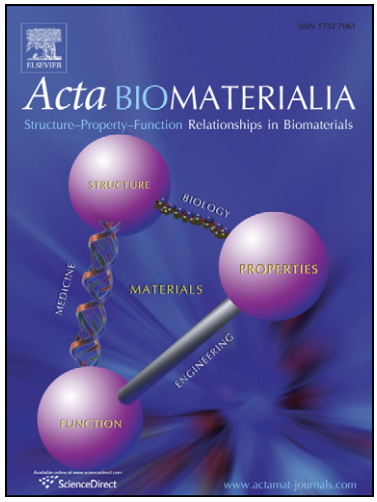

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Substrate stiffness and contractile behaviour modulate the functional maturation of osteoblasts on a collagen GAG scaffold

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Abstract:

Anchorage dependent cells respond to mechanical and physical properties of biomaterials. One such cue is a material's mechanical stiffness. We compared the osteogenic potential of collagen glycosaminoglycan (CG) scaffolds with varying stiffness up to 6 weeks in culture. The mechanical stiffness of CG scaffolds were varied by crosslinking via physical (dehydrothermal [DHT]) and chemical, 1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide [EDAC] and glutaraldehyde [GLUT]) methods. Results showed that all CG substrates allowed for cellular attachment, infiltration and osteogenic differentiation. CG scaffolds treated with EDAC and GLUT, were mechanically stiffer, retained their original scaffold structure and resisted cellular contraction. Consequently they facilitated a 2-fold greater cell number probably due to pore architecture being maintained allowing for improved diffusion of nutrients. On the other hand, the less stiff substrates crosslinked with DHT allowed for increased cell-mediated scaffold contraction; contracting by 70% following 6 weeks ($p<0.01$) of culture. This reduction in scaffold area resulted in cells reaching the centre of the scaffold quicker up to 4 weeks; however, at 6 weeks all scaffolds showed similar levels of cellular infiltration with higher cell numbers found on the stiffer EDAC and GLUT– treated scaffolds. Analysis of osteogenesis showed, that scaffolds crosslinked with DHT expressed higher levels of the late stage bone formation markers osteopontin and osteocalcin ($p<0.01$) and increased levels of mineralisation. In conclusion, the more compliant CG scaffolds allowed for cell-mediated contraction and supported a greater level osteogenic maturation of MC3T3 cells while the stiffer, non contractible scaffolds resulted in lower levels of cell maturation but higher cell numbers on the scaffold. Therefore, we find scaffold stiffness has different effects on differentiation and cell number whereby the increased cell-mediated contraction facilitated by the less stiff scaffolds positively modulates osteoblast differentiation while reducing cell numbers.

1. Introduction

Tissue engineering strives to provide solutions to create tissue *in vitro*, by culturing cells on supportive biomaterials (scaffolds) which act as a template for tissue formation. Scaffold properties such as pore structure, mechanical strength and degradation properties play important roles in cellular growth and function [1, 2]. One such property is scaffold stiffness and has been previously shown to

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affect cellular adhesion proliferation, infiltration and phagocytic cellular processes [3]. This is because cells respond to substrate stiffness via mechanoregulatory processes; osteoblasts are one cell type that are particularly sensitive to mechanical changes in their extracellular environment, which in turn effects their behavior [4].

Recently, interest has been drawn towards the mechanisms that are important for cell-substrate adhesion. *Hapotaxis* is a substrate adhesion model which refers to the tendency of cells to migrate along gradients of extracellular matrix (ECM) adhesion ligand density. Similarly, durotaxis refers to the tendency of cells to migrate along gradients of ECM stiffness [5]. Not all cells respond to substrate stiffness in the same way however, mesenchymal stem cells (MSCs), fibroblasts and epithelial cells show increased cell spreading, adhesion and proliferation on stiffer scaffolds. In 2D substrate studies it has been shown that a relationship exists between stiffness and cell differentiation; where soft substrates were shown to compel a neurogenic lineage whereas stiffer bone-like substrates (>34kPa) commit to osteogenic lineages [6, 7]. Other studies have shown that more compliant substrates appear to promote cellular differentiation over cell proliferation and motility e.g. MSC neural differentiation has been shown to be best at a low substrate stiffness of less than 7 kPa; stiffness levels higher than this resulted in an increased level of proliferation [3]. However, to date no long term 3D osteogenic studies have examined this relationship.

The collagen glycosaminoglycan (CG) scaffold is a highly porous (99.9%) sponge which exhibits excellent bioactivity, low antigenicity, biodegradability and high ligand density [8, 9]. It has been successfully used in dermal and neuronal regeneration [8, 10-13]. An important property of these scaffolds is their ability to contract during in vitro culture [12, 14-17]. Contraction is often clinically viewed as a potential limitation of a scaffold's properties, linked with structural instability within a defect. However, in vitro, contraction has been linked with the production and maintenance of ECM and may benefit tissue formation [14]. As a result, these contractible CG scaffolds are mechanoactive and have been shown to be beneficial for chondrogenesis and nerve wound healing but have not been assessed for long term osteogenesis [12, 18, 19].

