# Upregulated sirtuin 1 by miRNA-34a is required for smooth muscle cell differentiation from pluripotent stem cells

Yu, X; Zhang, L; Wen, G; Zhao, H; Luong, LA; Chen, Q; Huang, Y; Zhu, J; Ye, S; Xu, Q; Wang, W; Xiao, Q

**Tissue Engineering and Delivery Systems** **Biotechnology and Bioengineering**

**DOI 10.1002/bit.26273**

# Complex mechanical conditioning of cell-seeded agarose constructs can influence chondrocyte biosynthetic activity†

***Running head:*** *Biaxial loading influences chondrocytic activity*

**E. Di Federico[1,2], J.C. Shelton[1], D.L. Bader[1, 3]**

[1]Medical Engineering Division, School of Engineering and Materials Science, Queen Mary, University of London, London, UK

[2]Department of Bioengineering, Imperial College, London

[3]Faculty of Health Sciences, University of Southampton, UK

Corresponding author

Professor JC Shelton

School of Engineering and Materials Science, Queen Mary, University of London, Mile End Road, London E1 4NS, UK e-mail: j.shelton@qmul.ac.uk

†This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: [10.1002/bit.26273]



**Received October 4, 2016; Revision Received January 16, 2017; Accepted February 7, 2017**

**Abstract**

Articular cartilage with its inherently poor capacity for self-regeneration represents a primary target for tissue engineering strategies, with approaches focusing on the *in vitro* generation of neo-cartilage using chondrocyte-seeded 3D scaffolds subjected to mechanical conditioning. Although uniaxial compression regimens have significantly up-regulated proteoglycan synthesis, their effects on the synthesis of collagen have been modest. Articular cartilage is subjected to shear forces during joint motion. Accordingly, this study utilised an apparatus to apply biaxial loading to chondrocytes seeded within agarose constructs with endplates. The chondrocytes yielded a monotonic increase in proteoglycan synthesis both in free swelling culture up to day 8 and when the constructs were subjected to dynamic compression alone (15% amplitude at a frequency of 1Hz for 48 hours). However, when dynamic shear (10% amplitude at 1 Hz) was superimposed on dynamic compression, total collagen synthesis was also up-regulated, within 3 days of culture, without compromising proteoglycan synthesis. Histological analysis revealed marked collagen deposition around individual chondrocytes. A significant proportion (50%) of collagen was released into the culture medium, suggesting that it had only been partially synthesised in its mature state. The overall biosynthetic activity was enhanced more when the biaxial stimulation was applied in a continuous mode as opposed to intermittent loading. Results of the present study strongly suggest that proteoglycan and collagen synthesis may be triggered by uncoupled mechanosensitive cellular responses. The proposed *in vitro* model and the prescribed conditioning protocols demonstrated that a short pre-culture period is preferable to long free swelling culture condition as it enables a significantly higher up-regulation of collagen. 

**Keywords**: compression, shear, chondrocytes, cartilage, up-regulation, collagen, proteoglycans, bioreactor, agarose.

This article is protected by copyright. All rights reserved

## Introduction

Articular cartilage is a highly organised connective tissue consisting of chondrocytes surrounded by an extracellular matrix (ECM) composed of a proteoglycan gel, enclosed within a complex network of collagen fibres (Bader and Lee, 2000). Its primary role is to provide a bearing surface that can withstand large loads, permitting low friction movement at synovial joints over many decades of use. The interaction of both the solid ECM components critical in providing mechanical competence to support loading experienced during normal joint activities (Langelier and Buschmann 2003; Mow *et al*. 1999), which involves a combination of compressive, shear and tensile loads (Guo *et al*., 2015; Grodzinsky *et al.*, 2000; Sawae et al., 2004; Smith *et al.*, 2000; Frank *et al.*, 2000; Lee and Bader, 1997). Since articular cartilage is avascular it exhibits a very limited capacity to regenerate and to repair (Hunziker, 1999) and thus changes in the ECM composition can dramatically alter function can ultimately result in its total degradation (Mauck *et al.* 2000). Accordingly, a number of clinical strategies have been established to repair partial thickness cartilage defects, each of which provides the delivery of metabolically active cells to the defect site. However such interventions rarely provide long term functional stability, thereby motivating a number of tissue engineering strategies incorporating chondrocyte-seeded scaffolds for the *in vitro* development of neo-cartilage tissues prior to implantation (Buckwalter and Mankin, 1998). Although several studies have reported the temporal development of neo-cartilagenous tissue, which resemble the morphological and biochemical appearance of the native cartilage, although its functional competence has not been demonstrated (Schöne et al., 2016; Johnstone *et al.*, 2013; Ng *et al.*, 2009; Freyria et al. 2005; Joen et al. 2013).

Mechanical conditioning has often been introduced to enhance biosynthetic activity of chondrocytes within 3D constructs. These studies, involving animal models (Helminen *et al.*, 1992 Kurz *et al.*, 2001), tissue explants (Burton-Wurster *et al.*, 1993) and cell culture models

(Chowdhury *et al.*, 2008; Knight *et al.*, 2006; Lee & Bader, 1997), have each demonstrated that mechanical stimuli influence chondrocyte metabolism and consequently the temporal evolution of neo-cartilage structure and composition. Typically, the *in vitro* studies employed uniaxial compression to modulate chondrocytes, either in monolayer or 3D constructs (Chowdhury *et al.*, 2008; Lee and Bader, 1997; Mauck *et al.*, 2000; Kisiday *et al.*, 2004; Davisson *et al.*, 2002), and reported an up-regulation of proteoglycans with minimal associated collagen. A few reports have suggested that the superposition of direct tissue shear on uniaxial compression (Wimmer *et al.*, 2009; Guo *et al*., 2015) can enhance the temporal production of collagen in culture. In addition, the application of intermittent cyclic shear has been reported (Jin *et al.*, 2001; Waldman *et al.*, 2003) to produce matrix deformation that leads to a significant change in both the composition and mechanical properties of neo-cartilage formed *in vitro*. However, the nature of the application of shear stress varies considerably (Frank *et al.*, 2000; Bian *et al.*, 2010). In a recent paper the authors described a system which can apply well-defined biaxial loading to chondrocyte-agarose constructs (Di Federico *et al.*, 2014). It highlighted the novel design of constructs with endplates which could withstand the application of long-term biaxial loading.

The study tests the hypothesis that the imposition of shear superimposed on direct compression could influence the synthesis of both solid ECM constituents, using a model of isolated chondrocytes seeded in agarose constructs (Ofek *et al.*, 2010; Gooch *et al.*, 2001). Initially a free-swelling study was conducted to determine the suitability of the proposed culture model. Subsequently constructs were subjected to both continuous or intermittent loading regimens. Cell metabolism was investigated at various times in culture by the quantitative evaluation of the newly synthesised GAG and collagen, both retained within the construct and released into the culture medium. The histological appearance of the chondrocyte pericellular matrix (PCM) and its temporal evolution was also evaluated.

## Materials and Methods

### Sample preparation

The agarose-chondrocyte constructs were prepared using a series of established procedures (Lee and Bader, 1997). To review briefly, the metacarpophalangeal joints of the front feet of 18-month-old steers were opened aseptically, within 3 hours of slaughter at a local abattoir. Full-depth slices of cartilage were removed from the proximal surface and slices from at least five separate joints were pooled for each experiment. The slices were finely diced and incubated at 37°C for 1 hour in 10 ml of DMEM supplemented with 16.1% foetal calf serum (FCS) plus 34 U/ml pronase (Sigma-Aldrich, Poole, England). The supernatant was removed and replaced with 30 ml of DMEM + 16.1% FCS plus 100 U/ml of collagenase type IA (Sigma-Aldrich, Poole, England) and incubated at 37°C for 16 hours. The supernatants containing released chondrocytes were passed through a sieve with a pore size of 70 μm (Falcon, Oxford, England) to remove undigested tissue, washed twice in DMEM + 16.1% FCS, and resuspended at a concentration of $8 \times 10^6$ cells/ml. The chondrocyte suspension was added to an equal volume of 6% agarose (type VII; Sigma-Aldrich, Poole, UK) in Earle's balanced salt solution (EBSS, Sigma-Aldrich, Poole, UK) to obtain a final concentration of $4 \times 10^6$ cells/ml in 3%w/v agarose. Cell viability was determined using the trypan-blue exclusion test.

The agarose-chondrocyte suspension was poured in two different custom-made moulds to obtain both cylindrical constructs (Ø5 mm x 5 mm) and constructs provided with nylon endplates (Di Federico *et al.*, 2014) and allowed to gel at 4°C for 20 minutes (Fig. 1c). A total of 8 experiments were performed for the free swelling study, while the continuous and intermittent mechanical loading involved 5 and 3 experiments, respectively.

**Free Swelling study**

Chondrocyte-seeded agarose cylindrical constructs and constructs with endplates were individually inserted in 24-well culture plates (Corning Costar, Sigma-Aldrich, Poole, UK) and pre-cultured in 1 mL of DMEM supplemented with 16.1% foetal calf serum at 37°C/5% $CO_2$ (Heywood et al., 2004) for approximately 16 hours. Representative samples and corresponding culture medium were removed from culture at the end of this equilibration period, representing time-zero (T0) for all experiments and these samples were stored at -20 ˚C prior to subsequent analysis, performed 2 days after the end of each experiment i.e. 10 days following isolation.

