

Functional Properties of Cartilaginous Tissues Engineered From Infrapatellar Fat Pad Derived Mesenchymal Stem Cells

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Abstract

Articular cartilage has a poor intrinsic capacity for self repair. The advent of autologous chondrocyte implantation has provided a feasible method to treat cartilage defects. However, the associated drawbacks with the isolation and expansion of chondrocytes from autologous tissue has prompted research into alternative cell sources such as mesenchymal stem cells (MSCs) which have been found to exist in the bone marrow as well as other joint tissues such as the infrapatellar fat pad (IFP), synovium and within the synovial fluid itself. In this work we assessed the chondrogenic potential of IFP derived porcine cells over a six week period in agarose hydrogel culture in terms of mechanical properties, biochemical content and histology. It was found that IFP cells underwent robust chondrogenesis as assessed by glycosaminoglycan (1.47 ± 0.22 % w/w) and collagen (1.44 ± 0.22 % w/w) accumulation after 42 days of culture. The 1Hz dynamic modulus of the engineered tissue at this time-point was 272.8 kPa (± 46.8). The removal of TGF- β 3 from culture after 21 days was shown to have a significant effect on both the mechanical properties and biochemical content of IFP constructs after 42 days, with minimal increases occurring from day 21 to day 42 without continued supplementation of TGF- β 3. These findings further strengthen the case that the IFP may be a promising cell source for putative cartilage repair strategies.

Introduction

Autologous chondrocyte implantation (ACI) is an established technique for the treatment of articular cartilage defects and is now in clinical use (Brittberg et al., 1994; Peterson et al., 2000; Horas et al., 2003; Ruano-Ravina and Jato Diaz, 2006). However, limitations still exist with both first and second generation ACI techniques in relation to the cell source procured. Issues such as dedifferentiation during monolayer expansion of isolated chondrocytes (Benya et al., 1978; Diaz-Romero et al., 2005), age related capacity (Barbero et al., 2004) and poor chondrogenic potential of osteoarthritic (OA) derived cells (Tallheden et al., 2005) have prompted investigations into the therapeutic potential of utilising mesenchymal stem cells (MSCs) as an alternative cell source for cartilage repair (Wakitani et al., 1994; Bosnakovski et al., 2004; Bosnakovski et al., 2006; Mauck et al., 2006; Miyanishi et al., 2006; Coleman et al., 2007; Hannouche et al., 2007; Mauck et al., 2007; Huang et al., 2008; Kisiday et al., 2008).

MSCs possess the ability to proliferate extensively *ex vivo* while maintaining their multipotent differentiation capabilities (Bruder et al., 1997; Kadiyala et al., 1997; Barry et al., 2001), making them an attractive cell type for cell-based cartilage repair strategies. These cells can, for example, be isolated from bone marrow aspirates taken from the iliac crest and have the capacity to differentiate along a number of mesenchymal lineages including bone, cartilage and fat (Maniatopoulos et al., 1988; Caplan, 1991; Johnstone et al., 1998; Pittenger et al., 1999; Jones et al., 2002). To date, MSCs derived from the bone marrow have been the prime site of cell harvesting for cartilage therapy development (Yoo et al., 1998; Kuroda et al., 2007; Wakitani et al., 2007). More recently, interest has also extended to utilising MSCs from other locations, and in the case of knee joint

cartilage repair, from non-cartilaginous knee joint tissues. It has been demonstrated that tissues from this region including the joint infrapatellar fat pad (IFP) (Dragoo et al., 2003; Wickham et al., 2003; English et al., 2007; Khan et al., 2007; Lee et al., 2008), synovium (Nishimura et al., 1999; Sakaguchi et al., 2005; Mochizuki et al., 2006; Shirasawa et al., 2006; Ando et al., 2007; Marsano et al., 2007; Pei et al., 2008a; Pei et al., 2008b) and synovial fluid (Jones et al., 2008) possess significant chondrogenic potential and perhaps provide a more readily available and clinically feasible source of chondroprogenitor cells. Through *in vitro* experiments it has been demonstrated that 80% of OA derived IFP pellet cultures were more chondrogenic than control bone marrow MSCs, and in addition exhibited similar chondrogenic capacity to cartilage-derived cultures (English et al., 2007).