A scaffold's stiffness and ability to contract can be controlled by crosslinking methods [5, 20, 21]. Crosslinking strengthens substrates by introducing extra molecular bonds; in collagen for example this can increase stiffness by restricting collagen fibres from sliding over each other during stress. Different crosslinking methods exist and are classified into being either physical or chemical. Dehydrothermal (DHT) treatment is a physical cross-linking process which removes water molecules and creates cross-links by esterification and amide formation [22]. Glutaraldehyde (GLUT) is chemical crosslinker which has traditionally been used for reducing the antigenicity of bioprosthesis since the late 1960s and has been shown to be an effective cross-linker of collagen [23, 24]. It crosslinks collagen by bridging amine groups between adjacent polypeptide chains and has been shown to provide greater fibroblast infiltration and vascularisation [25]. GLUT-treated materials often have a tendency to calcify however, residual GLUT may also restrict cell growth and viability [18]. EDAC is a carbodiimide which acts as a catalyst in the presence of N-hydroxysuccinimide (NHS) and forms bonds between collagen fibres and glycosaminoglycans in CG scaffolds, it is not incorporated itself into the collagen scaffold but is converted to 1-ethyl-3-(3-dimethyl aminopropyl)-urea. Increased substrate stiffness can also slow down the overall degradation of a material thus improving the structural integrity of the scaffold.

To date however, it is not yet known how substrate stiffness and the ability of a scaffold to contract may affect *in vitro* bone formation and osteogenesis. Therefore, the objective of this study was to compare the effects of substrate stiffness and contractile behaviour on osteogenesis within 3D CG scaffolds. This study examines the effect of DHT, EDAC and GLUT-treated CG scaffolds on long term osteogenesis and mineralization using the mechanosensitive MC3T3 pre-osteoblast cell line which has been previously shown to sequentially develop into mature osteogenic mineralizing osteoblasts *in vitro* in the presence of ascorbate and a phosphate donor. Recently, these cells have also been shown to express the contractile protein smooth muscle actin-α (SMA-α) and can contract collagen matrices [12]. We examine osteogenesis by investigating early, mid and late stage bone formation markers and also investigate cell contractile related gene expression of MC3T3 cells on CG scaffolds with varying substrate stiffness.

2. Materials and methods

2.1 Scaffold fabrication

Collagen glycosaminoglycan (CG) scaffolds were fabricated using a lyophilisation technique as described previously [1]. 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) with chondroitin-6 sulfate (0.05%wt) (Sigma-Aldrich, Germany). After blending, (Ultra Turrax T18, NC) the slurry was degassed prior to lyophilisation. The slurry was freeze dried at a cooling rate of 0.9^{o}C/min and a final freezing temperature of -40^{o}C, 50mTorr for 24 hours. This produced CG sheets (thickness = 3.5mm; mean pore diameter = 96μm; porosity =99.5%).

2.2 CG scaffold cross-linking

Following fabrication CG scaffolds were aseptically cut to size (10x10mm) prior to physical or chemical crosslinking. For physical crosslinking via Dehydrothermal (DHT) treatment, scaffolds were placed in an aluminium foil bag in a vacuum oven (Fisher IsoTemp 201, Fisher Scientific, Boston, MA) at 50mTorr for 24 hours at 105^{o}C [26, 27].

Chemical crosslinking using glutaraldehyde (GLUT) (Sigma-Aldrich, Germany) was carried out by first pre-hydrating scaffolds in phosphate buffered saline (PBS) at 4^{o}C for 24 hours. Each pre-hydrated scaffold was immersed in a 2ml PBS solution containing 0.125g GLUT per gram of collagen and crosslinked for 24 hours at room temperature, scaffolds were rinsed several times using sterile PBS to remove residual GLUT.

1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide (EDAC) (Sigma-Aldrich, Germany cross-linking was carried out by first pre-hydrating scaffolds PBS at 4^{o}C for 24 hours before adding 2ml of filtered solution containing 6mM EDAC per gram of collagen and 2mol N-hydroxysuccinimide (NHS) (Sigma-Aldrich, Germany) in dH_2O for 2 hours at room temperature. Scaffolds were rinsed several times with sterile PBS prior to cell seeding [20].

2.3 Cell culture and cell seeding

MC3T3 pre-osteoblastic cells were cultured under standard conditions (5% CO_2, 37^{o}C). Cells were routinely grown to 80% confluency in T175 culture flasks (Sarstedt, Ireland) containing culture

media α-MEM (Sigma-Aldrich, Germany), 10% FBS (Sigma-Aldrich, Germany), 2% penicillin/streptomycin (Sigma-Aldrich, Germany) and 1% L-Glutamine (Sigma-Aldrich, Germany). $1x10^6$ MC3T3 pre-osteoblasts were seeded on both sides of DHT, EDAC and GLUT-treated scaffolds (10x10x3mm) [28]. Constructs were cultured under osteogenic conditions i.e. standard media supplemented with 50µg/ml ascorbic acid and 10mM β-glycerolphosphate (Sigma-Aldrich, Germany) for 2, 4 or 6 weeks.

