and static groups. In conclusion, a flow perfusion bioreactor improved construct homogeneity by preventing peripheral encapsulation whilst also providing an enhanced osteogenic phenotype over static controls.

Keywords: Flow Perfusion Bioreactor, Scaffold, Osteogenesis, Collagen, Tissue Engineering

Introduction

Bioreactors have been used to improve cellular activities in many biotechnological and bioengineering processes from fermentation and drug production to organ regeneration and tissue engineering. Similarly, they have been used to aid *in vitro* tissue formation of several organs including cartilage, prostate, gut, heart and liver formation (Kim et al. 2007; Margolis et al. 1999; Syedain and Tranquillo 2009; Yates et al. 2007). Several types of bioreactors exist including spinner flasks, rotating wall vessels, hydrostatic, dynamic compression and flow perfusion (Chowdhury et al. 2008; Elder and Athanasiou 2009; Frith et al. 2009; Jaasma and O'Brien 2008; Wang et al. 2009). They all aim to improve on traditional static culturing methods by providing a more efficient nutrient delivery and waste removal system under controlled settings. Disadvantages of long term static culture include the development of a cell capsule along the periphery of the construct which can cause limited nutrient and waste mass transfer resulting in central core degradation (Keogh et al. 2010; Keogh et al. 2009; Shea et al. 2000). Therefore, one objective of this study is to use a flow perfusion bioreactor to improve cell distribution within a collagen glycosaminoglycan (CG) scaffold in order to prevent peripheral encapsulation and subsequent core degradation developing during long term osteogenic culture.

Flow perfusion bioreactors act by directing the flow of media through the interior of a cell seeded scaffold. Different versions of these bioreactors exist for bone tissue engineering and are often specifically developed for a given scaffold type e.g. for stiffer scaffolds such as a titanium mesh (Bancroft et al. 2002) or for compliant scaffolds such as the CG scaffold. Flow perfusion bioreactors have been previously shown to improve cell seeding and distribution within 3-dimensional constructs (Glowacki et al. 1998; Goldstein et al. 2001; Janssen et al. 2006). In addition, they have also been shown to provide mechanical stimuli to cells which can be beneficial for improving cell activity and maturation (Owan et al. 1997; Zhu et al.), for

example, osteoblasts are a highly mechanosensitive cell that have been shown to respond to fluid flow resulting in altered gene expression levels of the bone formation markers alkaline phosphatase, osteopontin and osteocalcin (Partap et al. 2009; Wiesmann et al. 2004). Thus, the second objective of this study was to investigate the effect of the application of flow on the long term effects on osteoblast behaviour in terms of gene expression and cell viability.

Specifically, the hFOB 1.19 (human foetal osteoblast) cell line was chosen for the study (Harris et al. 1995). hFOB cells have been shown to undergo sequential osteogenesis by expressing high levels of routine bone formation markers and to mineralise in 2D and in 3D (Donahue et al. 2000; Harris et al. 1995). In a recent publication, we showed that the CG scaffolds supported the attachment, infiltration and viability of human osteoblast cells (hFOB 1.19) which demonstrated the ability of the CG scaffold to support human osteogenesis *in vitro* (Keogh et al. 2010). Whilst recent literature from our group has examined the effect of fluid flow on the behaviour of MC3T3 pre-osteoblast cells, we used a more clinically relevant cell line in this study as to date, the effects of fluid flow from a perfusion bioreactor on human cells within a CG scaffold have not been examined.

