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The effect of cyclic hydrostatic pressure on the functional development of
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Abstract

Mechanical signals can play a key role in regulating the chondrogenic differentiation of Mesenchymal Stem Cells (MSCs). The objective of this study was to determine if the long-term application of cyclic hydrostatic pressure could be used to improve the functional properties of cartilaginous tissues engineered using bone marrow derived MSCs. MSCs were isolated from the femora of two porcine donors, expanded separately under identical conditions, and then suspended in cylindrical agarose hydrogels. Constructs from both donors were maintained in a chemically defined media supplemented with TGF- β 3 for 42 days. TGF- β 3 was removed from a subset of constructs from day 21 to 42. Loaded groups were subjected to 10 MPa of cyclic hydrostatic pressurization at 1 Hz for one hour/day, five days/week. Loading consisted either of continuous hydrostatic pressure (CHP) initiated at day 0, or delayed hydrostatic pressure (DHP) initiated at day 21. Free-swelling (FS) constructs were cultured in parallel as controls. Constructs were assessed at days 0, 21 and 42. MSCs isolated from both donors were morphologically similar, demonstrated comparable colony forming unit-fibroblast (CFU-F) numbers, and accumulated near identical levels of collagen and GAG following 42 days of free swelling culture. Somewhat unexpectedly the two donors displayed a differential response to hydrostatic pressure. For one donor the application of CHP resulted in increased collagen and GAG accumulation by day 42, resulting in an increased dynamic modulus compared to FS controls. In contrast, CHP had no effect on matrix accumulation for the other donor. The application of DHP had no effect on either matrix accumulation or construct mechanical properties for both donors. **Variability in the response to hydrostatic pressure was also observed for three further donors. In conclusion, this study demonstrates that the application of long term hydrostatic pressure can be used to improve the functional properties of cartilaginous tissues engineered using bone marrow derived MSCs by enhancing collagen and GAG accumulation. The response to such loading however is donor dependant, which has implications for the clinical utilization of such a stimulus when engineering cartilaginous grafts using autologous MSCs.**

Introduction

Articular cartilage has a poor capacity for repair. Of the procedures available to the orthopaedic surgeon, osteochondral grafting is the only technique which reliably produces hyaline cartilage within a defect (Getgood et al. 2009). This suggests that if tissue engineering strategies could be used to develop cartilaginous grafts with mechanical properties approaching that of normal articular cartilage, then hyaline tissue could be regenerated. There has been increased interest in using Mesenchymal Stem Cells (MSCs) seeded onto scaffolds or hydrogels to engineer such functional grafts (Meinel et al. 2004; Li et al. 2005; Wang et al. 2005; Hofmann et al. 2006; Cheng et al. 2009; Erickson et al. 2009; Buckley et al. 2010; Huang et al. 2010). Of concern with such approaches are reports that the mechanical properties of cartilaginous tissues engineered using MSCs are inferior to that engineered using chondrocytes derived from articular cartilage (Mauck et al. 2006; Huang et al. 2009; Buckley et al. 2010), although recent studies have demonstrated that adult equine MSCs produce a cartilaginous tissue mechanically superior to that derived using animal-matched adult chondrocytes (Kopesky et al. 2010). Furthermore it has been demonstrated that aggrecan from MSC based engineered cartilage tissue exhibits ultrastructure and nanomechanical properties superior to native cartilage (Lee et al. 2010). While these results support the use of MSCs for cartilage tissue engineering, further effort is required to determine the combinations of biophysical and biochemical cues necessary to engineer grafts with the necessary mechanical functionality for implantation into load bearing defects.

Mechanical signals have been shown to play a key role in regulating the differentiation of MSCs (Kelly and Jacobs 2010). A number of authors have explored the use of dynamic compression as a means to enhance Mesenchymal Stem Cell (MSC) chondrogenesis and/or the subsequent mechanical properties of cartilaginous grafts (Angele et al. 2004; Huang et al. 2004; Campbell et al. 2006; Mouw et al. 2007; Thorpe et al. 2008; Li

et al. 2009; Li et al. 2009; Huang et al. 2010; Thorpe et al. 2010). The response of MSCs to such mechanical signals is complex. Dynamic compression has been shown to inhibit chondrogenesis if initiated at the onset of growth factor induced differentiation (Thorpe et al. 2008; Thorpe et al. 2010). Recently it has been shown that dynamic compression can be used to enhance the functional properties of cartilaginous tissues engineered using bone marrow derived MSCs, but only if applied after the initiation of robust chondrogenesis (Huang et al. 2010).

Cyclic hydrostatic pressure has been shown to enhance chondrogenesis of MSC aggregates, as evidenced by increases in type II collagen and aggrecan mRNA expression and/or proteoglycan and collagen accumulation (Angele et al. 2003; Miyanishi et al. 2006; Miyanishi et al. 2006; Luo and Seedhom 2007; Wagner et al. 2008). It has also been demonstrated that the magnitude of hydrostatic pressure (0.1, 1 or 10 MPa) differentially regulates chondrogenesis of MSC aggregates, with greater type II collagen mRNA expression and collagen accumulation at higher pressures (Miyanishi et al. 2006). In contrast, other studies report that hydrostatic pressure has little or no effect on chondrogenic gene expression or matrix accumulation in MSC aggregates, in either the presence or absence of TGF- β or BMP-2 (Zeiter et al. 2009). Furthermore, hydrostatic pressure has been shown to have no effect on aggrecan and collagen II mRNA expression for MSCs embedded in agarose hydrogels (Finger et al. 2007). Clearly some uncertainty exists in the literature as to the effect of hydrostatic pressure on chondrogenesis of MSCs, and for the role it might play in improving the functional properties of cartilaginous tissues engineered using this cell source.