2.4 Scaffold contraction and mechanical testing

Scaffold contraction was measured at 2, 4 and 6 weeks of culture, as the percentage change in diameter over time measured using a Vernier caliper (n=3).

Scaffolds were analysed for mechanical stiffness using a Z050 mechanical testing machine (Z050, Zwick/Reoll) fitted with a 5-N load cell at 0 and 6 weeks of culture (n=6). Unconfined wet compression testing was performed on cell seeded constructs that were immersed in PBS and tested at a rate of 10% strain/minute (n=3). The modulus was calculated from the slope of the stress-strain curve over the range 2-5% strain.

2.5 Cellular analysis

Cell number was assessed using the Hoechst 33258 DNA assay (Sigma-Aldrich, Germany). Constructs (n=3) were flash frozen in liquid nitrogen at each time point and stored at -80^{o}C. DNA was isolated from the constructs by homogenisation in RLT lysis buffer (Qiagen, UK) using a rotor-stator homogeniser (Omni International, Marietta, GA). 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.

Metabolic activity was assessed using the alamar blue assay. Alamar blue is a non toxic dye which is metabolized by viable cells resulting in a colour change. Specifically, media surrounding scaffolds was removed and replenished with that containing 10% alamar blue dye (Bioscience, Ireland) at days 21, 28 and 35 (n=6). Constructs 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.

2.6 Histological and mineralisation staining

Hematoxylin & Eosin (H&E) Staining: constructs (n=3) were fixed in 4% paraformaldehyde for 30 minutes. Dehydration and paraffin embedding was carried out using an automated tissue processor (ASP300, Leica) and cut into 10µm sections on a microtome (RM2255, Leica). Standard H&E staining was carried out on deparaffinised sections. Images were captured on a digital microscope (7500 systems software NIS Elements, Nikon).

Alizarin Red Staining: 10µm sections of scaffold were deparaffinised using xylene, rehydrated and stained with a 2% Alizarin red (Sigma-Aldrich, Germany) aqueous (pH 4.2) filtered solution for 2 minutes to stain calcium deposits. Sections were rinsed several times with distilled water dehydrated in xylene and mounted with DPX.

2.7 Gene expression analysis

Constructs (n=3) were flash frozen in liquid nitrogen at each time point and stored at -80^{o}C. RNA was isolated from the constructs by homogenisation in RLT lysis buffer (Qiagen, UK) using a rotor-stator homogeniser (Omni International, Marietta, GA). 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 quantified using a spectrophotometer abs 260nm. Following RNA extraction, trace genomic DNA was removed and the RNA sample was reverse transcribed using 400ng total RNA with an RT kit (QuantiTect RT Kit, Qiagen, UK) according to the manufacturer's instructions. Real time RT-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).

Analysis of bone formation marker expression was carried out, specifically, for early markers collagen type I and alkaline phosphatase mid/late stage markers, osteopontin and osteocalcin and the contractile marker smooth muscle actin-α (SMA-α). Expression levels were assessed using the relative quantification $\Delta\Delta$Ct method. 18S acted as a house keeping control.

2.8 Statistical analysis

Statistical analysis was determined 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 $p<0.05$.

3. Results

Crosslinking increased the mechanical stiffness of all CG scaffolds compared to the non-crosslinked control (0.4 kPa). Both EDAC and GLUT displayed similar levels of stiffness (EDAC 1.17 ± 0.89 kPa; and GLUT 1.37 ± 1.5 kPa) showing over a 2-fold significant increase over DHT treated scaffolds (0.48 ± 0.45 kPa) ($p<0.01$) (Fig. 1). There was a significant 2.65-fold increase in the stiffness of the DHT scaffold over the 6 week culture period ($p=0.01$); this difference was not observed in either EDAC or GLUT treated CG scaffolds. These stiffer scaffolds resisted substrate contraction retaining their shape and size after 6 weeks of culture with contraction observed only in the DHT groups (Fig. 2). DHT constructs contracted by 50% (from 10mm to 5mm) within 2 weeks of culture, by 6 weeks however, constructs had contracted by 70% (3mm) ($p<0.01$). A statistically significant difference was observed between DHT treatments and GLUT or EDAC treatments at 2, 4 and 6 weeks ($p<0.01$).

Cells remained viable on the scaffolds at all time points irrespective of crosslinking. DNA analysis found a lower cell number with DHT constructs than EDAC or GLUT ($p<0.003$) (Fig. 3a). All constructs remained viable at all time points although a similar trend of both EDAC and GLUT yielding a 2 fold greater viability than DHT scaffolds was observed ($p<0.001$) (Fig. 3b).