In this study, a flow perfusion bioreactor that was developed in house and specifically designed for a compliant CG scaffold was used (Jaasma et al. 2008). CG scaffolds have been used successfully for skin and nerve regeneration and show potential as an excellent bone graft substitute (Compton et al. 1998; Tierney et al. 2008). They are a highly biocompatible and highly porous sponge made via a lyophilisation process and has been shown to support the growth and development of several cells types including mesenchymal stem cells (MSCs) and the cell lines mouse calvarial (MC3T3s) and hFOB (Farrell et al. 2006; Keogh et al. 2010; Tierney et al. 2008). The use of the flow perfusion bioreactor was shown to be beneficial for osteogenesis by MC3T3 cells on the CG scaffold up to 28 days with increased levels of the mid stage marker osteopontin. However, it was also shown that longer term

bioreactor culture (>24 hours) resulted in decreased cell numbers which was hypothesised to be attributed to flow-induced cellular detachment (Plunkett et al. 2010) . As a result, in the current study the flow regime is altered, and the duration of bioreactor culture reduced, with cell-seeded scaffolds subjected to a bioreactor culture regime of 3x1hour bouts of fluid flow in a 24 period prior to a static culture in osteogenic conditions up to 28 days. The effects of this culture regime on cell distribution and osteogenesis by human cells is examined and compared to static culture conditions where cell-seeded scaffolds were not subjected to any period of bioreactor culture.

The running hypothesis is that the use of the flow perfusion bioreactor will result in a more homogenous cell distribution within a CG scaffold by preventing peripheral encapsulation; this coupled with mechanical stimulation of the cells may provide an improved osteogenic response by the hFOB cells cultured on CG scaffolds.

Materials and methods

Bioreactor design

A validated flow perfusion bioreactor system was used as described by Jaasma *et al.* (2008) (Fig.1). It consists of a syringe pump, a polycarbonate scaffold chamber and a media reservoir connected with gas permeable silicon tubing and quick release tubing connections (Cole-Parmer, Vernon Hills, IL). The bioreactor system was placed in a standard incubator. 12.7 mm diameter scaffolds (with a thickness of 3.5 mm) were held in the scaffold chamber using silicone O-rings. The syringe pump held six 50ml syringes to allow simultaneous culture of 6 scaffolds per run.

Scaffold fabrication

Collagen-glycosaminoglycan (CG) scaffolds were fabricated using a lyophilisation technique as described previously (Haugh et al. 2010; O'Brien et al. 2005). Briefly, CG slurry was prepared by blending bovine collagen type-1 (0.5% wt) (Integra Life Sciences, Plainsboro, NJ) with 0.05M acetic acid (pH 3.2) containing shark cartilage derived chondroitin-6 sulfate (0.05% wt) (Sigma-Aldrich, Germany). After blending (Ultra turrax), the slurry was degassed prior to lyophilisation. The slurry was freeze dried at a cooling rate of 0.9°C/min with a final freezing temperature of -40°C at 50 mTorr for 24 hours. Scaffolds were then sterilized and cross-linked by a dehydrothermal treatment (DHT) by placing in a vacuum oven at 105°C for 24 hours (VacuCell 22). CG sheets (thickness = 3.5mm; mean pore diameter = 96µm; porosity = 99.5%) (Haugh et al. 2010; O'Brien et al. 2004) were aseptically cut to size (12.7 mmϕ). Scaffolds were further cross-linked with a filtered solution of 6mM solution of 1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide (EDAC) and 2mol N-hydroxysuccinimide (NHS) in distilled water for 2 hours at room temperature. Scaffolds were rinsed several times in sterile phosphate buffer solution (PBS) prior to cell seeding.

Cell Culture, cell seeding and bioreactor conditions

hFOB 1.19 human pre-osteoblastic cells were cultured under standard conditions (5% CO₂, 37°C). Cells were routinely grown to 80% confluency in T175 culture flasks (Sarstedt, Ireland) containing a 1:1 ratio of HAMS F12 and DMEM (Gibco, UK), 10% FBS (Sigma-Aldrich, UK), 2% penicillin/streptomycin (Sigma-Aldrich, UK), 1% G418 (Gibco, UK) and 1% L-Glutamine (Sigma-Aldrich, UK).