The objective of this study is to determine the influence of cyclic hydrostatic pressure initiated either before or after TGF- β 3 induced chondrogenic differentiation on the functional maturation of cartilaginous grafts engineered using bone marrow derived MSCs encapsulated in agarose hydrogels. The hypothesis of this study was that the application of hydrostatic

pressure initiated after chondrogenic differentiation of MSCs embedded in agarose hydrogels would improve the functional properties of the resulting engineered graft. MSCs were isolated from the femora of two porcine donors, expanded separately under identical conditions, and then suspended in cylindrical agarose hydrogels. MSCs isolated from both donors accumulated nearly identical levels of cartilage specific matrix components, although somewhat surprisingly, displayed a differential response to hydrostatic pressure. These results raise the possibility that the response of stem cells to mechanical signals is donor dependent.

Methods

Cell isolation and construct assembly

Bone marrow was aseptically harvested from the femoral diaphysis of two four-month old pigs (~50 kg). Porcine MSCs were isolated from the bone marrow, and expanded according to a modified method developed for human MSCs (Lennon and Caplan, 2006). Cells were seeded at a density of 50,000 cells/cm² in T-75 cm² flasks in high-glucose DMEM GlutaMAX supplemented with 10% foetal bovine serum and 100 U/mL penicillin/streptomycin (all GIBCO, Biosciences, Dublin, Ireland). MSCs were subcultured at a density of 5,000 cells/cm² following colony formation and expanded to passage three. Cells were suspended in 2% agarose (Type VII, Sigma-Aldrich, Arklow, Ireland) at a density of 15 million cells/ml and cast in a stainless steel mould to produce cylindrical constructs (Ø5 mm x 3 mm thickness). Constructs were maintained in 2.5 mL of a chemically defined chondrogenic medium consisting of hgDMEM supplemented with 100 U/mL penicillin/streptomycin (both GIBCO), 100 µg/ml sodium pyruvate, 40 µg/ml L-proline, 50 µg/ml L-ascorbic acid-2-phosphate, 1.5 mg/ml BSA, 1× insulin-transferrin-selenium, 100 nM dexamethasone (all Sigma-Aldrich) and 10ng/ml of TGF-β3 (ProSpec-Tany TechnoGene

Ltd., Israel). Medium was changed twice per week for six weeks. MSCs from both donors were expanded and cultured separately from cell isolation to the final day of loading (i.e. no cell pooling); at the same time using identical batches of serum, growth factors and other supplements. MSCs from three further donors were isolated and expanded under similar conditions at different time points and used to replicate certain arms of the experiments described below.

Colony-forming unit-fibroblast (CFU-F) assay

For the CFU-F assay 5×10^6 live cells were seeded into a 100mm diameter dish following isolation from the bone marrow. Triplicate wells were seeded for all conditions. At the end of the assay, wells were fixed with phosphate buffered formaldehyde, stained with 1% crystal violet (Sigma) and colony numbers (>50 cells) counted.

Application of hydrostatic pressure

Constructs from each group were removed from their culture dishes and sealed into sterile bags with 1 mL of medium per construct during the loading period. Cyclic hydrostatic pressure was carried out in a custom bioreactor placed within a 37°C incubator. The sealed construct-containing bags in the hydrostatic pressure groups were placed into a water-filled pressure vessel while the free swelling (FS) control groups were placed into an open water bath (Fig. 1a). The cyclic hydrostatic pressure protocol consisted of 0 to 10 MPa amplitude at a frequency of 1 Hz. This loading regime was employed for a period of 1 hour/day, 5 days/week. Continuous hydrostatic pressure (CHP) constructs were loaded for six weeks, initiated at day 0, while delayed dynamic compression (DHP) constructs were only loaded

during day 22-42. These constructs were continuously supplemented with TGF- β 3 for the duration of the experiment (TGF- β 3+). An additional arm of the study explored withdrawal of TGF- β 3 from the culture media at day 21 (TGF- β 3-) while at the same time initiating the application of DHP (Fig. 1b).

Mechanical analysis

Constructs were assessed at days 0, 21, and 42 as previously described (Buckley et al. 2009). Constructs from each group were mechanically tested in unconfined compression between impermeable platens using a standard materials testing machine with a 5N load cell (Zwick Z005, Roell, Germany). Stress relaxation tests were performed, consisting of a ramp and hold cycle with a ramp displacement of 1 μ m/s until 10% strain was obtained and maintained until equilibrium was reached (30 min). Dynamic tests were performed immediately after the stress relaxation cycle. A cyclic strain amplitude of 1% superimposed upon the 10% strain was applied for 10 cycles at 1 Hz. The compressive equilibrium modulus and dynamic modulus were determined from these tests.

Quantitative biochemical analysis

Following mechanical testing, constructs were cored using a 3 mm biopsy punch, the wet mass of the annulus and core recorded and frozen for subsequent biochemical analyses. Annulus and core samples were digested with 125 μ g/mL papain in 0.1 M sodium acetate, 5 mM L-cysteine-HCl, 0.05 M EDTA, pH 6.0 (all from Sigma-Aldrich) at 60 °C under constant rotation for 18 h. DNA content was quantified using the Hoechst Bisbenzimidazole 33258 dye assay as described previously (Kim et al. 1988), with a calf thymus DNA standard.