Histologically, all scaffolds allowed for MC3T3 cell attachment and infiltration with time (Fig. 4 a-i). At 2 weeks, histological analysis showed a similar level of infiltration for all construct types; the cells were residing mainly on the periphery of the constructs (Fig. 4a-c). The DHT constructs at 4 weeks showed a noticeable visual reduction in porosity as a result of cell mediated contraction (Fig. 4d). At 4

weeks, the EDAC and GLUT treated constructs showed cells predominantly near the surface of the constructs; however, these groups retained a highly porous scaffold structure (Fig. 4 e-f). At 6 weeks, cells were distributed throughout all scaffold types. Alizarin red staining for mineralization was positive for all scaffolds and reflected cellular distribution. EDAC and GLUT scaffolds showed positive staining at the periphery which increased with time as distribution proceeded. EDAC levels of staining increased up to 6 weeks (Fig. 5h) with GLUT showing equivalent levels after 4 weeks (Fig. 5f). However, the most intense staining was observed in the DHT treated groups evident after 2 weeks and peaking at 4 weeks and maintained at 6 weeks (Fig. 5a, d and g).

Expression of osteogenic markers was detected in all scaffolds (Fig. 6). Levels of the early bone formation marker alkaline phosphatase increased with time, with all scaffold treatments showing greatest expression at 6 weeks. This increase was 3 fold higher at 6 weeks than at 2 weeks for both EDAC and GLUT groups ($p<0.005$). Another early stage bone formation marker collagen type-1 showed no significant difference in expression levels over time; however, DHT scaffolds showed a decrease between 2 and 6 weeks. The mid/late stage marker osteopontin increased significantly in DHT-treated scaffolds at 4 weeks ($p<0.001$); this increase was 24 fold higher than EDAC or GLUT at this time point. Similarly, the late stage marker osteocalcin levels increased 6 fold at 4 weeks for DHT ($p<0.001$). DHT scaffolds produced significantly higher levels of osteocalcin than GLUT and EDAC at 4 and 6 weeks ($p<0.05$). A 3.8 fold decrease in the expression levels of the contractile related SMA-α gene was observed from 2 to 4 weeks in DHT groups ($p<0.05$), whereas the non contractile EDAC and GLUT scaffolds maintained higher levels of SMA-α up to 6 weeks (Fig. 7). At 6 weeks DHT gave the lowest expression levels in comparison with EDAC or GLUT which resulted in a 1.2 fold increase and 2.1 fold increase respectively.

4. Discussion

Recent studies have shown that cells can sense differences in 2D matrix elasticity by sensing the effort required to pull against a matrix using stimulated cellular mechano-transducers [29]. Although several forces such as fluid flow, hydrostatic pressures, compression, tension which and cell shape are all important in activating mechanical cues for differentiation, the actual mechanisms involved in activating mechano-transducers to direct a cell towards osteogenic remains under discussion what is more certain is that scaffold properties have also been shown to influence cell behaviour [6, 30]. This study examined the correlation between substrate stiffness, cell-mediated contraction and long term osteogenesis of MC3T3 pre-osteoblasts. Using three established crosslinking methods, DHT, EDAC and GLUT on collagen glycosaminoglycan (CG) scaffolds, the study found that more compliant CG scaffolds allowed for cell-mediated contraction and supported a greater osteogenic maturation of MC3T3 cells, while the stiffer non-contractible scaffolds displayed a lower osteogenic cell phenotype but higher cell numbers. Specifically, DHT treated scaffolds which allowed cell based scaffold contraction improved osteogenic maturation over the stiffer, non-compliant EDAC and GLUT scaffolds which demonstrated increased cell number.

The results demonstrate that there is a significant 2.65-fold increase in the stiffness of the DHT scaffold over the 6 week culture period. We propose that the reasons for this are due to the increased density (reduced porosity) which results from a combination of cell-mediated contraction and increased osteogenesis (ECM deposition & mineralization) in the DHT group. This is validated by the fact that no

significant change in stiffness occurs in the EDAC and GLUT scaffolds over the culture period where lower levels of contraction and osteogenesis were found.

2D substrate studies have shown that matrix elasticity can direct cell lineage; with soft tissue like neuronal tissue cells preferring soft substrate and hard tissues like bone preferring stiffer substrates [5-7]. The results from this study suggest that although substrate stiffness influences cell phenotype and differentiation; a compliant mechanically active scaffold can also direct the maturity of osteoblasts to a well defined mineralizing phenotype.

This study has shown that stiffer non-contractible EDAC and GLUT scaffolds resulted in a 2-fold greater cell number and metabolic viability. This may be attributed to higher scaffold porosity which allowed for greater diffusion of nutrients and waste as well as providing a greater scaffold surface area and therefore higher ligand density for cell adhesion than to DHT scaffolds over time [1, 31].