One of the key challenges for any cartilage tissue engineering strategy is ensuring sufficient functional properties so that the engineered construct can be implanted in a load bearing environment. Bone marrow derived MSCs undergoing chondrogenesis have been shown to synthesise less extracellular matrix ECM than chondrocytes (Worster et al., 2001). Furthermore bone marrow derived MSC's generate an ECM that is mechanically inferior to that produced by fully differentiated articular chondrocytes maintained in identical hydrogel culture conditions (Mauck et al., 2006; Erickson et al., 2009; Huang et al., in press). It has been suggested that this may in part be due to some critical structural ECM components not being expressed and deposited by bone marrow derived MSCs (Erickson et al., 2009). There is therefore a need to assess the functional properties of cartilaginous tissues engineering using MSCs isolated from other tissue

sources. The objective of this study is to determine the functional properties of cartilaginous tissues engineered from MSCs isolated from the IFP.

Materials and methods

Cell Isolation and expansion

Porcine IFPs were harvested from the knee joint capsule of two 4 month old porcine donors (~50 kg) within 3 hours of sacrifice. IFPs were washed thoroughly in phosphate buffered saline (PBS) and diced followed by overnight incubation under constant rotation at 37°C with high-glucose Dulbecco's Modified Eagle Medium (hgDMEM, GlutaMAX™) (GIBCO, Biosciences, Ireland) containing collagenase type II (0.5mg/ml, Sigma–Aldrich, Ireland) and 1% penicillin (100 U/mL)-streptomycin (100 µg/mL). After tissue digestion, cells were washed in PBS, filtered through a 40 µm nylon cell strainer and centrifuged at 650g for 5 min. Mononucleated cells (MNCs) were counted using a haemocytometer and plated in T-75cm² flasks (Sarstedt, Wexford, Ireland) at a density of 5×10³ cells/cm² and cultured in expansion medium (EM) consisting of hgDMEM containing 10% foetal bovine serum and 1% penicillin (100 U/mL)-streptomycin (100 µg/mL) (all from GIBCO, Biosciences, Dublin, Ireland). Non-adherent cells were removed during the first medium change after 72 hours. After colony formation (~8 days), cells were detached with 0.05% trypsin-EDTA (Sigma-Aldrich, Arklow, Ireland) for 5 min at 37°C and re-plated at 5×10³ cells/cm² (P1). Cultures were expanded to passage three (P3, ~21days from initial isolation) at a seeding density of 5×10³ cells/cm² at each passage in EM. Complete medium exchanges were performed twice weekly. Cells were pooled from two donors prior to agarose encapsulation.

Hydrogel construct fabrication and culturing

IFP MSCs (P3) were encapsulated in agarose (Type VII) at ~40°C, to yield a final gel concentration of 2% and a cell density of 15×10^6 cells/mL. The agarose-cell suspension was cast in a stainless steel mould to produce cylindrical discs (\varnothing 5mm \times 3mm thickness). Agarose hydrogel constructs were maintained in a chemically defined chondrogenic medium (CDM) consisting of DMEM GlutaMAX™ supplemented with penicillin (100 U/mL)-streptomycin (100 μ g/mL) (both GIBCO, Biosciences, Ireland), 100 μ g/ml sodium pyruvate, 40 μ g/ml L-proline, 50 μ g/ml L-ascorbic acid-2-phosphate, 1 mg/ml BSA, $1 \times$ insulin–transferrin–selenium, 100 nM dexamethasone (all from Sigma-Aldrich, Ireland) and 10 ng/ml recombinant human transforming growth factor- β 3 (TGF- β 3; R&D Systems, UK). Hydrogel constructs were cultured in standard 6 well plates (Sarstedt, Ireland) with two-three constructs per well. Each construct was maintained in 2.5mL of complete medium with complete medium exchanges performed every 3-4 days for the total culture duration of 42 days. The surfaces of wells were monitored and changed when outgrowth occurred (~weekly) to avoid excessive nutrient demands from monolayer cells.

The effect of removal of TGF- β 3 from culture after 21 days was also examined in a parallel study. Agarose hydrogels were maintained in fully supplemented CDM with TGF- β 3 for either the total culture period of 42 days (TGF+) or for the first 21 days, with subsequent removal of TGF- β 3 (TGF-) from days 21 to 42.

Assessment of functional properties

Constructs were mechanically tested in unconfined compression at days 0, 21 and 42 using a standard materials testing machine with a 5N load cell (Zwick Z005, Roell, Germany). A preload of 0.01N was applied to ensure that the surfaces of the gel constructs were in direct contact with the impermeable loading platens. Stress relaxation tests were performed, consisting of a ramp and hold cycle with a ramp displacement of 0.001mm/s until 10% strain was obtained and maintained until equilibrium was reached. Dynamic tests were performed immediately after the stress relaxation cycle. A cyclic strain of 1% was applied at 0.1Hz and 1Hz. After mechanical testing, constructs were weighed wet and the total mass recorded. Constructs were cored using a 3mm biopsy punch and separated from the annulus; the wet mass of both the core and annulus was recorded and frozen for subsequent biochemical analyses.