Sulphated glycosaminoglycan (sGAG) content was quantified using the dimethylmethylene blue dye-binding assay (Blyscan, Biocolor Ltd., Northern Ireland), with a chondroitin sulphate standard. Total collagen content was determined by measuring the hydroxyproline content. Samples were hydrolysed at 110 °C for 18 hrs in 38% HCl and assayed using a chloramine-T assay (Kafienah and Sims 2004) with a hydroxyproline:collagen ratio of 1:7.69 (Ignat'eva et al. 2007). Each biochemical constituent was normalised to the tissue wet weight.

Histology and immunohistochemistry

At each time point, two additional samples per group were fixed in 4% paraformaldehyde overnight, dehydrated, and embedded in paraffin. The constructs were sectioned with 6 µm thickness perpendicular to the disc face. Sections were stained with 1% alcian blue 8GX (Sigma-Aldrich) in 0.1 M HCl for sGAG, and picro-sirius red for collagen. Type II collagen was identified through immunohistochemistry. Briefly, sections were treated with peroxidase (between steps slides were washed in PBS) followed by treatment with chondroitinase ABC (Sigma-Aldrich) in a humidified environment at 37 °C to enhance permeability of the extracellular matrix. After incubation with goat serum to block non-specific sites, the primary antibody for type II collagen (mouse monoclonal, Abcam, Cambridge, UK) was applied on the sections overnight at 4 °C. Next, the secondary antibody (Anti-Mouse IgG biotin conjugate, Sigma-Aldrich) was added for one hour, followed by incubation with ABC reagent (Vectastain PK-4000, Vector Labs, Petersbough, UK) for 45 minutes. Finally, the slides were developed with DAB peroxidase (Vector Labs) for five minutes. Negative and positive controls of porcine ligament and cartilage were also assessed.

Statistical analysis

Mechanical and biochemical properties are expressed in the form of mean \pm standard deviation, with a minimum of $n = 3$ for each group and each donor at each time point. Differences in mechanical and biochemical properties with loading and either time-in-culture, location (annulus and core) or growth factor supplementation (\pm TGF- β 3) were determined by two-way ANOVAs with Bonferroni post-tests. A level of $p < 0.05$ was considered significant.

Results

Both donors displayed comparable CFU-F numbers and morphologically cells appeared similar during monolayer culture (data not shown). Following 42 days of free swelling (FS) culture in the presence of TGF- β 3, both donors also displayed near comparable levels of GAG and collagen accumulation (Fig. 2). For donor 1, the application of either continuous hydrostatic pressure from day 0 (CHP), or delayed hydrostatic pressure from day 21 (DHP), did not result in changes to either collagen (FS: 0.253 ± 0.005 %w/w; CHP: 0.196 ± 0.021 %w/w; 0.258 ± 0.043 %w/w) or GAG (FS: 0.598 ± 0.017 %w/w; CHP: 0.596 ± 0.015 %w/w; 0.587 ± 0.049 %w/w) accumulation within the constructs (Fig. 2a). In contrast, for donor 2 the application of CHP was observed to increase both collagen (FS: 0.255 ± 0.033 %w/w; CHP: 0.452 ± 0.026 %w/w; 0.233 ± 0.042 %w/w) and GAG (FS: 0.542 ± 0.032 %w/w; CHP: 0.771 ± 0.020 %w/w; 0.51 ± 0.059 %w/w) accumulation by day 42 (Fig. 2b). Collagen, but not GAG, accumulation was also observed to be significantly higher in the CHP group by day 21 for this donor (data not shown). The application of DHP from day 21 did not result in any significant changes in either collagen or GAG accumulation by day 42 for either donor. The withdrawal of TGF- β 3 from the culture media at day 21 had a significant effect on both collagen and GAG accumulation by day 42 ($p < 0.001$), reducing values compared to

constructs continuously supplemented with TGF- β 3. The application of DHP did not influence this finding (Fig. 2).

Collagen and GAG accumulation were significantly higher in the core of engineered constructs compared to the annulus for both donors. For donor 2, where CHP increased overall levels of matrix accumulation, it was found that CHP did not affect collagen or GAG accumulation in the annulus of the construct, but dramatically increased matrix levels in the core of the constructs (Fig. 3). These increases in the core due to CHP were also observed if matrix accumulation was normalised to DNA. The application of DHP was not observed to alter spatial patterns of matrix accumulation compared to FS conditions (data not shown).

Histological sections stained with alcian blue generally confirmed the findings of the biochemical assays (Fig. 4). Matrix accumulation appeared greater in the centre of each construct compared to the annulus, and this difference appeared to increase with the application of CHP. All groups stained positive for type II collagen (data not shown).

For donor 2, there was no significant difference between the equilibrium moduli or dynamic moduli of FS and CHP constructs at day 21 (Fig. 5). Both the equilibrium and dynamic modulus significantly increased with time for FS and CHP conditions ($p < 0.01$). By day 42, there was no difference in the equilibrium moduli between either FS or CHP constructs, however the dynamic moduli of CHP constructs was significantly higher than the other groups ($p < 0.001$). The application of DHP did not alter the mechanical properties compared to FS conditions.

To further explore the donor dependent response to hydrostatic pressure, we subjected MSCs isolated from three further donors to hydrostatic pressure for 3 weeks following encapsulation in agarose hydrogels. (It should be noted that these MSCs were expanded and loaded at different times in separate experiments, so other sources of experimental variability

that were accounted for in the main part of the study by expanded and loading MSCs at the same time may also be playing a role in the variable response to loading.) As was observed for the first two donors, no significant increase in sGAG accumulation was observed for any of these three donors by week 3, while one of the three additional donors demonstrated a 1.3 fold increase in collagen synthesis in response to hydrostatic pressure.