Previous studies have also shown the importance of pore structure and how pore size can directly regulate osteoblast adhesion and growth [1, 32-34]. This study demonstrated that as the pore structure was not contracted there was a greater surface area available for cell proliferation and cell infiltration thus cells retained in a proliferative stated increasing in number for longer; coupled with it being a stiffer scaffold. Similar findings were reported by Lee et al. (2001) where it was shown that chondrocytes remained in a proliferative state on a stiffer CG scaffolds [18].

On the other hand, scaffold contraction can cause changes in pore size and void space; this results in new mechanical environments which can affect cellular behaviour [21]. Similarly, as the scaffolds contract they can increase cell-cell contact and confluency within pores; an important factor which has been shown to enhance cellular differentiation [35]. We show that although contraction did result in a reduced porosity for cells to proliferate in DHT scaffolds; hence lower cell numbers, there were still sufficient cells present to maintain biological activity and osteoblast maturation; as observed by the greater levels of the mid to late stage bone formation markers osteopontin (24 fold increase) and osteocalcin (6 fold increase) coupled with greater levels mineralization as observed after 4 weeks of culture.

Of particular note was MC3T3 SMA-α expression levels. SMA-α has been shown to be associated with cellular contraction of CG matrices [16]. We found that SMA-α levels were maintained in both EDAC and GLUT however, they decreased in DHT constructs following a contraction phase. Therefore, scaffolds which resisted contraction had consistent levels of SMA-α expression as these cells were still trying to contact the scaffold. In the DHT treated scaffolds, once contraction occurred a decrease in SMA-α expression was observed. This down-regulation in SMA-α following a contraction phase in culture has been previously identified in CG scaffolds [17]. We find the down-regulation of SMA-α coincided with the end of substrate contraction and an increase in the expression levels of late stage osteogenic markers and mineralization. As crosslinking the scaffold does not change the composition of the scaffold we hypothesize that the greater levels of osteogenesis observed in DHT treatments was a result of cell-mediated contraction.

Recent studies have demonstrated the importance of mechano-active poly(D,L-lactic-co-glycolic acid) matrices for tendon remodelling [36]. Similarly, physical properties of scaffolds can exert forces on cells which respond via mechano-sensitive pathways [6]. Therefore, the ability of the scaffold to allow for cellular contraction may be beneficial for differentiation in 3D structures by providing greater cell-cell interactions as well as inducing mechanostimulation [35, 37, 38]. Contractile scaffolds may act

as alternative option than using bioreactors to provide biophysical cell responses on scaffolds during the cultivation of a construct in vitro. They may also be more beneficial than using bioactive (growth factor loaded) scaffolds which may be limited in chemical release due to temporal degradation [4, 39].

Cell-scaffold contraction can have a negative connotation, often linked with collapsed pores and limited nutrient/waste diffusion. However, we show that although contraction changed the pore architecture dramatically coupled with a lower cell number, it did not prevent cellular viability or osteogenesis. In the clinical setting contraction may result in gap formation at a defect site. However, although DHT scaffolds contracted greatly *in vitro*, 50% of the contraction occurred in the first 2 weeks, after which there was only a 10% change in scaffold size. This limit in contraction was reached following osteogenesis and could be a more appropriate time for implantation into a defect at this point.

One caveat of the high contraction rate in DHT scaffolds however, could be possible restriction of angiogenesis once the scaffold has completely contracted. An alternative may be to use a DHT scaffold with a greater initial pore size (~300µm), one large enough to retain a high porosity thus providing nutrient diffusion and vascular in-growth whilst also providing mechano-sensitivity to cells during *in vitro* growth cultures.

There are a number of limitations with the current study. We investigated how the crosslinking techniques used affected the compressive modulus of the scaffolds and thus, how compressive modulus affects cellular behaviour. While numerous previous studies have analysed the mechanical properties of the CG studies scaffolds in detail beyond the scope of this study, it would nonetheless be useful to obtain further information on how the specific crosslinking techniques used affect parameters such as the tensile modulus [40], strut modulus [41] as well as the nonlinear elastic properties [42] and to determine in turn how changes in all of these properties affect individual cell behaviour.

5. Conclusion

In conclusion, the results from this study demonstrate that different crosslinking techniques result in different scaffold stiffness values which modulate the functional maturation of osteoblasts on a CG scaffold. The stiffness of the CG scaffold and its ability to contract displayed a converse effect on cell number and osteoblast differentiation, with the more compliant DHT CG scaffolds which allowed for cell-mediated contraction displayed a greater osteogenic maturation while the stiffer, non contractible scaffolds resulted in a lower osteogenic phenotype but higher cell numbers. Therefore, we find scaffold stiffness has different effects on differentiation and proliferation whereby the increased cell-mediated contraction facilitated by the less stiff scaffolds positively modulates osteoblast differentiation while reducing cell numbers.