2x10⁶ hFOB pre-osteoblasts were seeded dropwise on each side of the CG scaffolds (total of 4x10⁶ cells per construct). Cell-seeded constructs were pre-cultured in standard media for 6 days to allow for cell attachment, infiltration and proliferation (34°C optimal proliferation)

(Donahue et al. 2000; Harris et al. 1995) Constructs were then placed in the flow perfusion bioreactor and exposed to 3 x 1hr bouts of steady flow (1ml/min) with each bout being followed by 7 hrs of no flow (to prevent cellular desensitization) for 25 hrs (bioreactor groups). Constructs in the bioreactor were cultured in standard media. Once removed from the bioreactor, the constructs were cultured statically in osteogenic conditions for a further 28 days (39°C optimal differentiation). Osteogenic media consisted of standard media supplemented with 50µg/ml ascorbic acid, and 10mM β-glycerolphosphate, 10nM vitamin D₃, 50nM vitamin K₃, 10ng/ml transforming growth factor-β₁ for 7 days, reduced to 0.2ng/ml thereafter (Sigma-Aldrich, UK). The static control group consisted of constructs that were placed in osteogenic media after the 6 day pre-culture period and maintained in static conditions in parallel with the bioreactor group. This group was not exposed to the bioreactor at any point and remained in static culture for the entire duration of the experiment. In addition, two other control groups were used; these were the ‘unseeded static’ and ‘unseeded bioreactor’ groups. These groups were cultured in identical conditions to the static and bioreactor groups except for the absence of cells.

Viability of hFOB cells on CG scaffold

Metabolic activity was analysed using the alamar blue assay. Media was removed and replenished with that containing 10% alamar blue dye (Bioscience, Ireland) at each end point. Scaffolds were incubated on an orbital shaker for 4 hours. 100µL of media was read using a spectrophotometer at 570nm and 610nm. The percentage of reduced dye was calculated in accordance with manufacturer’s recommendations.

Cell number was assessed by measuring DNA content using the Hoechst 33258 DNA assay (Sigma-Aldrich, UK). Cell-seeded constructs were flash frozen in liquid nitrogen at each time point and stored at -80°C. DNA was isolated from the constructs by

homogenisation in RLT lysis buffer (Qiagen, UK) using a rotor-stator homogeniser (Omni International, Germany). Cell lysates were centrifuged using QI Shredder columns (Qiagen, UK). Fluorescence was measured (excitation: 355 nm, emission: 460 nm; Wallac Victor2, PerkinElmer, Waltham, MA) on samples in triplicate and readings were converted to cell number using a standard curve.

Histological staining for cell distribution on CG scaffold

Tissue engineered constructs were fixed in 4% paraformaldehyde (Sigma-Aldrich, UK) for 30 minutes. Dehydration and paraffin embedding was carried out using an automated tissue processor (ASP300, Leica) and cut into 10µm sections (RM2255, Leica). Standard haematoxylin & eosin staining was carried out on deparaffinised sections. Alizarin S Red staining for mineralisation was carried out using a 2% Alizarin red (Sigma-Aldrich, UK) solution for 2 minutes. Sections were rinsed several times with dH₂O, dehydrated in xylene and mounted with DPX. Images were captured on a digital microscope (NIS Elements, Nikon).

Gene expression analysis

RNA isolation: Cell seeded constructs were flash frozen in liquid nitrogen at each time point and stored at -80°C. RNA was isolated from the constructs by homogenisation in RLT lysis buffer (Qiagen, UK) using a rotor-stator homogeniser (Omni International, Germany). Cell lysates were centrifuged using QI Shredder columns (Qiagen, UK) and RNA extracted using the RNeasy Mini Kit (Qiagen, UK) according to the manufacturer's instructions. RNA concentration was determined using a spectrophotometer at an absorbance of 260 nm.

Following RNA extraction, Real time Reverse Transcription PCR was carried out for gene expression analysis. Trace genomic DNA was removed and RNA reverse transcribed

using 400ng total RNA with an RT kit (QuantiTect RT Kit, Qiagen, UK) according to the manufacturer's instructions. Real-time PCR was then carried out using the 7500 Real-Time PCR System (Applied Biosystems, UK). The QuantiTect SYBR Green PCR Kit (Qiagen, UK) was used, according to the manufacturer's instructions, with QuantiTect Primers (Qiagen, UK). β -actin acted as a house keeping control and results were quantified for the early stage (alkaline phosphatase and osteonectin) and mid to late stage (osteopontin and osteocalcin) bone formation markers using the $2^{-\Delta\Delta C_t}$ relative quantification method.