Discussion

It has been proposed that hydrostatic pressure is one of the most important stimuli for cartilage tissue engineering (Elder and Athanasiou 2009). The initial hypothesis of this study was that the application of hydrostatic pressure initiated after chondrogenic differentiation had occurred would improve the functional properties of cartilaginous grafts engineered using bone marrow derived MSCs. Our hypothesis was initially motivated by our previous findings that other forms of mechanical stimulation, specifically dynamic compression, can inhibit chondrogenesis of MSCs if applied before chondrogenesis has occurred (Thorpe et al. 2008; Thorpe et al. 2010). In addition, it has been shown that dynamic compression can improve the mechanical functionality of cartilaginous constructs engineered using MSCs if initiated following three weeks of free swelling culture (Huang et al. 2010). In contrast to these results, we found that hydrostatic pressure applied at the onset of cytokine induced chondrogenic differentiation improved the functional properties of such cartilaginous grafts, and that delayed application of hydrostatic pressure did not improve construct functionality. It may be that 3 weeks of delayed hydrostatic pressure was of insufficient duration, as previous studies have also suggested that multiple days of hydrostatic pressure may be required to enhance chondrogenesis of bone marrow derived MSCs (Angele et al. 2003; Luo and Seedhom 2007; Elder and Athanasiou 2009). Therefore it would appear that MSCs are

responding differentially to the signals they experience when encapsulated in agarose hydrogels and subjected to either cyclic hydrostatic pressure or dynamic compression. It could be speculated that in the absence of a well developed pericellular matrix that the deviatoric (shape changing) strain within dynamically compressed hydrogels, absent in constructs subjected to hydrostatic pressure, is playing a role in the inhibition of chondrogenesis.

Another unexpected result from this study was the donor dependent response to hydrostatic pressure, with one donor from the main part of the study apparently not responding to this form of mechanical stimulation, despite the fact that MSCs from both donors were expanded and maintained under identical conditions. **A further analysis of MSCs isolated from three additional donors and subjected to hydrostatic pressure for three weeks only was also undertaken. When combined with the results from the first two donors, the data demonstrates that two out of five donors respond to hydrostatic pressure by week 3 as quantified by an increase in collagen synthesis.** There is significant variability in the reported response of MSCs to hydrostatic pressure in the literature, from increases in cartilage-specific gene expression and matrix accumulation (Angele et al. 2003; Miyanishi et al. 2006; Miyanishi et al. 2006; Wagner et al. 2008), to transient increases in Sox-9 expression but no subsequent increases in aggrecan or collagen II mRNA expression (Finger et al. 2007), to no effect on chondrogenic gene expression or matrix accumulation (Zeiter et al. 2009). The finding in this study that the response to loading is donor dependent may in part explain some of this variability. **Perhaps this result is to be expected given the well documented variability in the response of MSCs from different donors to cytokine induced chondrogenic differentiation (Cicione et al. 2010), and the fact that animal model studies investigating mechanically induced chondrogenesis *in vivo* often report dramatic donor dependant response to loading (Khayyeri et al. ; Tägil and Aspenberg 1999; De Rooij et al. 2001). Further studies**

are required to better understand donor variability in the response to mechanical signals that clearly cannot be determined based on an analysis of only a small number of donors. For experiments where it is not practical to run multiple replicates using cells from multiple donors, an alternative approach may be to pool MSCs from multiple donors to create a ‘superlot’, which will provide information on the average response of a sample of a given population to a certain stimulus. Of course, the inherent limitation of such an approach is that it will not provide any information on the variability in the response to that given stimulus within the population.

In the case of donor 2, where CHP increased extracellular matrix accumulation, an interesting observation was the finding that both collagen and GAG content only increased in the core of the construct compared to the annulus. This is despite the fact that theoretically each MSC within the construct should experience the same magnitude of hydrostatic pressure. Previous studies have reported increased matrix condensation in chondrogenic aggregates subjected to hydrostatic pressure (Miyanishi et al. 2006; Miyanishi et al. 2006). Hydrostatic pressure may be playing a similar role here, resulting in compaction of extracellular matrix into the core of the cell seeded hydrogel (Fig. 4). An alternative explanation is that hydrostatic pressure acts synergistically with certain magnitudes of oxygen tension or nutrient/growth factor concentrations that are known to vary spatially within cell seeded constructs. For example, oxygen tension is lower within the core of MSC seeded constructs (Demol et al. 2011). Oxygen tension is a key regulator of MSC chondrogenesis within agarose hydrogels (Buckley et al. 2010; Meyer et al. 2010), and previous studies have shown that hydrostatic pressure and low oxygen tension act synergistically to promote chondrogenesis (Hansen et al. 2001). If hydrostatic pressure as a chondrogenic stimulus is more potent at certain levels of biochemical cues, it may also explain some of the discrepancy in the reported response of MSCs to hydrostatic pressure

described in the literature. This is because different studies typically use different scaffold materials and geometries, as well as different cell seeding densities, which will all lead to altered gradients in the regulatory factors throughout the developing tissues. A third explanation for the spatial response of MSCs to hydrostatic pressure is that it depends on local levels of ECM accumulation and associated cell-matrix interactions. MSCs do not directly adhere to agarose, rather they are encapsulated within it, only adhering to their own pericellular matrix once it is synthesised. Both collagen and GAG accumulation were greater in the core, the region of the construct where hydrostatic pressure enhanced matrix accumulation, possibly suggesting that certain levels of extracellular matrix may need to be secreted into the pericellular space before an anabolic response to this type of loading occurs.