The remaining constructs were conditioned in free-swelling culture (FSC for cylindrical constructs and FSE for constructs with nylon endplates) in 24 well plates for up to 8 days with medium changes every other day. At various time intervals chondrocyte viability of representative constructs was assessed. On days 3, 6 and 8 both constructs and corresponding culture medium, were collected and separately stored in 1.5 mL Eppendorf tubes at -20 ˚C. In the case of constructs with endplates, only the cylindrical central section (Fig. 1c), was removed and subjected to metabolic and histological studies. The effects of culture time on the temporal profiles of DNA, GAG and collagen production with particular reference to that retained within both sets of constructs were evaluated. The temporal evolution of the PCM around individual chondrocytes was observed by analysing their histological and immuno-histological appearance using Haematoxylin and Eosin (HE), Picrosirius Red and keratan sulphate antibodies (Novus Biological, Cambridge, UK).

**Mechanical Conditioning study**

A cell-straining apparatus, developed by the authors (Di Federico *et al.*, 2014), was employed to apply dynamic compressive and shear strains simultaneously to constructs with endplates (Fig. 1a). Twelve of these were centered individually in the outer wells of a 24 well culture

plate, which itself was positioned within a sterile Perspex chamber such that each construct was immediately beneath a corresponding PTFE cup-shaped loading pin (Fig. 1b). One ml of DMEM + 16.1% FCS was introduced into each well. In this position, the constructs were considered to be subjected to a tare strain resulting from the pin mass, referred to as tare load. The set-up enables the prescription of biaxial loading regimens to chondrocytes-seeded agarose constructs. Shear strain was applied within the entire construct using a motorised linear stage. A porous viscoelastic biphasic FE model of the constructs with embedded chondrocytes, developed to determine the cells deformations within the agarose gel (Di Federico *et al.*, 2015) revealed that chondrocytes in the centre of the construct with endplates deform slightly more compared to cells in the top and bottom sections, supporting a previous study (Sawae et al. 2004). Any differences related to metabolic activity were examined by performing histological analysis on both, top and middle sections of the construct.

For each experiment a second chamber was employed to apply dynamic compressive and tare strains to an identical set of chondrocytes-seeded constructs. At the end of the mechanical conditioning period, both constructs and corresponding culture medium of each group were removed and stored separately at -20°C prior to biochemical analysis.

The constructs were prepared and pre-cultured unstrained in 1 ml of DMEM + 16.1% (FCS) at 37°C/5% $CO_2$, for 16 hours (short-term experiment) or 6 days (long-term experiment). Three loading conditions, involving both continuous and intermittent regimens were prescribed each for a period of 48 hours, namely,

- *Tare Load (TL):* 0.2% tare compressive strain arising from the mass of the loading pin.
- *Dynamic Compression (DC):* sinusoidal cyclic compressive strain at a maximum amplitude of 15% at a frequency of 1 Hz, superimposed on the tare load.

- *Dynamic Compression & Dynamic Shear (DC&DS):* 15% sinusoidal compressive strain superimposed on a 10% dynamic shear strain (Sawae *et al.*, 2004) using a triangular waveform at 1 Hz.

In the case of intermittent mechanical stimulation, the cyclic strains were applied continuously for 10 minutes followed by an unstrained period of 350 minutes. This temporal profile was repeated for a total of 8 times. A schematic of the mechanical conditioning protocols is illustrated in Fig. 2. Intermittent and continuous conditioning experiments were not performed in parallel. Therefore, the experiments were based on different cell pools.

**Chondrocyte viability**

Chondrocyte viability of both control and experimental constructs was assessed over the 8 day culture period. Vertical slices were obtained from the centre of representative constructs and stained by incubation in 1mL solution of 5 μM of calcein AM and Ethidium homodimer-1 in DMEM + 16.1% FCS at 37ºC/5% $CO_2$ for 30 minutes. The cells were viewed on an epifluorescence microscope (Leica DMI4000B) and the percentage of viable cells was determined.

**Biochemical Analysis**

Cell-seeded agarose constructs were subjected to a digestion procedure, with an initial incubation in 1 ml of digest buffer at 70°C until molten. The suspension was cooled to 37°C, 5 μl/mL of papain and 10 μl/mL of agarase suspension (Sigma–Aldrich, Poole, UK) were added, and the solution was incubated for 16 hours at 37°C and for a further 1 hour at 60 °C to ensure the removal of papain. The digested constructs were cooled to room temperature prior to performing the biochemical assays. The digests and culture media samples were assayed for glycosaminoglycan using the dimethyl-methylene blue dye-binding method (Farndale *et al.*, 1982) with a chondroitin sulphate standard. The collagen content was estimated by measuring the hydroxyproline content using a modification of the method

described by Bergman and Loxley (1963). Samples were hydrolysed at 110°C for 18 h in 38% HCl and assayed using a chloramine-T assay with a hydroxyproline : collagen ratio of 1 : 7.69 (Ignat'eva *et al.*, 2007). Total DNA, determined with use of the Hoechst 33258 method with a calf thymus DNA standard (Rao and Otto, 1992), was used to normalise both glycosaminoglycan and collagen contents.

**Histological and Immuno-histological investigations**

Representative constructs were fixed in 10% (w/v) neutral buffered formalin with 0.1 M $CaCl_2$ for 24 h at room temperature, dehydrated using a conventional ethanol gradient dehydration procedure (70%, 95% and 100%), cleaned with toluene at 30 ˚C and embedded in molten paraffin using the Excelsior ES Tissue Processor machine (Fisher Scientific UK Ltd, Loughborough , UK). The embedded constructs were divided into 3 μm thick sections, which were deparaffinised in xylene and rehydrated through graded alcohols than stained with HE (Gills Haematoxylin and 1% Eosin) and Picrosirious red for GAG and collagen staining, respectively.

In the case of free swelling, constructs sections from the embedded specimens were also stained with a polyclonal antibody to visualize the presence of keratan sulphate (KS), to determine the presence and distribution of proteoglycans around the cells and verify that the chondrocyte phenotype was maintained throughout the culture period. Indeed, several studies have demonstrated that when cell types are maintained in *vitro*, they adopt a fibroblastic morphology thus losing their ability to synthesise KS (Funderburgh, 2000).

The deparaffinised and rehydrated sections were incubated in 5% serum in PBS-T (PBS with 0.4% Triton X-100) for 30 minutes at room temperature to block any non-specific binding and then incubated overnight at 4 °C with the primary antibody (Rabbit polyclonal Keratan Sulfate Antibody, Novus Biological, Cambridge, UK) diluted (1:100) in PBS, whose penetration and activity has been reported to be unaffected in the presence of an agarose gel

(Steward *et al.*, 2012; Thorpe *et al.*, 2008). The staining was developed by incubating the sections with 3, 3′-diaminobenzidine tetrahydrochloride (DAB) solution (0.5 mg/mL DAB, 1 mL of 30% $H_2O_2$, 50 mM Tris-HCl, pH 7.6) for 1 h. The reaction was stopped with TBS buffer. The nuclei were counterstained with HE. Specimens were then mounted for imaging. Bright field images of the stained sections with each dye were recorded using a light microscope (Leica Microsystems GmbH Leica Microsytems GmbH, Wetzlar, Germany) with a 63× objective lens and analysed with the NDP View software.

**Statistical Analyses**

The data are presented as mean +/- standard deviation. The normality of distribution and the equality of variance of all data sets were assessed using Shapiro-Wilk normality test and Brown-Forsythe's test, respectively. Differences in the synthesis of GAG and collagen and their retention within the construct were analysed for each of the test conditions involving both control and experimental groups by one-way ANOVA, if distribution of all data sets and the equal variance among them were confirmed to be normal. A Kruskal-Wallis test was performed for non-normal distribution or data of unequal variance. If the effect was shown to be significant ($p < 0.05$) Mann-Whitney comparisons tests were employed. A level of 5% was considered statistically significant in all statistical tests ($p < 0.05$).

**Results**

**Free Swelling study**

The mean values of chondrocyte viability were maintained above 90% for all the free swelling culture periods with values ranging between 93 ± 2 % and 97 ± 3 % for cylindrical constructs (FSC) at day 0 (T0) and day 8, respectively. The corresponding viability for construct with endplates (FSE) was 94 ± 2 % (T0) and 93 ± 4 % (day 8). The differences in viability with culture period were not statistically significant ($p > 0.05$).

The mean values of DNA content for each of the free swelling culture periods are detailed in Table I. Although there were small differences in the DNA content with culture period for each of the construct morphologies, none were found to be statistically significant ($p > 0.05$). However, the DNA content in constructs with endplates (FSE3, 3.96 ± 0.50 μg) was significantly higher than the corresponding values in cylindrical constructs (FSC3, 3.09 ± 0.61 μg). Both the absolute GAG and collagen contents and the values normalised by DNA retained in the construct and released into the medium are detailed in Table I. There was a clear increase of GAG retained in the construct with free swelling culture period, such that by day 8, there was a 4.25 and 3.25 fold increase for cylindrical constructs and constructs with endplates, respectively. The corresponding increase in GAG released into the medium was small and generally constant and thus represented a significant decrease in the total GAG synthesised with culture period for both morphologies. Table I also reveals a small increase of collagen synthesis with time in culture, although the differences between values in free swelling were not statistically significant for constructs of either morphology ($p > 0.05$).