Mechanical stiffness

Mechanical stiffness of the tissue engineered scaffolds was analysed to determine if changes in cell/matrix distribution following bioreactor exposure caused variation in stiffness to that of static cultures. Stiffness of the constructs was determined using a Z050 mechanical testing machine (Z050, Zwick/Reoll) fitted with a 5-N load cell. Unconfined wet compression testing was performed on constructs that were immersed in PBS and tested at a rate of 10% strain/minute. The modulus was calculated from the slope of the stress-strain curve over the range 2-5% strain.

Statistical analysis

Statistical analysis was performed using sigma statistical software package SigmaStat 3.0. The statistical differences between 2 groups were calculated using the Students t test and multiple groups were calculated using Kruskal-Wallis One Way Analysis of Variance on Ranks (ANOVA). Statistical significance was declared at $p < 0.05$.

Results

The number of hFOB cells increased up to 2×10^6 cells per scaffold by 21 days of culture in both the static and bioreactor groups with no significant difference observed between groups over time (Fig. 2a). Similarly, for both groups, metabolic activity was maintained over the culture duration, with no significant difference between the groups (Fig. 2b).

Histological analysis showed that cells infiltrated the constructs over 28 days of culture in both the static and bioreactor groups (Fig. 3). However, differences in cell distribution were observed; cells in the constructs following bioreactor exposure appeared initially in clusters throughout the construct which increased in both number and distribution over time whereas cells on the static group resided predominantly along the scaffold periphery where by 14 days a cellular capsule had developed. Mineralisation reflected cell distribution where the greatest degree of staining was observed in the static groups but this mineralisation was found predominantly along the scaffold periphery (Fig. 4). Quantification of this revealed that the level of mineralisation was significantly greater in the static group than the bioreactor group ($p < 0.007$). By day 28, levels of mineral were 3.8 fold higher in the static group compared to the bioreactor group (Fig. 5).

Osteogenesis was supported in both static and bioreactor groups on the CG scaffold. Real time PCR showed that gene expression was altered in constructs following bioreactor culture compared to the statically cultured constructs (Fig. 6). A 3 fold increase in the expression of the early bone formation marker, alkaline phosphatase was seen in the bioreactor group versus the static group at 21 days. The mid stage markers osteopontin and osteonectin showed similar trends, with bioreactor groups providing higher expression levels earlier than the static groups; osteopontin expression increased by 1.6 fold after 1 day post-flow whereas the maximum expression levels of static groups occurred after 14 days. Similarly, osteonectin levels peaked after 14 days with a 1.7 fold increase for the bioreactor group ($p < 0.01$) whereas the level of osteonectin in static cultures peaked later at 21 days. At this stage the levels of

osteonectin were comparable in both groups. Expression levels of the late stage marker of bone formation, osteocalcin increased 2 fold after 1 day of bioreactor culture compared to constructs cultured statically ($p < 0.01$). The levels of osteocalcin peaked at 21 days for both the static and bioreactor groups, but within that period, the expression levels increased 8 and 12 fold ($p < 0.001$) in static culture and bioreactor culture groups respectively.

The compressive modulus of constructs in both the static and bioreactor groups increased with culture duration (Fig. 7). At the end of the 28 day culture period, the compressive modulus of constructs in the bioreactor group was 1.6 times greater than the statically cultured constructs. Also, an increase in stiffness was observed in cell-seeded constructs versus unseeded controls.

Discussion

Long term static culture conditions can lead to a heterogeneous cell distribution, often resulting in the development of a peripheral cellular capsule which may result in the development of a necrotic/acellular central core caused by a reduction in mass transfer conditions (Partap et al. 2010). Bioreactors can improve mass transfer of nutrients and wastes via fluid flow and may also be used to encourage cell growth and maturation (Bjerre et al. 2008; Yu et al. 2004). This study investigated the ability of a previously designed and validated flow perfusion bioreactor system (Jaasma et al. 2008) to enhance human hFOB cell distribution and osteogenic activity in a CG scaffold.