Hydrostatic pressure was observed to increase the dynamic modulus but not the equilibrium Young's modulus for donor 2, despite the fact that CHP increased both GAG and collagen accumulation by day 42. The fact that CHP had a more pronounced effect on collagen as opposed to GAG content may partially explain this result, as the short-term dynamic properties are strongly dependent on the collagen network (Kiviranta et al. 2006; Julkunen et al. 2008; Julkunen et al. 2009). The formation of a more inhomogeneous tissue with the application of CHP may also contribute to this finding (specifically the formation of an annular ring around the periphery of the CHP constructs staining weakly for sGAG, see Fig. 4), as heterogeneity of an engineered tissue has been shown to influence the equilibrium modulus values obtained from compression tests that assume construct homogeneity (Kelly and Prendergast 2004).

In conclusion, we have demonstrated that cyclic hydrostatic pressure can be used to enhance the functional properties of cartilaginous tissues engineered using bone marrow derived MSCs isolated from donors that display an anabolic response to loading. The application of hydrostatic pressure can result in a two-fold increase of the dynamic modulus

of the tissue compared to FS controls. While promising, it should be noted that these values are still an order of magnitude lower than that of normal articular cartilage, so clearly further work is required to optimise the environmental cues to which MSCs are exposed to in order to engineer truly mechanically functional cartilaginous grafts. At one level this will involve identifying the most appropriate isolation and expansion conditions for a given source of MSCs, as well as selecting the most appropriate pro-chondrogenic scaffold matrix. At another level it will involve determining the optimal type, magnitude and temporal application of chondrogenic growth factors and biophysical signals, and understanding how they interact, with the aim of promoting cartilage-specific extracellular matrix production and organization. Only through careful consideration of all of these factors can we engineer an environment that leads to the development of cartilaginous grafts with near native mechanical properties.

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Figure Legends

Fig. 1. (a) Schematic of hydrostatic pressure bioreactor. (b) Experimental design. For the first three weeks of the experiment, all constructs were supplemented with TGF- β 3. At day 21, TGF- β 3 was withdrawn from selected experimental arms. MSC seeded constructs were subjected to either continuous cyclic hydrostatic pressure (CHP) from day 0 or delayed cyclic hydrostatic pressure (DHP) from day 21. (FS: Free swelling constructs).

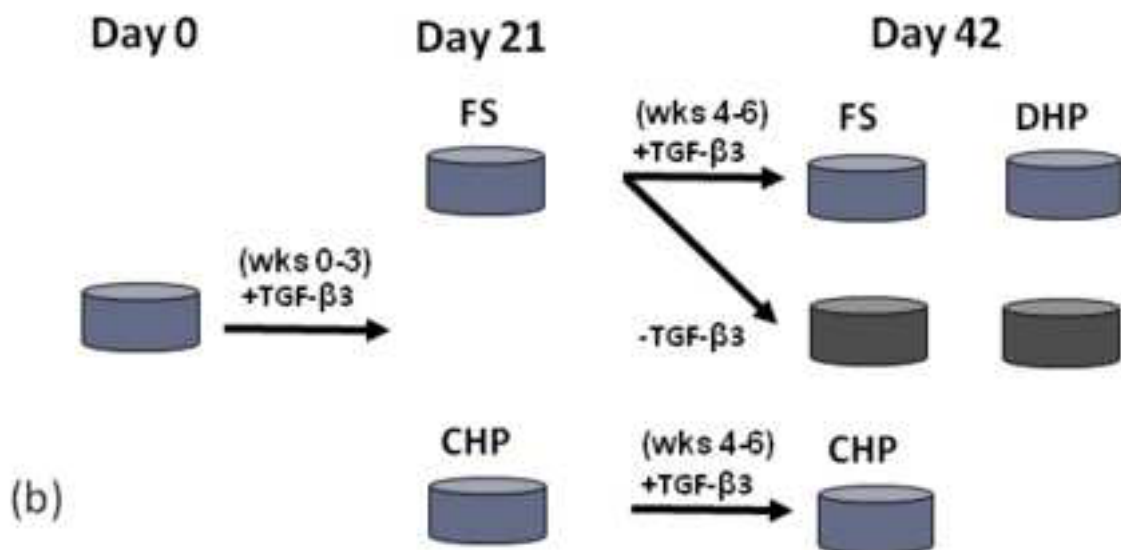
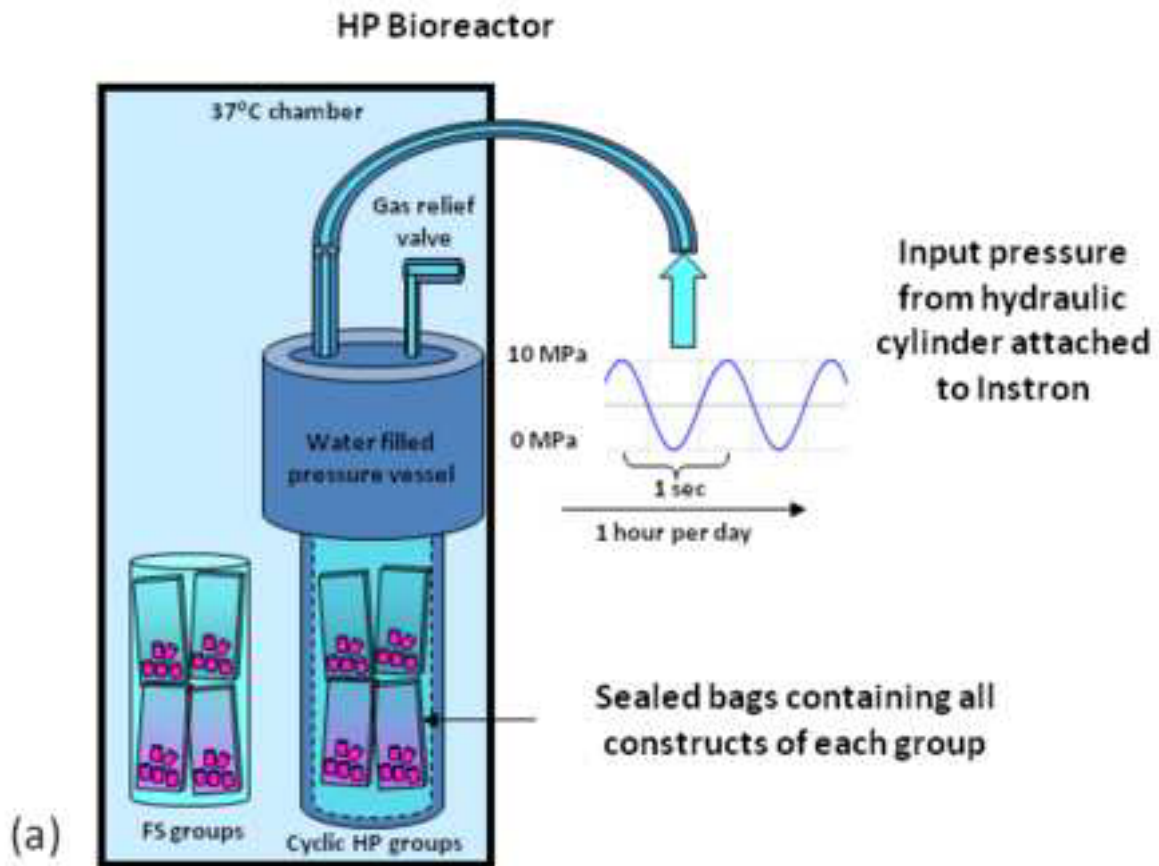
Fig. 2. GAG and collagen accumulation within MSC seeded constructs subjected to either continuous cyclic hydrostatic pressure (CHP) or delayed cyclic hydrostatic pressure (DHP) for (a) donor 1 and (b) donor 2. TGF- β 3 was either supplemented to the media for the duration of the experiment (+ groups), or was withdrawn from the media after 21 days (- groups). * Indicates significant difference between CHP and both free swelling (FS) and DHP ($p < 0.01$). ! Indicates withdrawal of TGF- β 3 had a significant effect on both collagen and GAG accumulation compared to comparable group continuously supplemented with TGF- β 3 ($p < 0.01$).