The mean values of synthesized GAG/DNA and collagen/DNA for constructs with endplates were compared to the condition at T0 (Fig. 3). These revealed a statistically significant increase of GAG synthesis retained in the construct with period in free swelling culture (Fig. 3a), with mean fold values increasing with time from between 1.5 (FSE3), 2.5 (FSE6) and 3.2 (FSE8). By contrast, there was only a small percentage collagen increase after 6 and 8 days in free swelling cultures retained within constructs (Fig. 3b) and also released into the culture medium (Table I) ($p > 0.05$ in each case). Indeed the collagen released into the medium represented between 47 and 58% of the total synthesised across all free swelling culture periods (Fig. 3d). Typical images of the ECM deposition around individual chondrocyte-seeded constructs with endplates are illustrated in Fig. 4. HE staining revealed an increase in cell diameter following free swelling culture (FSE8 versus T0). This change is associated

with the appearance of a PCM, forming a chondron-like structure in free swelling culture at both 6 (not shown) and 8 days. Analysis also revealed the presence of substantial amounts of keratan sulphate in the pericellular region in samples at FSE8, which was absent at T0. The presence of collagen, as indicated by Picrosirius red staining, was weak for each free swelling period, although it appeared to be slightly more intense at FSE8 (Fig. 4).

**Biomechanical Conditioning study**

The mean values of cell viability of constructs with endplates exposed to both loading regimens were maintained above 90% for both control and experimental constructs, over both 3 and 8 day culture periods. There were no statistically significant differences between any of the treatments (TL, DC and DC&DS) groups ($p > 0.05$ in all cases).

Both the absolute GAG and collagen contents and the values normalised by DNA retained in the construct and released into the medium for each loading regimens are detailed in Table II. Statistical analysis revealed no significant differences in DNA content, with the exception of small decreases in the short-term continuous loading group when both were compared with TL3 ($p < 0.05$) and when the DC3 groups for continuous and intermittent loading regimens were compared (Table II). The mean values of synthesized GAG/DNA for each of the prescribed loading conditions were compared to the free swelling conditions values (FSE3 or FSE8), as shown in Fig. 5. It is evident that for a pre-culture period of 16 hours, GAG synthesis was enhanced for the strained groups (DC and DC&DS) with both continuous and intermittent loading (Fig. 5a, c). For the short-term experiments, the mean GAG content retained in the constructs increased from between 116% and 127% for both continuous and intermittent loading groups (Figs 5a, c). By contrast, there was no systematic trend in the GAG synthesis in the long-term experiment (Figs. 5b, d) with no significant difference between both loading and control (FSE8) groups ($p> 0.05$) in the case of continuous loading regimens. Indeed, for intermittent loading regimens, the amount of GAG was significantly

lower than the value of the control group (FSE8) (Fig. 5d). It is interesting to note that the percentage of total proteoglycans released into culture medium decreased significantly with pre-culture time for for both loading regimens (Table III).

The corresponding mean values of newly synthesised collagen/DNA retained within the construct or released into culture medium were compared to the corresponding control group values (FSE) for each of the prescribed conditions are presented in Fig. 6. In short-term continuous loading experiments (Fig. 6a), results indicated a significant up-regulation in collagen both retained in the construct and released into the medium during biaxial loading (DC&DS3) compared to both the control group (FSE3) and the experimental groups (TL3 and DC3) with an up-regulation of total collagen of approximately 82% (Table II). Statistically significant differences were evident between the experimental groups (TL3, DC3 and DC&DS3) and the control group (FSE3) for constructs exposed to short-term intermittent loading regimens (Fig. 6c). In the long-term loading experiments the biaxial loading regimen produced a small increase in the collagen retained within constructs for both loading regimens, although the differences did not attain statistically significance for all of the comparisons (Fig. 6b, d). The newly synthesised collagen released into the medium ranged between 53 and 63% of the total across all the pre-culture periods and experimental conditions (Table III), with a small decrease in the long-term experiments ($p > 0.05$). Typical results of the histological analysis performed on control and strained chondrocyte-seeded constructs subjected to continuous loading regimens are illustrated in Fig. 7. Results indicated an increase in chondrocyte diameter as a result of extended pre-culture periods. HE staining reveals the formation of a significant amount of PCM around chondrocytes, which is more pronounced after 6 days of pre-culture period. In addition, constructs subjected to continuous biaxial loading presented a broader and more intensely stained halo around the cell when compared to both TL and DC groups, for both short and long-term experiments (Fig. 7).

Results from the Picrosirus red staining demonstrated that histological sections of TL control group exhibited a weak staining intensity, corresponding to the minimal collagen synthesis, for both pre-culture periods (Fig. 7). For both experiments involving continuous compression alone (DC), the staining for collagen was relatively modest and mainly localized around the cell membrane (Fig. 7a). By contrast, a considerably higher amount of collagen was observed in the pericellular microenvironment of constructs subjected to biaxial loading (DC&DS). This was particularly marked in long term cultures where an intense band immediately adjacent to the cell membrane was surrounded by a diffuse less intense region. This is seen in both single (Fig. 7a) and multiple chondrocytes in all sections of the constructs (Figs. 7b-c).

**Discussion**

The chondrocyte-agarose system is a well-established model to investigate cell proliferation, ECM production and biochemical composition (Lee and Bader, 1997; Benya and Shaffer, 1982; Chang and Poole, 1996). Histological examination of constructs in the present study confirmed that when in free swelling conditions the system maintained a high viability, a relatively low proliferation rate and characteristic chondrocyte morphology throughout the 8 day culture period. Findings indicated that the DNA content in chondrocyte seeded agarose constructs was not strongly influenced by time in culture (Table I). Histochemical data highlighted a rapid synthesis of proteoglycans deposited in the pericellular region. These features were evident within three days of culture and generally continued to progress with free swelling culture. Proteoglycan production represents the primary event in the constitution of freshly synthesized ECM (Loredo *et al.*, 1996; Archer *et al.*, 1990). Concomitantly with the progressive increase of matrix deposition, a gradual increase in the chondrocyte diameter was observed (Fig. 4). It has been previously suggested that temporal modifications of chondrocyte volume in culture may reflect altered regulatory mechanisms triggered by variations in the cell niche and associated adaptations to its external environment

(Quinn *et al.*, 2002). Staining of construct sections confirmed a consistent production of keratan sulphate and its localisation mainly in the pericellular capsule (Fig. 4). By contrast, collagen synthesis and retention within the construct was not up-regulated in free swelling culture for either construct morphology, which confirms previous reports (Hunter *et al.*, 2004). Indeed results indicated that approximately 55% of the synthesised collagen was released into the culture medium (Table III). This may be a direct result of the production of immature collagen fragments, characterised by a low molecular weight, which can easily diffuse out of the agarose constructs (Kjaer, 2004; Lamande and Bateman, 1993).

The newly designed constructs with endplates (Di Federico *et al.*, 2014) proved ideal for the current study involving biaxial loading regimens. The design enabled the chondrocyte viability to remain consistently high for each loading regimen up to 8 days culture. Findings revealed a consistent synthesis of proteoglycans and their deposition in the PCM of single and multiple chondrocytes (Figs. 7 a-c), with a more significant GAG up-regulation in the case of constructs exposed to both forms of continuous dynamic regimens (DC and DC&DS). It is interesting to note that there is a small but significant difference between the GAG content in constructs subjected to tare loads alone at 3 (TL3) and 8 days (TL8) for the continuous and intermittent loading experiments (Table II). This can be attributed to the inherent variability in cell response between these experiments, as all other experimental parameters remained identical.

The temporal evolution of GAG content within the construct was associated with a reduction in the proportion of total GAG released into the culture medium (Table III). The observed GAG up-regulation and localisation around chondrocytes, forming a chondron-like structure (Fig. 7), will alter both the magnitude and the modality by which the mechanical loads are perceived by the chondrocytes modifying their biosynthetic response. In particular, the chondrocyte deformation will be reduced by the presence of the PCM (Knight et al., 1998),

which may explain why the mechanical conditioning effect was more pronounced in short-term experiments (Figs. 5-6). Additionally, the remodelling of the PCM may represent a priority in chondrocytes seeded in agarose constructs, particularly when exposed to dynamic compressive loads. The superposition of dynamic shear on dynamic compression (DC&DS) significantly increased collagen content without marked compromise to the GAG synthesis. These effects were more pronounced with continuous biaxial loading in the short-term experiments, compared to intermittent biaxial loading (Figs. 6). These findings contrast with previous studies in which intermittent strain regimes were reported to be beneficial to the biosynthetic activity of chondrocytes (Burton-Wurster *et al.*, 1993) and also bone cells (Robling *et al.*, 2001). This effect may be related to the increased diffusion of nutrients within the scaffold induced by continuous loading. Furthermore, since chondrocytes are not directly linked to the agarose gel, higher level of strain delivered continuously may be necessary to trigger collagen biosynthetic pathways.

The free swelling pre-culture period prior to biomechanical conditioning appeared to greatly influence collagen synthesis (Table II). Regardless of the extent of the collagen up-regulation, its retention rate within the construct was moderate (Table III). Further studies would be required to examine whether biaxial loading (DC&DS) either partially promotes the maturation of the low molecular weight collagen or upregulates catabolic processes involving MMPs. Noticeably, the application of dynamic compression (DC) alone to the constructs with endplates revealed no major effects on the rate of collagen synthesis (Table II) nor in its segregation into the construct (Chowdhury *et al.*, 2003; Bader and Lee, 2000).

The present series of experiments strongly suggest that proteoglycan and collagen synthesis may be triggered by uncoupled mechanosensitive cellular responses. Indeed, GAG synthesis appear to be up-regulated predominantly by intermittent dynamic compressive loads (DC3, DC&DS3) prescribed on constructs cultured in free-swelling conditions for only 16 hours

(Table II). By contrast, enhanced collagen synthesis associated with slightly lower GAG content is evident in constructs and medium after exposure to continuous biaxial loading regimen (Fig. 6, Table II). Moreover, these uncoupled mechanisms necessitate the use of tuned conditioning protocols, which vary with time, dependent on the status of the ECM solid components and their structural interactions to evolve an ECM composition and structure to match that of native cartilage.