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Figure Legends

Fig. 1 Compressive modulus of CG scaffolds crosslinked with DHT, EDAC and GLUT treatments (n=6). Cross-linking increased mechanical stiffness in all groups; with EDAC (1.17 ± 0.89 kPa) and GLUT (1.37 ± 1.5 kPa) showing over a 2-fold significant increase in mechanical stiffness over DHT scaffolds (0.48 ± 0.45 kPa) ($p<0.01$). A 2.65 fold increase in compressive modulus was observed in DHT groups following 6 weeks culture ($p=0.01$) however, no significant change in mechanical stiffness was noted in EDAC or GLUT.

Fig. 2 Construct contraction was expressed as a percent change of diameter from the original size over time. Contraction occurred in DHT treated constructs only; contracting by 50% the original after 2 weeks culture, up to 70% of original size by 6 weeks ($p<0.01$). A statistically significant difference was observed between DHT and GLUT or EDAC treatments at 2, 4 and 6 weeks ($p<0.01$) (n=3).

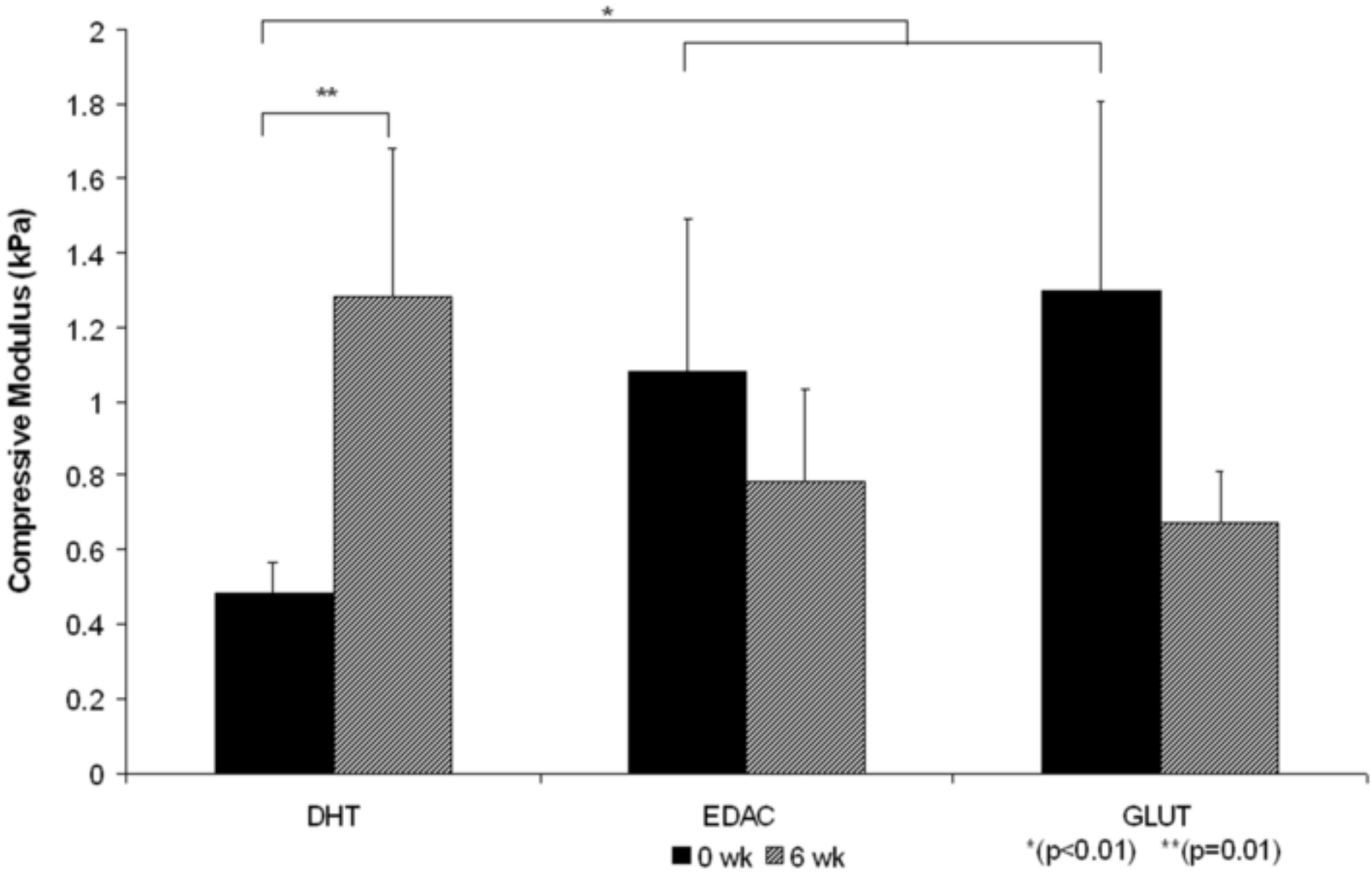
Fig. 3 (a-b) Shows cell viability on CG scaffolds with varying crosslinking methods (n=3); (a) illustrates Hoechst DNA analysis of cell number on CG scaffold over time, which resulted in a lower cell number with DHT treated constructs than EDAC or GLUT treated constructs ($p<0.05$) (b) demonstrated metabolic viability using alamar blue and found a similar trend of both EDAC and GLUT yielding a 2 fold greater viability than DHT scaffolds ($p<0.01$). All constructs remained viable at all time points.