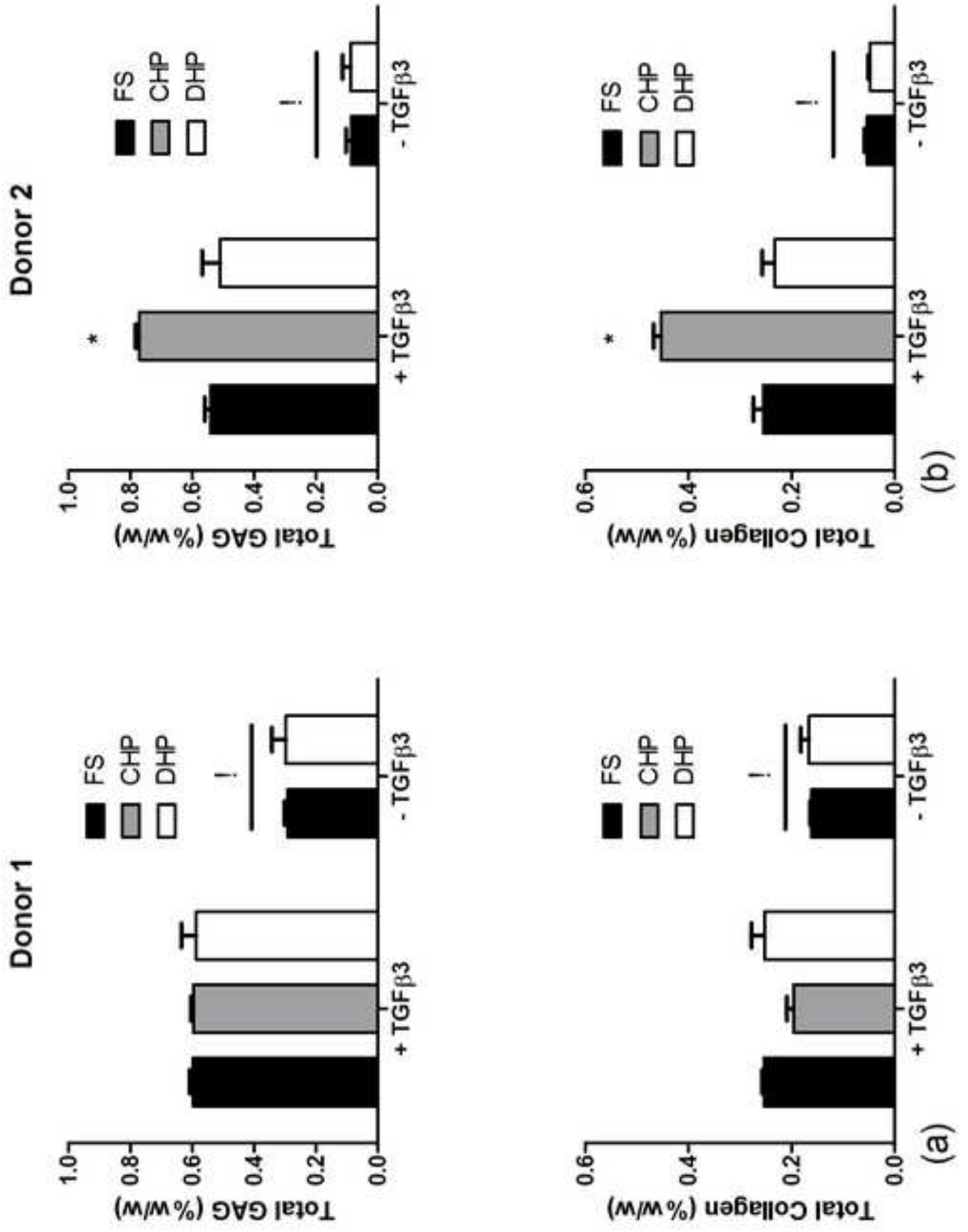
Fig. 3. Spatial accumulation of (a) GAG and (b) collagen within MSC seeded constructs subjected to either continuous cyclic hydrostatic pressure (CHP) or delayed cyclic hydrostatic pressure (DHP) for donor 1. Matrix accumulation was measured separately in the core and annulus of each construct. * Indicates significant difference between CHP and free swelling (FS) ($p < 0.01$). 'a' indicates significant difference ($p < 0.001$) between corresponding core and annuli. 'b' indicates significant difference ($p < 0.05$) between corresponding core and annuli.