The present work offers the potential for a more effective pre-conditioning of cell-seeded constructs with physiologically-relevant loading regimens for use in resolving localised defects in joint cartilage. The investigation of the response of chondrocytes to a complex physiologically relevant deformation profile will lead to the development of neo-cartilage characterised by organized ECM with a ratio of collagen to proteoglycan matching that of native tissue structural competency and long term functionality.

**Disclosure of potential conflict of interest**

The authors indicate no potential conflicts of interest.

**Acknowledgements**

The support of Orthopaedic Research UK is gratefully acknowledged. The authors also wish to thank Mr Douglas Thomson for his invaluable technical advice and assistance in the fabrication of the bioreactor and the Pathology Core Department at the Blizard Institute, part of the School of Medicine and Dentistry at QMUL, where the histological processes were performed.

**References**

Archer CW, Mcdowell J, Bayliss MT, Stephens MD, Bentley G. 1990. Phenotypic modulation in subpopulations of human articular chondrocytes *in vitro*. J Cell Sci 97:361-371.

Bader DA, Lee DL. 2000. Structural Biological Materials: Design and structure-property relationships. Oxford:Pergamon. 165 p.

Benya PD, Shaffer JD. 1982. Dedifferentiated chondrocytes re-express the differentiated collagen phenotype when cultured in agarose gels. Cell 30:215-224.

Bergman I, Loxley R. 1963. Two improved and simplified methods for the spectrophotometric determination of hydroxyproline. Anal Chem 35:1961-1965.

Bian L, Fong JV, Lima EG, Stoker AM, Ateshian GA, Cook JL, Hung CT. 2010. Dynamic mechanical loading enhances functional properties of tissue-engineered cartilage using mature canine chondrocytes. Tissue Eng Part A 16:1781-1790.

Buckwalter JA, Mankin HJ. 1998. Articular cartilage repair and transplantation. Arthritis Rheumatol 41:1331-1342.

Burton-Wurster N, Vernier-Singer M, Farquhar T, Lust G. 1993. Effect of compressive loading and unloading on the synthesis of total protein, proteoglycan, and fibronectin by canine cartilage explants. J Orthop Res 11:717-729.

Chang J, Poole CA. 1996. Sequestration of type VI collagen in the pericellular microenvironment of adult chondrocytes cultured in agarose. Osteoarthritis Cartilage 4:275-285.

Chowdhury TT, Akanji OO, Salter DM, Bader DL, Lee DA. 2008. Dynamic compression influences interleukin-1 beta-induced nitric oxide and prostaglandin E-2 release by articular chondrocytes via alterations in iNOS and COX-2 expression. Biorheology 45:257-274.

Chowdhury TT, Bader DL, Shelton JC, Lee DA. 2003. Temporal regulation of chondrocyte metabolism in agarose constructs subjected to dynamic compression. Arch Biochem Biophys 417:105-111.

Davisson T, Kunig S, Chen A, Sah R, Ratcliffe A. 2002. Static and dynamic compression modulate matrix metabolism in tissue engineered cartilage. J Orthop Res 20:842-848.

Di Federico E, Bader DL, Shelton JC. 2014. Design and validation of an *in vitro* loading system for the combined application of cyclic compression and shear to 3D chondrocytes-seeded agarose constructs. Med Eng Phys 36:534-540.

Di Federico E. 2015 Complex mechanical conditioning of cell-seeded constructs can influence chondrocyte activity. PhD Thesis, Queen Mary University of London.

Farndale RW, Sayers CA, Barrett AJ. 1982. A direct spectrophotometric micro-assay for sulfated glycosaminoglycans in cartilage cultures. Connect Tissue Res 9:247-248.

Frank EH, Jin M, Loening AM, Levenston ME, Grodzinsky AJ. 2000. A versatile shear and compression apparatus for mechanical stimulation of tissue culture explants. J Biomech 33:1523-1527.

Freyria AM, Yang Y, Chajra H, Rousseau CF, Ronzière MC, Herbage D, El Haj AJ. 2005. Optimization of dynamic culture conditions: effects on biosynthetic activities of chondrocytes grown in collagen sponges. Tissue Eng. 11:674-84.

Funderburgh JL. 2000. Keratan sulfate: structure, biosynthesis, and function. Glycobiology 10:51–958.

Goldring MB, Marcu KB. 2009. Cartilage homeostasis in health and rheumatic diseases. Arthritis Res Ther 11:224-239.

Gooch KJ, Kwon JH, Blunk T, Langer R, Freed LE, Vunjak-Novakovic G. 2001. Effects of mixing intensity on tissue-engineered cartilage. Biotechnol. Bioeng. 72:402-407.

Grodzinsky AJ, Levenston ME, Jin M, Frank EH. 2000. Cartilage tissue remodeling in response to mechanical forces. Annu Rev Biomed Eng 2:691-713.

Grodzinsky AJ, Roth V, Myers E, Grossman WD, Mow VC. 1981. The significance of electromechanical and osmotic forces in the nonequilibrium swelling behavior of articular cartilage in tension. J Biomech Eng 103:221-231.

Guo T, Yu L, Lim CG, Goodley AS, Xiao X, Placone JK, Ferlin KM, Nguyen B, Hsieh AH, Fisher JP. 2015. Effect of dynamic culture and periodic compression on human mesenchymal stem cell proliferation and chondrogenesis. Ann Biomed Eng 44:2103–2113.

Helminen HJ, Kiviranta I, Saamanen AM, Jurvelin JS, Arokoski J, Oettmeier R, Abendroth K, Roth AJ, Tammi MI. 1992. Effect of motion and load on articular-cartilage in animal-models. In: Kuettner KE, Schleyerbach R, Peyron J, Hascall VC (eds) Articular Cartilage and Osteoarthritis. New York:Raven Press. 501 p.

Heywood HK, Sembi PK, Lee DA, Bader DL. 2004. Cellular utilization determines viability and matrix distribution profiles in chondrocyte-seeded alginate constructs. Tissue Eng. 10:1467-79.

Hunter CJ, Mouw JK, Levenston ME. 2004. Dynamic compression of chondrocyte-seeded fibrin gels: effects on matrix accumulation and mechanical stiffness. Osteoarthritis Cartilage 12:117-130.

Hunziker EB, Quinn TM, Hauselmann HJ. 2002. Quantitative structural organization of normal adult human articular cartilage. Osteoarthritis Cartilage 10:564-572.

Ignat'eva EV, Klimova NV, Oshchepkov DY, Vasil'ev GV, Merkulova TI, Kolchanov NA. 2007a. Search for new binding sites for the transcriptional factor SF-1 by the SITECON method: experimental verification and analysis of regulatory regions of orthologous genes. Dokl Biochem Biophys 415:165-169.

Ignat'eva NY, Danilov NA, Averkiev SV, Obrezkova MV, Lunin VV, Sobol' EN. 2007b. Determination of hydroxyproline in tissues and the evaluation of the collagen content of the tissues. J Anal Chem 62:51-57.

Jeon JE, Schrobback K, Meinert C, Sramek V, Hutmacher DW, Klein TJ. 2013. Effect of preculture and loading on expression of matrix molecules, matrix metalloproteinases, and cytokines by expanded osteoarthritic chondrocytes. Arthritis Rheum. 65:2356-67.

Jin M, Frank EH, Quinn TM, Hunziker EB, Grodzinsky AJ. 2001. Tissue shear deformation stimulates proteoglycan and protein biosynthesis in bovine cartilage explants. Arch Biochem Biophys 395:41-48.

Johnstone B, Alini M, Cucchiarini M, Dodge GR, Eglin D, Guilak F, Madry H, Mata A, Mauck RL, Semino CE, Stoddart MJ. 2013. Tissue Engineering for Articular Cartilage Repair - the State of the Art. Eur Cell Mater 25:248-267.

Kisiday JD, Jin MS, DiMicco MA, Kurz B, Grodzinsky AJ. 2004. Effects of dynamic compressive loading on chondrocyte biosynthesis in self-assembling peptide scaffolds. J Biomech 37:595-604.

Kjaer M. 2004. Role of extracellular matrix in adaptation of tendon and skeletal muscle to mechanical loading. Physiol. Rev. 84:649-698.

Knight MM, Lee DA, Bader DL. 1998. The influence of elaborated pericellular matrix on the deformation of isolated articular chondrocytes cultured in agarose. Biochimica et Biophysica Acta, 1405:67-77.

Knight MM, Toyoda T, Lee DA, Bader DL. 2006. Mechanical compression and hydrostatic pressure induce reversible changes in actin cytoskeletal organisation in chondrocytes in agarose. J Biomech 39:1547-1551.

Kuettner KE, Pauli BU, Gall G, Memoli VA, Schenk RK. 1982. Synthesis of cartilage matrix by mammalian chondrocytes *in vitro*.1. isolation, culture characteristics, and morphology. J Cell Biol 93:743-750.

Kurz B, Jin M, Patwari P, Cheng DM, Lark MW, Grodzinsky AJ. 2001. Biosynthetic response and mechanical properties of articular cartilage after injurious compression. J Orthop Res 19:1140-1146.

Lamande SR, Bateman JF. 1993. A type I collagen reporter gene construct for protein engineering studies. Functional equivalence of transfected reporter COL1A1 and endogenous gene products during biosynthesis and *in vitro* extracellular matrix accumulation. Biochem J 293:387-394.

Langelier E, Buschmann MD. 2003. Increasing strain and strain rate strengthen transient stiffness but weaken the response to subsequent compression for articular cartilage in unconfined compression. J Biomech 36:853-859.

Lee DA, Bader DL. 1997. Compressive strains at physiological frequencies influence the metabolism of chondrocytes seeded in agarose. J Orthop Res 15:181-188.