Results of this study showed that just 3 hours of fluid flow culture prior to 28 days of static culture improved cell distribution and enhanced osteogenesis. Static and bioreactor groups displayed similar cell numbers and metabolic activity over time. The cell number was

similar in the bioreactor and static groups using this flow regime of 3 hours whereas longer durations have shown a loss of cells following bioreactor culture (Jaasma and O'Brien 2008; Partap et al. 2009). However, histologically, there was a difference in cellular distribution between those constructs cultured statically or in the bioreactor. Constructs exposed to the bioreactor, displayed a more homogenous cell distribution throughout the construct compared to those cultured statically where the cells mainly resided along the construct periphery leading to peripheral encapsulation. In this study, static cultures developed peripheral encapsulation after 14 days in culture, a result that is consistent with other observations (Glowacki et al. 1998; Goldstein et al. 2001; Jaasma and O'Brien 2008; Janssen et al. 2006; Yu et al. 2004). The presence of a peripheral capsule can inhibit nutrient and waste mass transfer resulting in a necrotic core region. However, this was prevented by the use of the flow perfusion bioreactor which led to a redistribution of the cells. As a result of the spatial redistribution of the hFOB cells from the edge of the construct towards the centre due to fluid flow, the cells were visible in clusters throughout the constructs. Over time, these clusters became less evident (14 days post-flow) as a more homogenous cell distribution developed by the end of the culture period. Cells were evident in the centre of the construct by 21 days using the bioreactor but not until 28 days in static cultures. Therefore, it is clear that the short period of bioreactor culture (3 x 1 hr bouts) was beneficial for improving cell distribution within the construct.

Levels of mineralization reflected cell distribution with greatest levels identified along the construct periphery of static cultures where cell aggregation occurred, a finding similar to previous reports using rat mesenchymal stem cells (Farrell et al. 2006). Although, significantly higher levels of mineralisation were found in the statically cultured constructs, this is not necessarily a negative indictment of the bioreactor. Even though it is of interest to produce large levels of calcified tissue, there is a need to produce a construct *in vitro* with

homogeneously distributed cells that express the desired genes for osteogenesis that will not restrict mass transfer *in vivo* after implantation into a defect. It is envisaged that the data collected from this study can be translated to other cell types such as mesenchymal stem cells. Interestingly, no significant decrease was seen in the biomechanical properties of the CG scaffold exposed to bioreactor culture when compared to constructs cultured under standard conditions. Thus, even though there was more mineral in the static groups, its poor distribution (predominantly along the construct periphery) did not manage to improve the mechanical properties. However, both sets of cell-seeded constructs were stiffer than the unseeded scaffolds demonstrating the improvement in mechanical properties as a result of cell-mediated matrix deposition and mineralisation.

Gene expression analysis showed that exposing the constructs to just 3 hours of fluid flow in the bioreactor produced an altered pattern of gene expression compared to that observed in constructs cultured statically. The results show an increase in all early, mid and late stage bone formation markers investigated following bioreactor culture. Importantly, the effects of mechanical stimulation on gene expression were still detected in the long term cultured constructs even after 28 days of bioreactor culture. Specifically, the levels of the early bone formation marker alkaline phosphatase increased over time with greatest expression levels observed at 21 days in the bioreactor group. The mid stage marker osteonectin showed similar trends between the static and bioreactor groups, its levels increased up to 21 days although greatest expression occurred at 14 days in the bioreactor groups ($p < 0.01$). However, levels of osteopontin in the bioreactor groups increased following 3 hours of flow and declined thereafter. The late stage marker osteocalcin increased 8 fold in static cultures up to 21 days, but greatest expression (1.3 fold higher) was observed in bioreactor groups ($p < 0.01$). As the only difference between the culture groups was the effects of the bioreactor; the difference in gene expression levels appears to be due to the mechanostimulatory response

provided by 3 hours of fluid flow prior to static culture, this mechanostimulatory response is particularly notable for OPN, where it is significantly upregulated after the application of flow. Whilst it is known that OPN is affected by a mechanical stimulus, its molecular mechanisms within the mechanotransduction pathways are still not completely understood.