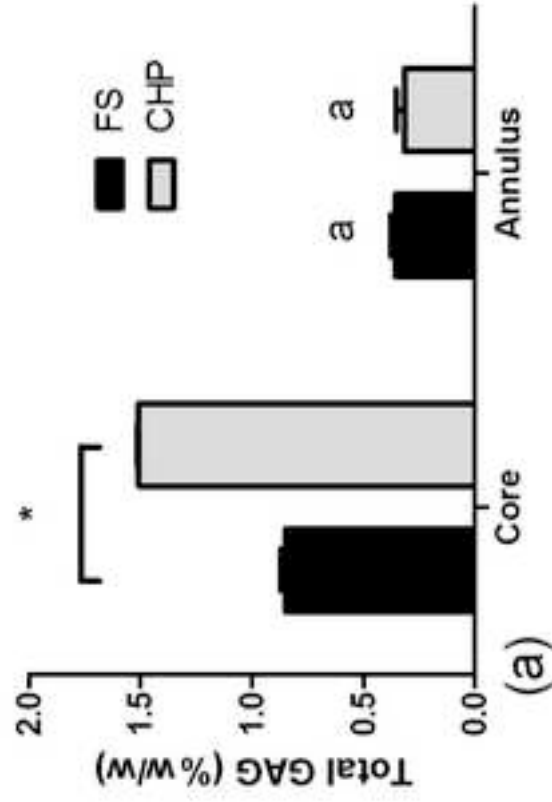
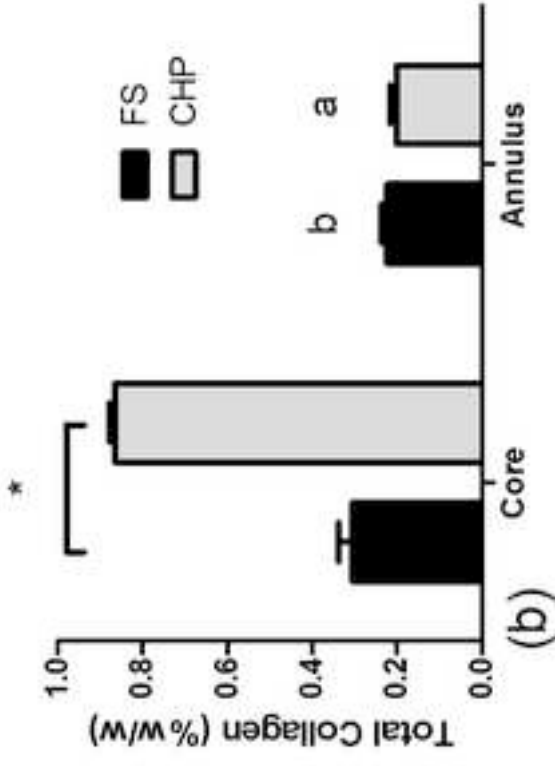
Fig. 4. Alcian blue staining for sulphated proteoglycan and picro-sirius red staining for total collagen for donor 2 (TGF- β 3+ group) at day 42. (FS: Free swelling constructs; CHP: Cyclic hydrostatic pressure; DHP: Delayed cyclic hydrostatic pressure).