Loredo GA, MacDonald MH, Benton HP. 1996. Regulation of glycosaminoglycan metabolism by bone morphogenetic protein-2 in equine cartilage explant cultures. Am J Vet Res 57:554-559.

Mauck RL, Soltz MA, Wang CC, Wong DD, Chao PH, Valhmu WB, Hung CT, Ateshian GA. 2000. Functional tissue engineering of articular cartilage through dynamic loading of chondrocyte-seeded agarose gels. J Biomech Eng 122:252-260.

Mow VC, Wang CC, Hung CT. 1999. The extracellular matrix, interstitial fluid and ions as a mechanical signal transducer in articular cartilage. Osteoarthritis Cartilage 7:41-58.

Ng KW, Ateshian GA, Hung CT. 2009. Zonal chondrocytes seeded in a layered agarose hydrogel create engineered cartilage with depth-dependent cellular and mechanical inhomogeneity. Tissue Eng Part A 15:2315-2324.

Ofek G, Dowling EP, Raphael RM, McGarry JP, Athanasiou KA. 2010. Biomechanics of single chondrocytes under direct shear. Biomech Model Mechanobiol 9:153-162.

Poole CA, Ayad S, Gilbert RT. 1992. Chondrons from articular-cartilage .5. immunohistochemical evaluation of type-VI collagen organization in isolated chondrons by light, confocal and electron-microscopy. J Cell Sci 103:1101-1110.

Poole CA, Honda T, Skinner SJM, Schofield JR, Hyde KF, Shinkai H. 1990. Chondrons from articular-cartilage 2. Analysis of the glycosaminoglycans in the cellular microenvironment of isolated canine chondrons. Connect Tissue Res 24:319-330.

Quinn TM, Schmid P, Hunziker EB, Grodzinsky AJ. 2002. Proteoglycan deposition around chondrocytes in agarose culture: Construction of a physical and biological interface for mechanotransduction in cartilage. Biorheology 39:27-37.

Rao J, Otto WR. 1992. Fluorometric DNA Assay for Cell-Growth Estimation. Anal Biochem 207:186-192.

Robling AG, Burr DB, Turner CH. 2001. Recovery periods restore mechanosensitivity to dynamically loaded bone. J Exp Biol 204:3389-3399.

Sawae Y, Shelton JC, Bader DL, Knight MM 2004. Confocal analysis of local and cellular strains in chondrocyte-agarose constructs subjected to shear. Annals of Biomed. Eng., 32:860-70.

Schöne M, Männicke N, Somerson JS, Marquas B, Henkelmann R, Aigner T, Raum K, Schulz RM. 2016. 3D ultrasound biomicroscopy for assessment of cartilage repair tissue: volumetric characterisation and correlation to established classification systems. eCells & Material 31:119 - 135.

Shahin K, Doran PM. 2012. Tissue engineering of cartilage using a mechanobioreactor exerting simultaneous mechanical shear and compression to simulate the rolling action of articular joints. Biotechnol Bioeng. 109:1060-73.

Smith RL, Lin J, Trindade MCD, Shida J, Kajiyama G, Vu T, Hoffman AR, Van der Meulen MCH, Goodman SB, Shurman DJ, Carter DR. 2000. Time-dependent effects of intermittent hydrostatic pressure on articular chondrocyte type II collagen and aggrecan mRNA expression. J Rehabil Res Dev 37:153-161.

Steward A, Chen Q, Chapman RI, Borgia MB, Rogers JM, Wojtala A, Wilmanns M, Clarke J. 2012. Two immunoglobulin tandem proteins with a linking beta-strand reveal unexpected differences in cooperativity and folding pathways. J Mol Biol 416:137-147.

Thorpe SD, Buckley CT, Vinardell T, O'Brien FJ, Campbell VA, Kelly DJ. 2008. Dynamic compression can inhibit chondrogenesis of mesenchymal stem cells. Biochem Biophys Res Commun 377:458-462.

Waldman SD, Couto DC, Grynpas MD, Pilliar RM, Kandel RA. 2007. Multi-axial mechanical stimulation of tissue engineered cartilage: review. Eur Cell Mater. 13:66-73.

Waldman SD, Spiteri CG, Grynpas MD, Pilliar RM, Kandel RA. 2003. Long-term intermittent shear deformation improves the quality of cartilaginous tissue formed *in vitro*. J Orthop Res 21:590-596.

Wimmer MA, Alini M, Grad S. 2009. The effect of sliding velocity on chondrocytes activity in 3D scaffolds. J Biomech 42:424-429.

## FIGURE CAPTION:

**FIGURE 1:** Global view and close-up of the assembled tissue culture sterile chamber positioned in the incubator a), b). Image of cylindrical agarose construct (Ø 5 mm x 5 mm, c right) and construct provided with custom designed nylon endplates (top endplate: Ø 10 mm x 5 mm; bottom endplate: Ø 15.5 mm x 5 mm, c left).

**FIGURE 2:** Schematic representing the protocols designed to examine the metabolic response of chondrocyte seeded in agarose constructs under a) continuous and b) intermittent uniaxial and biaxial loading.

**FIGURE 3:** Percentage of control (T0) of GAG (a, c) and collagen (b, d) synthesis either retained within the construct (dark blue and dark red, respectively ) or released in culture medium (light blue and light red, respectively) by chondrocytes seeded in constructs with endplates cultured in free swelling (FS) (FSE) condition for up to 8 days, (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

**FIGURE 4:** Typical microscope images of single sections labelled with HE, Keratan sulphate antibodies and Picrosirius red stains showing chondrocytes seeded in constructs with endplates, cultured in free swelling conditions (FSE) for 16 h (T0) and 8 days. Arrows indicate development of pericellular matrix around chondrocytes.

**FIGURE 5:** Percentage of controls (FS3 and FS8) of GAG synthesis retained within the construct by chondrocytes seeded in constructs pre-cultured for either 16 hours (a, c) or 6 days (b, d) and subjected to continuous (a, b) or intermittent (c, d) loading regimens, (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$).

**FIGURE 6:** Microscope images of single optical sections labelled with HE and Picrosirius red stains illustrating individual chondrocytes seeded in constructs with endplates, pre-cultured for 16 h and 6 days subjected to TL, DC and DC&DS (continuous loading regimen). Arrows indicate development of pericellular matrix around chondrocytes (a). Microscope images of multiple DC&DS8 chondrocytes stained with both HE (b) and Picrosirius red (c) illustrating the deposition of a significant amount of GAG and collagen in adjacent cells and in the territorial matrix between chondrocytes.