At present, the ideal flow regime to produce a viable construct *in vitro* has not yet been identified. In an attempt to find the ideal flow regime, the particular flow profile used in this study was based on a combination of the different flow regimes used in our group (Jaasma and O'Brien 2008; Plunkett et al. 2010). A limitation that we have previously reported is that constructs cultured in the bioreactor for prolonged periods exhibit flow-induced cellular detachment. Therefore, a significant finding of this study is that reducing culturing times within the bioreactor still elicits an osteogenic response and improves cellular homogeneity without significantly reducing cell numbers. This suggests that constructs do not need to be cultured continuously within a bioreactor for long periods of time. This is particularly important as long term bioreactor culture is often associated with being more time consuming, labour intensive and less cost effective than static culturing techniques.

In conclusion, the flow perfusion bioreactor improved hFOB cell infiltration by discouraging peripheral encapsulation on CG scaffolds and induced a superior osteogenic response within the constructs under long term culture compared to static controls. Even though there was more mineral in the static groups, its poor distribution did not improve the mechanical properties of the constructs. Taking together the histological and gene expression results, this study demonstrates that just 3 hours of flow perfusion bioreactor culture prior to a 28 day static culture period was beneficial for osteogenesis by human cells on a CG scaffold.

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List of Figures

Fig. 1 Flow perfusion bioreactor for CG scaffolds. Reprinted from Journal of Biotechnology, 133/4, Jaasma, M.J., N.A. Plunkett, and F.J. O'Brien, Design and validation of a dynamic flow perfusion bioreactor for use with compliant tissue engineering scaffolds, 490-6 □ (2008), with permission from Elsevier.

Fig. 2 Bioreactor and static culture cell number and metabolic activity of hFOB on CG scaffold. Similar levels of (a) cells numbers and (b) metabolic activity were observed at all time points in both static and bioreactor groups of hFOB cells on CG scaffold. Results are expressed as the mean \pm standard deviation (n=5).

Fig. 3 Histological analysis (H&E staining) of cell distribution within a CG scaffold; static control versus bioreactor culture over time. Cells attach to CG scaffolds and gradually infiltrate with time. Static groups develop a cellular capsule around the construct (white arrows) this; however, is prevented when using the bioreactor.

Fig. 4 Alizarin red staining of CG constructs up to 28 days post bioreactor treatment. Staining reflected cell distribution found mineralisation in static groups predominantly at the scaffold edge (white arrows) following 14 days incubation. Lower levels of staining were observed in bioreactor treated groups.

Fig. 5 A quantification of the levels of alizarin red staining. It was found that there was a significant increase in mineralisation in static groups compared to the bioreactor groups (* $p < 0.007$). Results are expressed as the mean \pm standard deviation (n=3).

Fig. 6 Gene expression results for static and bioreactor groups. Alkaline phosphatase expression levels in the bioreactor peaked at 21 days compared to the static group. Osteonectin levels increased up to 21 days for both the static and bioreactor groups and then decreased by day 28, however, osteonectin levels peaked earlier (by day 14) in the bioreactor group when compared to the same timepoint in the static group (* $p < 0.01$). The greatest levels of osteopontin were observed at 1 day post bioreactor culture when compared to the same timepoint in the static group (* $p < 0.05$). When compared to static culture, osteocalcin levels were significantly higher in the bioreactor groups at day 1 (* $p < 0.01$) and by day 21 where a 1.3 fold higher expression was observed (** $p < 0.01$). Results are expressed as the mean \pm standard deviation (n=5).

Fig. 7 Biomechanical analysis cell free and cell seeded CG constructs following bioreactor and static culture up to 28 days. Cell seeded constructs were stiffer than unseeded scaffolds; however, no difference was observed between static or bioreactor groups. Results are expressed as the mean \pm standard deviation (n=3).