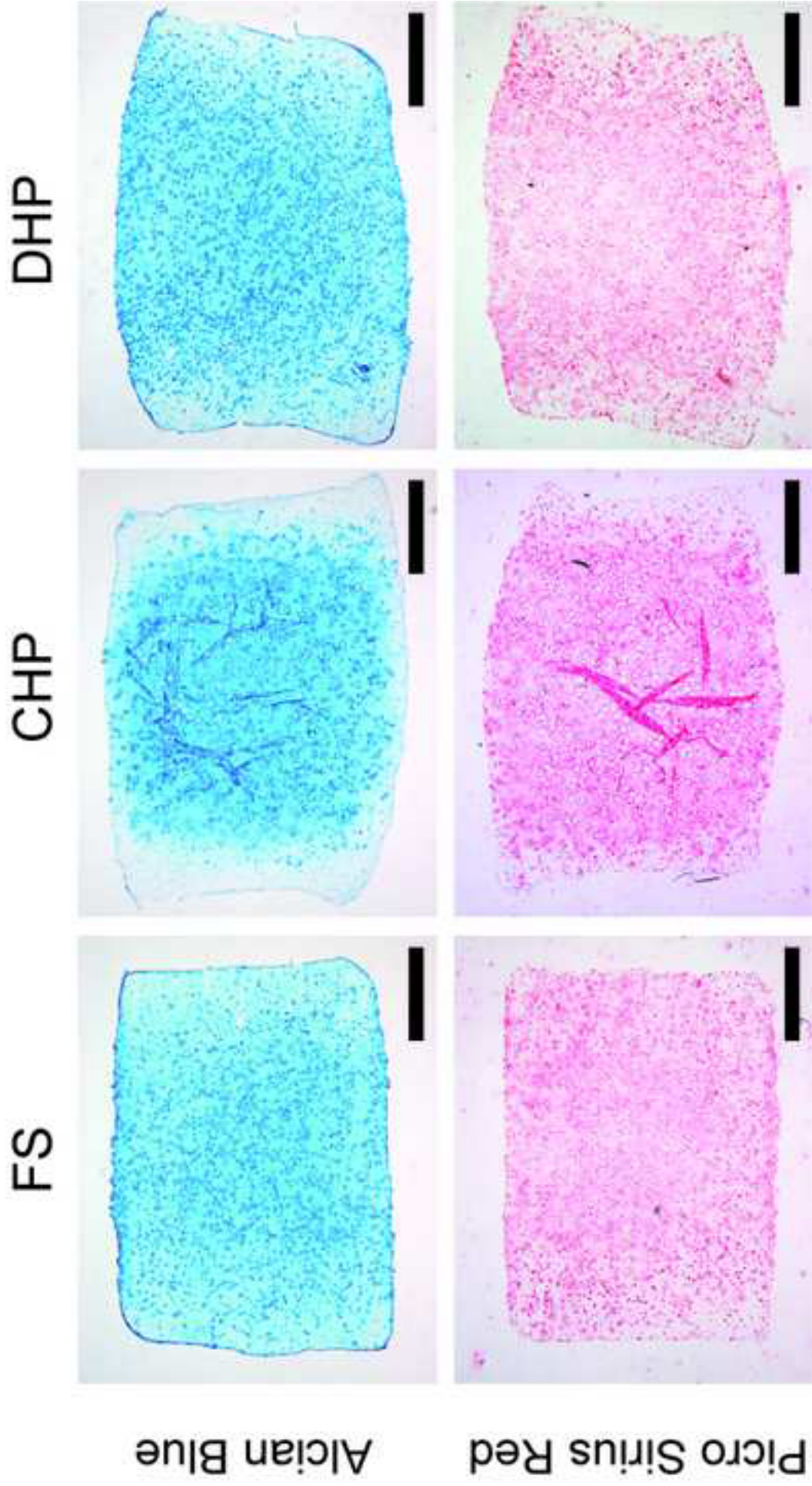
Fig. 5. (a) Equilibrium Young's modulus and (b) Dynamic Modulus for MSC seeded constructs subjected to either continuous cyclic hydrostatic pressure (CHP) or delayed cyclic hydrostatic pressure (DHP) for donor 2. * Indicates significant difference between CHP and free swelling (FS) ($p < 0.001$). 'a' indicates significant difference ($p < 0.01$) between day 21 and 42 for a given culture condition. 'b' indicates significant difference ($p < 0.001$) between day 21 and 42 for a given culture condition.

Figure 1
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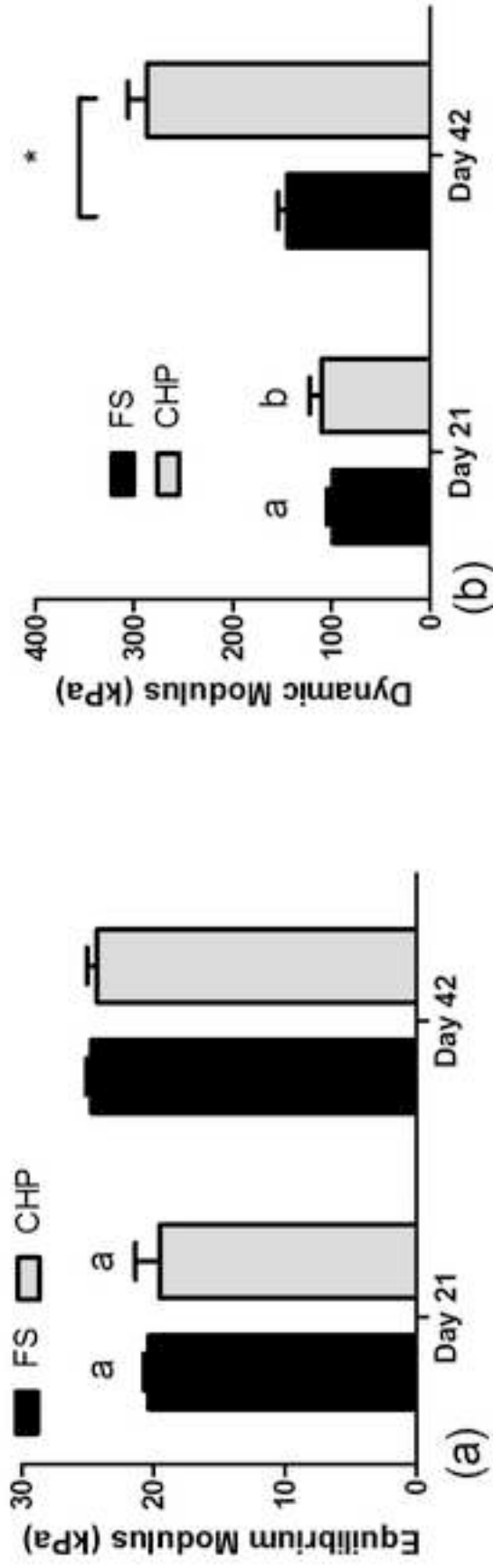


Figure 5
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