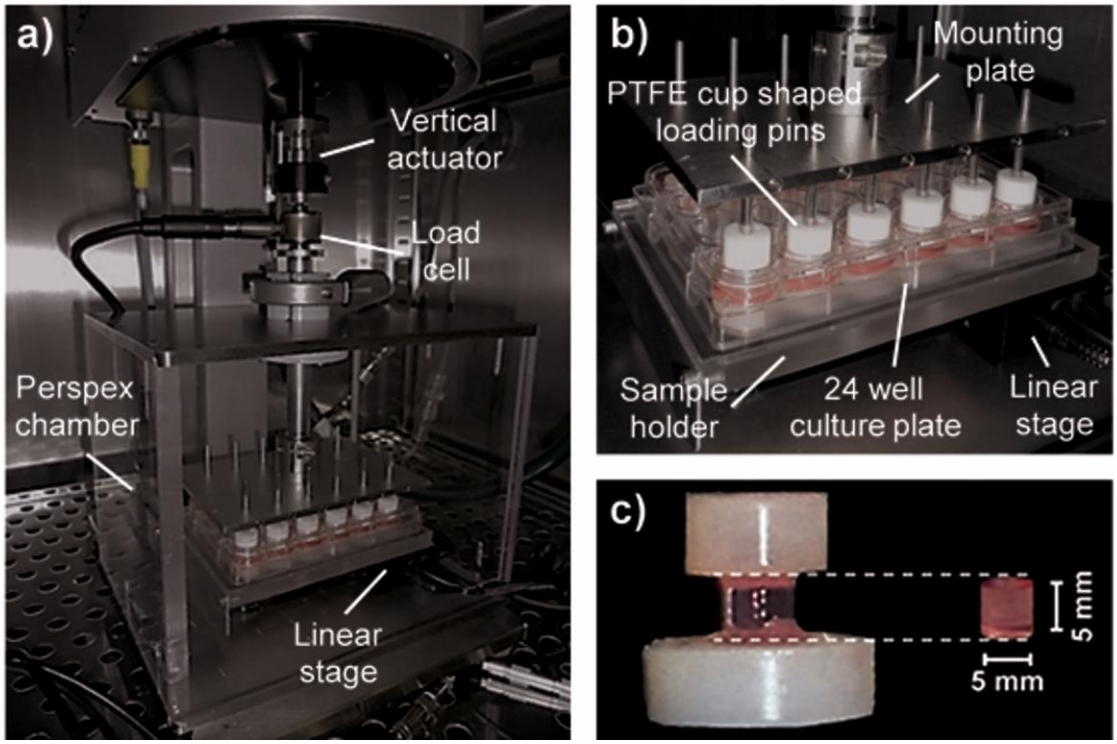


*Figure 1*

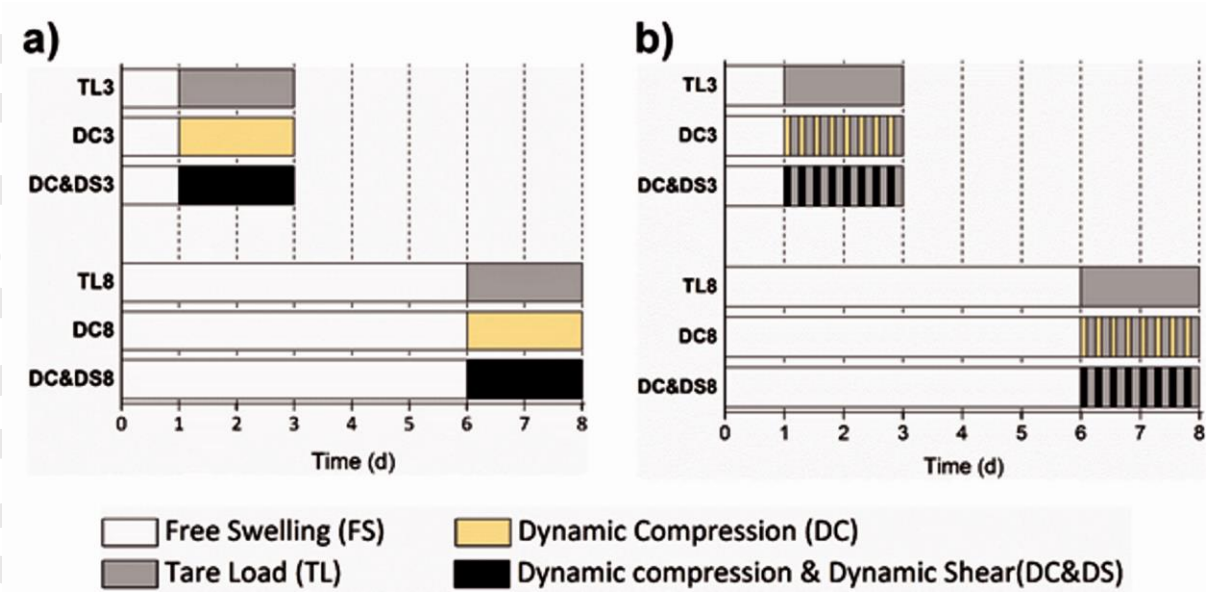


*Figure 2*

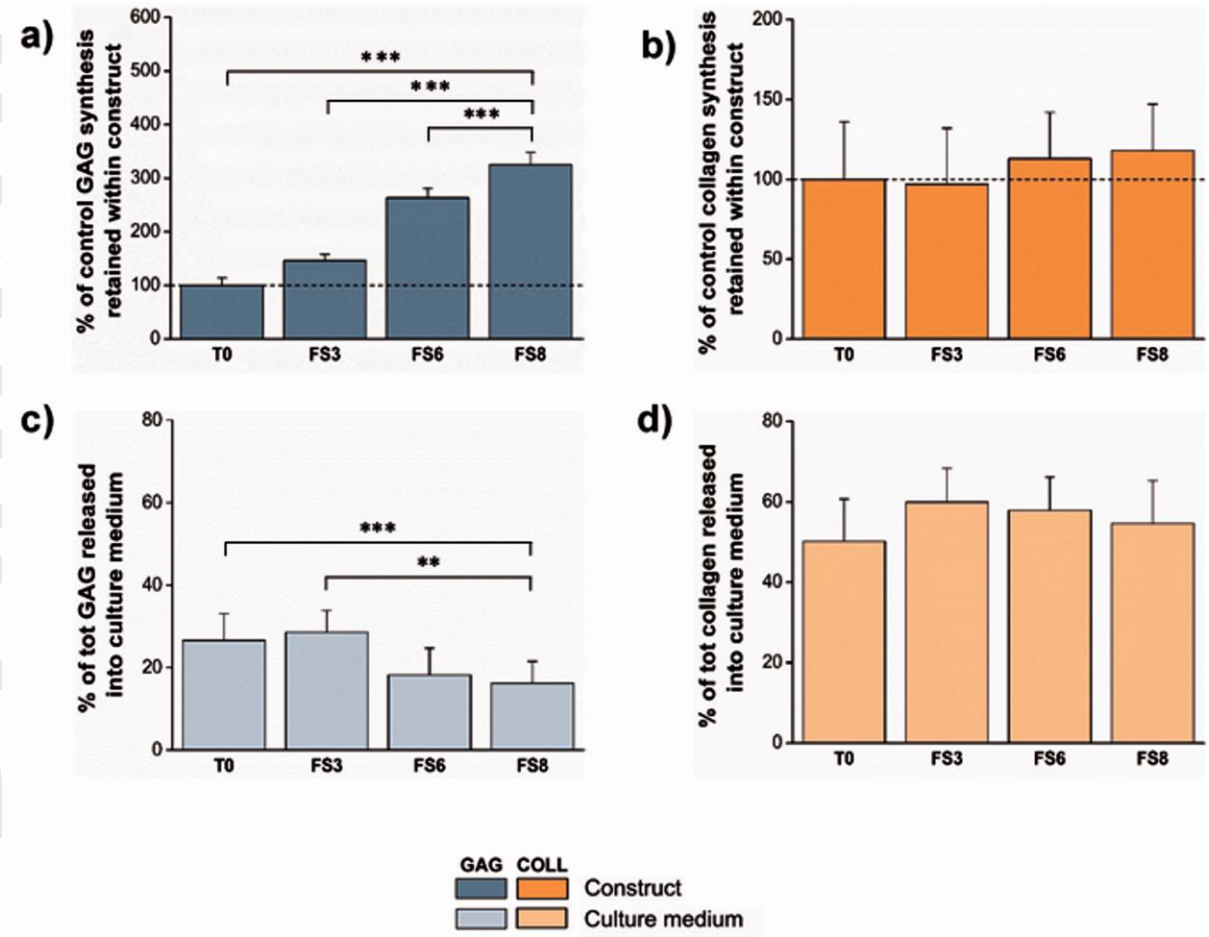


*Figure 3*

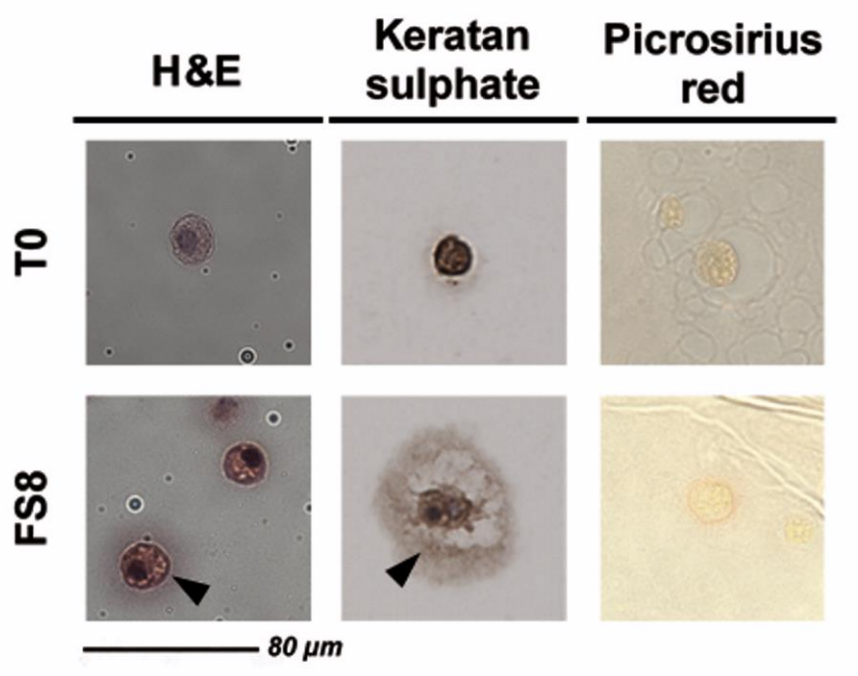


*Figure 4*

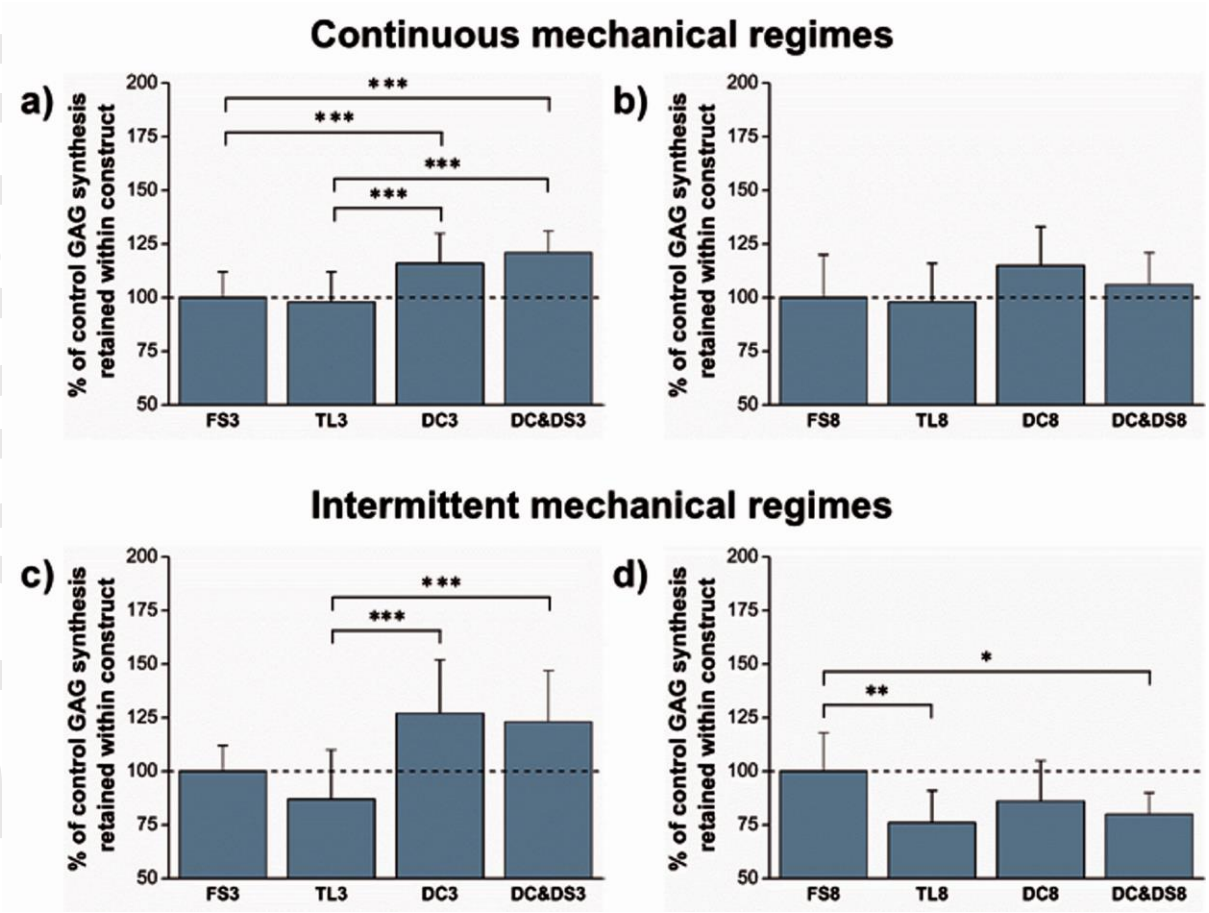


*Figure 5*

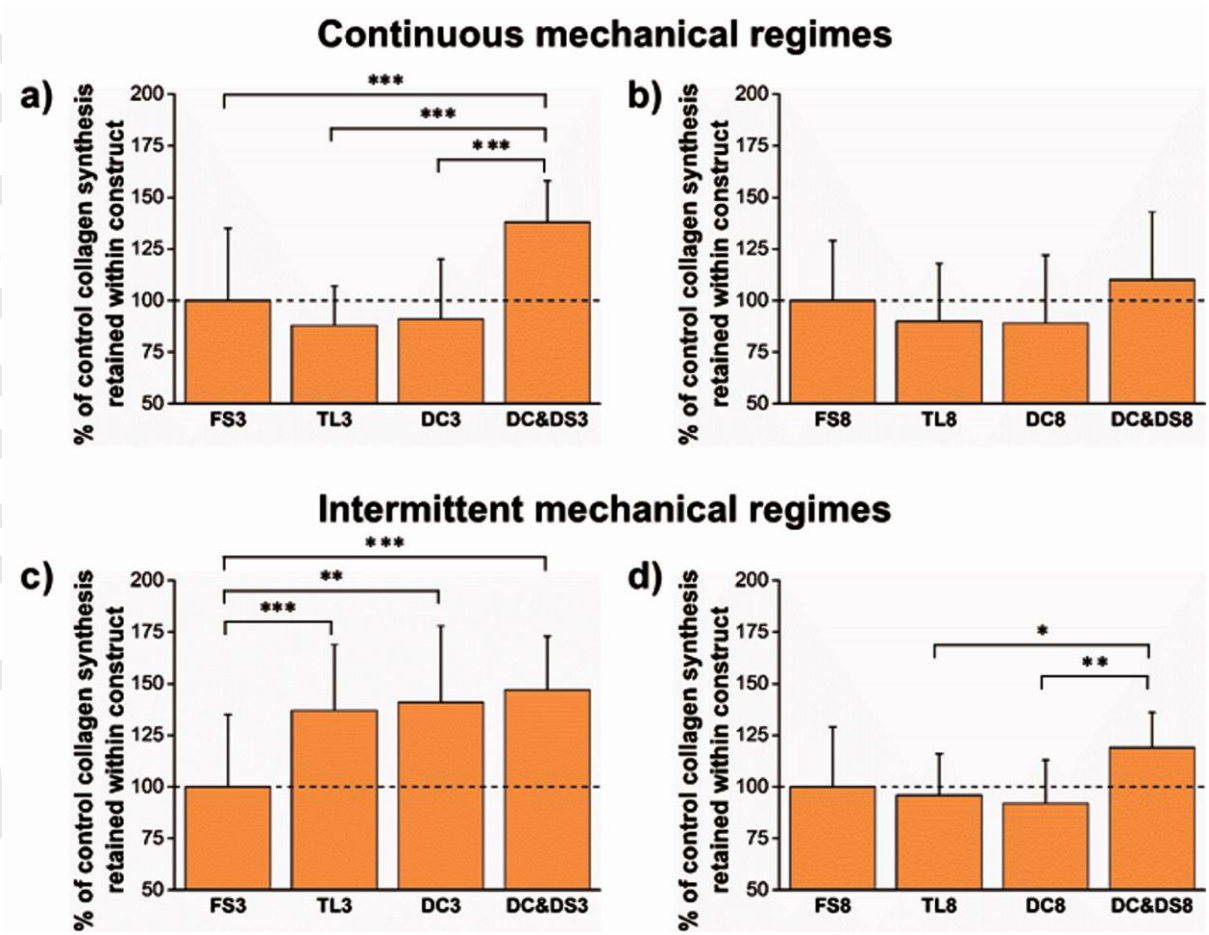