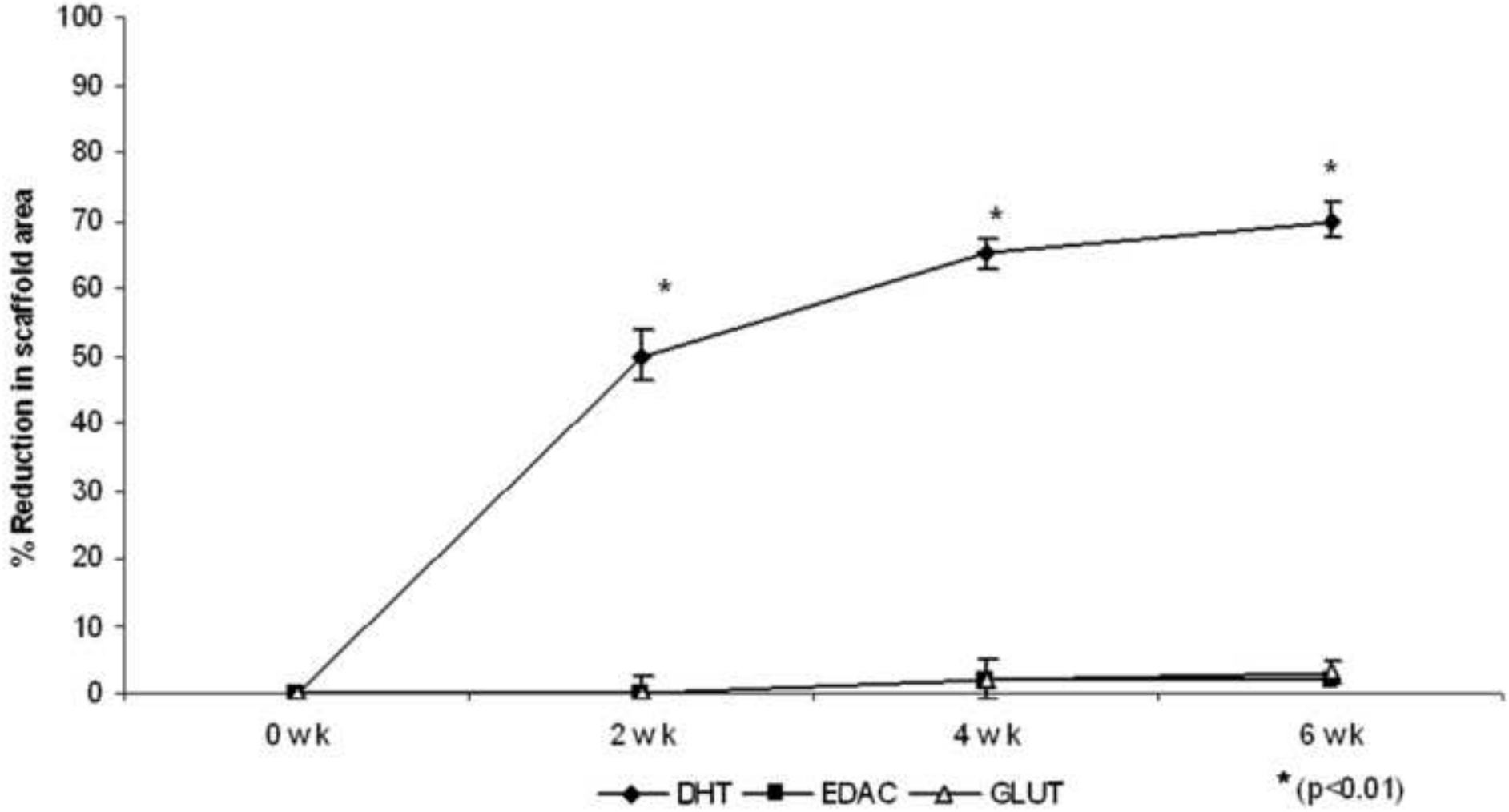
Fig. 4 Histological analysis of cells on scaffolds showed attachment and infiltration of MC3T3 cells throughout all scaffold treatments. DHT scaffolds (a, d, g) showed greatest cell distribution and cell-cell interactions. EDAC (b, e, h) and GLUT (c, f, i) scaffolds showed cells predominately at the edge of the scaffold. Pore structure and integrity remained unchanged in EDAC and GLUT scaffolds; however a reduction in pore size was evident in DHT groups. All sections were taken ~40-50% into the scaffold; sections of 1 scaffold representative of n=3, 100μm scale bar.