*Figure 6*

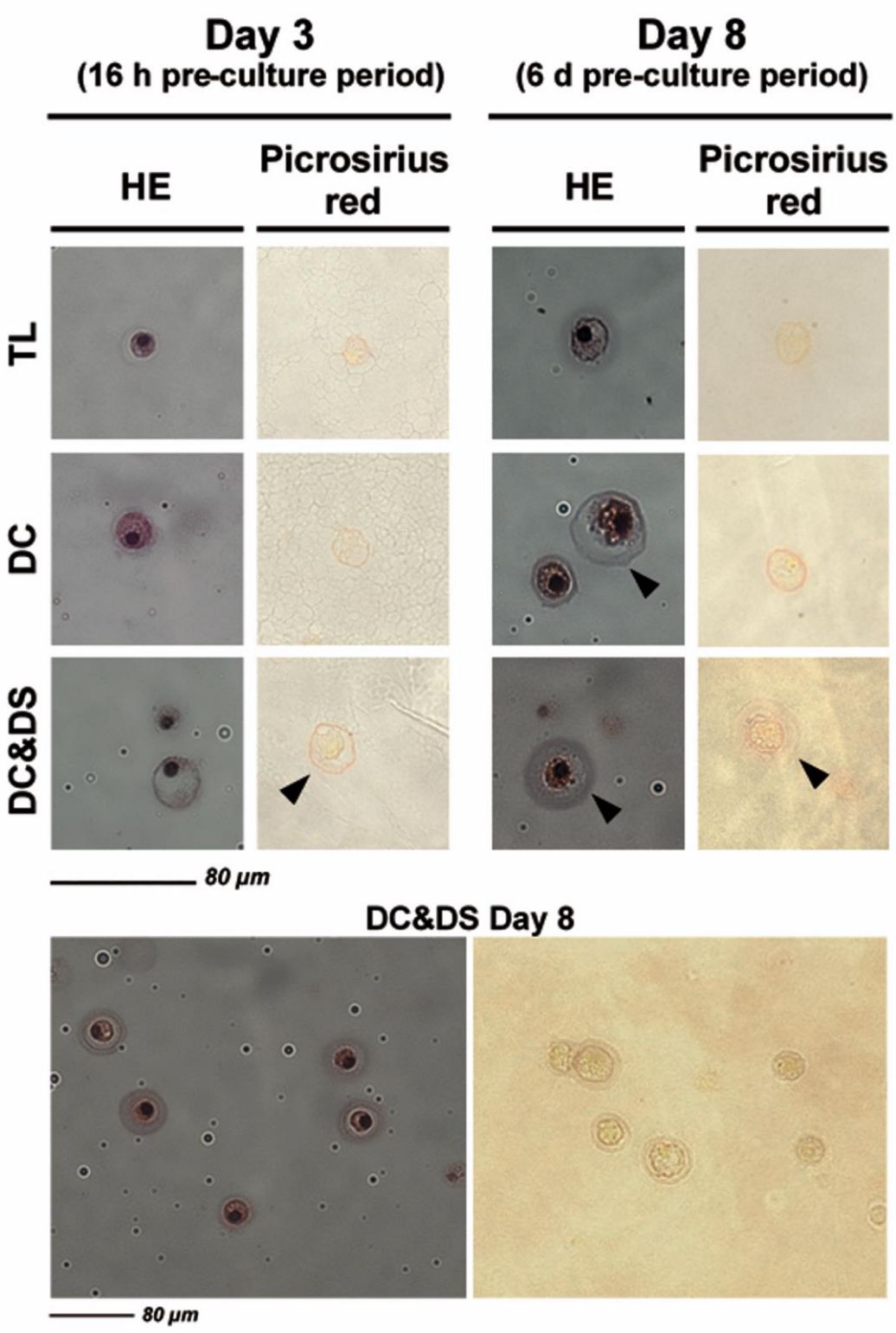


*Figure 7*

**Table I:** DNA, GAG/DNA and collagen/DNA content in chondrocytes seeded in constructs cultured in free swelling for up to 8 days (FSxn with x = E or C for constructs with endplates and cylindrical constructs respectively and n = culture period).

| | | DNA (µg) | GAG (µg) | | GAG (µg/µg DNA) | | Collagen (µg) | | Collagen (µg/µg DNA) | |
|---|---|---|---|---|---|---|---|---|---|---|
| | *n°* | *Construct* | *Construct* | *Medium* | *Construct* | *Medium* | *Construct* | *Medium* | *Construct* | *Medium* |
| **Cylindrical Constructs** | ***32*** ***T0*** | 2.99 ± 0.52 | 18.53 ± 1.74 | 8.96 ± 2.70 | 5.97 ± 1.60 | 3.12 ± 0.93 | 10.95 ± 3.64 | 11.76 ± 4.32 | 3.92 ± 1.38 | 4.00 ± 1.38 |
| | ***28*** ***FSC3*** | 3.09 ± 0.61 | 34.61 ± 2.35 | 9.45 ± 0.64 | 10.49 ± 2.18 | 3.14 ± 0.75 | 10.03 ± 2.01 | 12.38 ± 2.83 | 3.46 ± 1.23 | 4.00 ± 0.54 |
| | ***32*** ***FSC6*** | 3.15 ± 0.42 | 64.88 ± 10.02 | 10.33 ± 1.60 | 21.79 ± 7.25 | 3.56 ± 0.87 | 12.17 ± 3.52 | 12.30 ± 3.69 | 4.38 ± 1.61 | 4.54 ± 1.54 |
| | ***32*** ***FSC8*** | 3.25 ± 0.75 | 82.42 ± 12.99 | 9.68 ± 1.59 | 25.37 ± 8.36 | 3.20 ± 0.95 | 13.40 ± 4.03 | 12.66 ± 4.39 | 4.69 ± 2.23 | 4.00 ± 1.23 |
| **Constructs endplates** | ***38*** ***T0*** | 3.97 ± 0.41 | 21.84 ± 2.49 | 7.66 ± 2.04 | 5.43 ± 0.78 | 2.03 ± 0.64 | 12.50 ± 3.91 | 13.25 ± 6.23 | 3.22 ± 1.16 | 3.43 ± 1.66 |
| | ***46*** ***FSE3*** | 3.96 ± 0.50 | 31.39 ± 3.78 | 12.22 ± 2.79 | 7.95 ± 0.95 | 3.12 ± 0.89 | 12.15 ± 3.58 | 17.96 ± 4.25 | 3.12 ± 1.11 | 4.55 ± 1.01 |
| | ***28*** ***FSE6*** | 3.73 ± 0.38 | 53.35 ± 10.13 | 10.54 ± 2.92 | 14.27 ± 2.63 | 2.90 ± 0.93 | 13.51 ± 4.11 | 17.82 ± 2.50 | 3.62 ± 1.05 | 4.79 ± 0.59 |
| | ***32*** ***FSE8*** | 3.67 ± 0.40 | 64.68 ± 14.92 | 11.57 ± 2.85 | 17.63 ± 4.11 | 3.14 ± 0.79 | 13.95 ± 4.39 | 16.35 ± 3.53 | 3.80 ± 1.12 | 4.47 ± 0.75 |

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

**Table II**: DNA, GAG/DNA and collagen/DNA content in chondrocytes seeded in constructs with endplates subjected to different mechanical conditioning protocols (TLn=tare load, SCn=static compression, DCn=dynamic compression, DC&DSn=dynamic compression & dynamic shear with n=pre-incubation period+2 days loading regimen).

| | | | DNA (µg) | GAG (µg) | | Collagen (µg) | |
|---|---|---|---|---|---|---|---|
| | n° | | *Construct* | *Construct* | *Medium* | *Construct* | *Medium* |
| Continuous loading regimen | ***26*** | ***TL3*** | 4.23 ± 0.42 | 32.56 ± 3.07 | 11.36 ± 3.74 | 11.53 ± 1.54 | 19.64 ± 2.86 |
| | ***30*** | ***DC3*** | 4.17 ± 0.41 | 37.84 ± 2.09 | 14.09 ± 4.96 | 11.66 ± 2.69 | 19.76 ± 2.77 |
| | ***33*** | ***DC&DS3*** | 3.98 ± 0.40 | 37.55 ± 1.70 | 14.85 ± 5.00 | 17.00 ± 2.73 | 25.81 ± 5.78 |
| | ***21*** | ***TL8*** | 4.20 ± 0.60 | 70.84 ± 4.34 | 9.30 ± 4.41 | 13.92 ± 2.82 | 16.58 ± 2.15 |
| | ***21*** | ***DC8*** | 4.32 ± 0.65 | 84.98 ± 4.07 | 10.27 ± 4.91 | 14.02 ± 2.93 | 15.59 ± 2.67 |
| | ***21*** | ***DC&DS8*** | 4.30 ± 0.61 | 78.59 ± 1.83 | 9.87 ± 4.73 | 17.33 ± 3.94 | 19.24 ± 2.83 |
| Intermittent loading regimen | ***18*** | ***TL3*** | 3.91 ± 0.55 | 26.67 ± 4.74 | 11.42 ± 6.50 | 16.42 ± 4.14 | 19.89 ± 2.87 |
| | ***17*** | ***DC3*** | 3.84 ± 0.52 | 37.88 ± 6.34 | 11.58 ± 6.62 | 16.38 ± 4.94 | 18.98 ± 3.63 |
| | ***18*** | ***DC&DS3*** | 3.90 ± 0.48 | 37.23 ± 6.02 | 12.78 ± 5.16 | 17.58 ± 3.31 | 23.11 ± 2.87 |
| | ***22*** | ***TL8*** | 3.96 ± 0.46 | 52.99 ± 9.76 | 11.07 ± 2.61 | 14.35 ± 2.31 | 19.19 ± 2.08 |
| | ***22*** | ***DC8*** | 4.09 ± 0.89 | 59.87 ± 7.83 | 10.77 ± 1.24 | 14.04 ± 3.00 | 18.37 ± 2.69 |
| | ***22*** | ***DC&DS8*** | 4.28 ± 0.61 | 59.68 ± 4.98 | 9.82 ± 2.11 | 19.23 ± 1.87 | 21.43 ± 3.54 |

| | | | GAG (µg/µg DNA) | | Collagen (µg/µg DNA) | |
|---|---|---|---|---|---|---|
| | n° | | *Construct* | *Medium* | *Construct* | *Medium* |
| Continuous loading regimen | ***26*** | ***TL3*** | 7.77 ± 1.05 | 2.66 ± 0.81 | 3.62 ± 1.50 | 5.44 ± 2.16 |
| | ***30*** | ***DC3*** | 9.19 ± 1.27 | 3.35 ± 1.14 | 3.72 ± 1.94 | 5.58 ± 2.58 |
| | ***33*** | ***DC&DS3*** | 9.60 ± 0.94 | 3.72 ± 1.27 | 5.17 ± 1.99 | 7.32 ± 2.77 |
| | ***21*** | ***TL8*** | 17.26 ± 3.14 | 2.16 ± 0.91 | 4.04 ± 2.37 | 4.56 ± 2.06 |
| | ***21*** | ***DC8*** | 20.19 ± 3.73 | 2.31 ± 0.94 | 4.00 ± 2.44 | 4.26 ± 2.19 |
| | ***21*** | ***DC&DS8*** | 18.65 ± 2.80 | 2.23 ± 0.92 | 4.75 ± 2.37 | 5.04 ± 1.95 |
| Intermittent loading regimen | ***18*** | ***TL3*** | 6.95 ± 1.63 | 3.04 ± 1.90 | 4.29 ± 1.37 | 5.16 ± 0.90 |
| | ***17*** | ***DC3*** | 10.11 ± 2.49 | 3.11 ± 1.92 | 4.40 ± 1.61 | 5.24 ± 0.94 |
| | ***18*** | ***DC&DS3*** | 9.78 ± 2.39 | 3.40 ± 1.56 | 4.60 ± 1.18 | 5.99 ± 0.97 |
| | ***22*** | ***TL8*** | 13.39 ± 2.03 | 2.81 ± 0.64 | 3.67 ± 0.74 | 4.89 ± 0.68 |
| | ***22*** | ***DC8*** | 15.11 ± 3.18 | 2.80 ± 0.90 | 3.51 ± 0.72 | 4.72 ± 1.43 |
| | ***22*** | ***DC&DS8*** | 14.07 ± 1.37 | 2.37 ± 0.75 | 4.52 ± 0.77 | 5.03 ± 0.57 |

**,† p < 0.05; **, †† p < 0.01; ***, †††p < 0.001.*

**Table III:** Percentage of newly synthesised GAG and collagen released into culture medium for constructs with endplates pre-cultured for 16 hours and 6 days and subjected to tare load (TL), dynamic compression (DC) and dynamic shear superimposed to dynamic compression (DC&DS) for both intermittent (I) and continuous (C) loading regimens.

| | TL3 | DC3 | DC&DS3 | TL8 | DC8 | DC&DS8 |
|---|---|---|---|---|---|---|
| **% of total GAG released into culture medium** | | | | | | |
| **C** | 25 ± 6 | 27 ± 8 | 27 ± 8 | 12 ± 5$^{***}$ | 11 ± 5$^{***}$ | 11 ± 5$^{***}$ |
| **I** | 28 ± 9 | 22 ± 7 | 25 ± 5 | 18 ± 4 | 15 ± 3 | 14 ± 4$^{***}$ |
| **% of total collagen released into culture medium** | | | | | | |
| **C** | 63 ± 5 | 63 ± 7 | 60 ± 6 | 55 ± 7 | 53 ± 8 | 53 ± 9 |
| **I** | 55 ± 6 | 56 ± 7 | 57 ± 4 | 57 ± 4 | 57 ± 6 | 53 ± 4 |

Differences for corresponding groups between day 3 and day 8 values. $^{*}$ $p < 0.05$, $^{**}$ $p < 0.01$, $^{***}$ $p < 0.001$.