Fig. 5 Alizarin red staining for mineralization was positive for all scaffolds; with the most intense staining in DHT (a, d, g) treated groups evident after 2 weeks. Greatest staining reflected the high levels of calcification in the DHT constructs by 4 weeks (d). Staining of EDAC (b, e, h) and GLUT (c, f, i) remains positive at the edge of the scaffold where the cells reside. EDAC showed most staining at 6 weeks (h) with GLUT showing equivalent levels after 4 weeks (f). All sections were taken ~40-50% into the scaffold; sections of 1 scaffold representative of n=3, 500μm scale bar.

Fig. 6 Gene expression levels of osteogenic markers for constructs with varying stiffness. Alkaline phosphatase increased with time in EDAC and GLUT groups; greatest expression levels occurred at 6 weeks ($p<0.005$) (n=3). No differences were observed between groups for collagen type 1. Osteopontin increased at 4 weeks by 24 fold in DHT groups ($p<0.001$). Osteocalcin levels increased 6 fold between 2 and 4 weeks for DHT treated constructs ($p<0.001$). DHT treated constructs produced significantly higher levels of osteocalcin over GLUT and EDAC at 4 and 6 weeks ($p<0.05$).

Fig. 7 Smooth muscle actin alpha (SMA-α) gene expression levels over time (n=3). A 3.8 fold reduction in gene expression was observed in the contracted DHT scaffolds between 2 and 4 weeks ($p<0.05$), non-contractible scaffolds maintained greater levels of expression at all time points. At 6 weeks DHT gave the lowest expression levels in comparison with EDAC (1.2 fold increase) or GLUT (2.1 fold increase).





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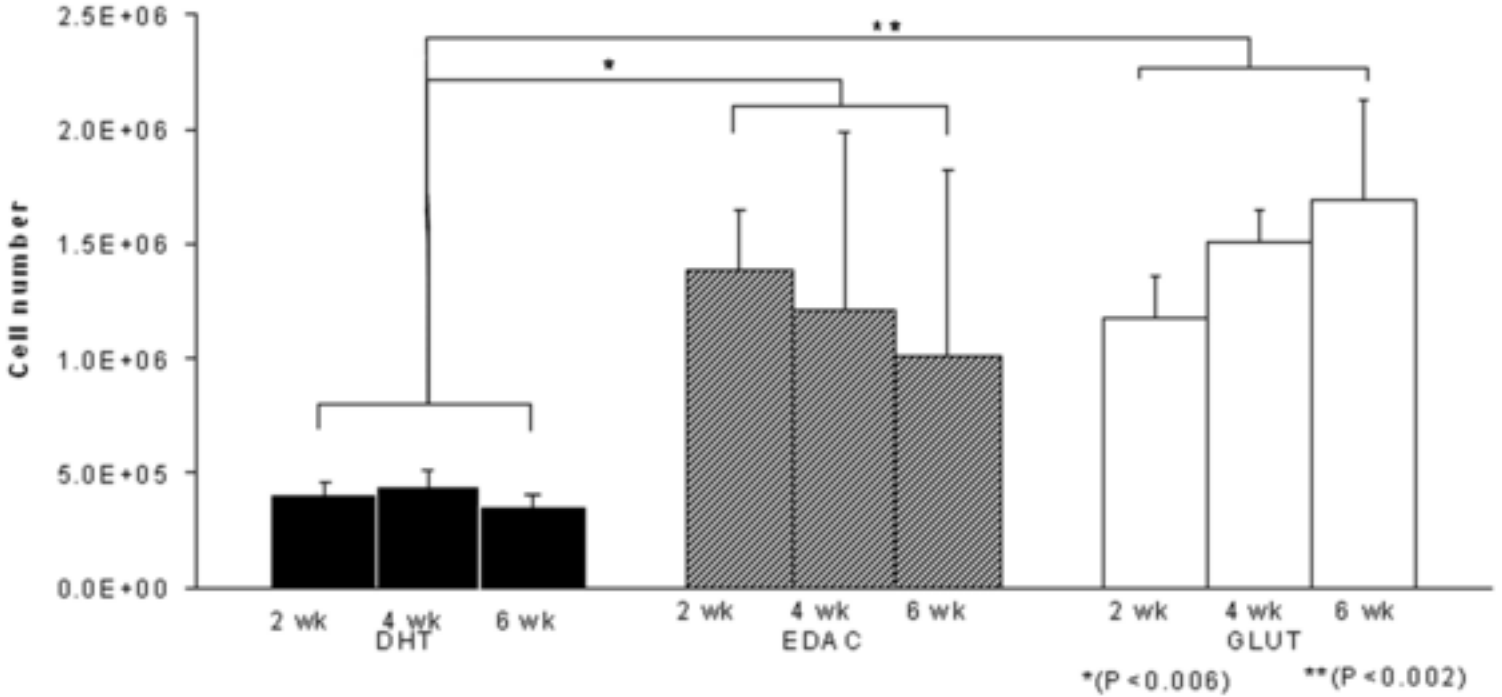

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