Quantitative biochemical analysis

Samples were digested at days 0, 21 and 42 in papain (125µg/ml) in 0.1 M sodium acetate, 5 mM cysteine HCl, 0.05 M EDTA, pH 6.0 (all from Sigma–Aldrich, Ireland) at 60°C under constant rotation for 18 hours. Total DNA content was measured using a Quant-iT™ PicoGreen® dsDNA kit (Molecular Probes, Biosciences) with a lambda DNA standard. The proteoglycan content was estimated by quantifying the amount of sulfated glycosaminoglycan (sGAG) in constructs using the dimethylmethylene blue dye-binding assay (Blyscan, Biocolor Ltd., Northern Ireland), with a chondroitin sulfate standard. Total collagen content was determined by measuring the hydroxyproline content. Samples were hydrolysed at 110°C for 18hrs in concentrated HCL (38%) and

assayed using a chloramine-T assay (Kafienah and Sims, 2004) with a hydroxyproline-to-collagen ratio of 1:7.69 (Ignat'eva et al., 2007).

Histology and Immunohistochemistry

Constructs were fixed in 4% paraformaldehyde, wax embedded and sectioned at 5 µm to produce a cross section perpendicular to the disc face. Sections were stained with 1% alcian blue 8GX (Sigma–Aldrich, Ireland) in 0.1M HCl which stains sGAG, and picrosirius red to stain collagen. The deposition of collagen types I and II were identified through immunohistochemistry. Briefly, sections were quenched of peroxidase activity, rinsed with PBS before treatment with chondroitinase ABC (Sigma-Aldrich, Ireland) in a humidified environment at 37°C to enhance permeability of the extracellular matrix by removal of chondroitin sulphate. Slides were rinsed with PBS and non-specific sites were blocked with goat serum. Sections were then incubated overnight at 4°C with the primary antibody; either mouse monoclonal collagen type I anti-body (1:400; 5.4 mg/mL; Abcam, Cambridge, UK) or mouse monoclonal anti-collagen type II (1:100; 1 mg/mL; Abcam, Cambridge, UK). After washing in PBS, sections were incubated for 1 hour in the secondary antibody for type I and type II collagen, anti-mouse IgG biotin antibody produced in goat (1:400; 1 mg/mL; Sigma-Aldrich, Arklow, Ireland). Colour was developed using the Vectastain ABC reagent (Vectastain ABC kit, Vector Laboratories, Peterborough, UK) followed by exposure to peroxidase DAB substrate kit (Vector Laboratories Ltd., Peterborough, UK). Negative and positive controls of porcine ligament and cartilage were included for each batch.

Statistical analysis

Statistical analysis was performed using one way ANOVA with Tukey post-hoc tests to enable comparisons between groups. Tests were performed using SigmaStat® (Version 3.1) software with 3 samples analysed for each group at each time point. Numerical and graphical results are displayed as mean \pm standard deviation. Significance was accepted at a level of $p < 0.05$. The experiment was also replicated with cells pooled from different animals (n=2 donors). Comparable results were observed in both studies, and the results of the first replicate are presented in the manuscript.

Results

Constructs increased in mechanical properties from an equilibrium modulus of 7.0 kPa (± 0.6) at day 0 reaching 26.2 kPa (± 1.1) by day 42 and a final 1 Hz dynamic modulus, of 272.8 kPa (± 46.8) (Fig. 1A).

Increases in total biochemical content (Fig. 1B-D) were also observed with increasing time in culture ($p < 0.001$). In terms of percentage wet weight (% w/w), sGAG content reached a maximum of 1.47% w/w (± 0.22) with collagen contents of 1.44% w/w (± 0.22) attained by day 42 (Fig. 1B). Similarly, normalisation of the biochemical constituents by DNA content resulted in sGAG accumulation of 85.93 ± 13.57 ($\mu\text{g}/\mu\text{g}$) and 84.28 ± 14.08 ($\mu\text{g}/\mu\text{g}$) for collagen (Fig. 1C). DNA content increased with time in culture from 88.15 ng/mg w/w (± 6.64) at day 0, to 112.78 ng/mg w/w (± 15.57) at day 21 reaching 171.65 ng/mg w/w (± 3.17) by day 42 (~1.9 fold increase), indicating significant cellular proliferation had occurred (Fig. 1D).

To gain an appreciation of the spatial distribution of biochemical constituents, the annuli and cores of samples were assessed separately (Fig. 2). By day 42, the core regions of constructs contained greater sGAG accumulation compared to the corresponding annular region when expressed in terms of percentage of wet weight (annulus= 1.37 ± 0.20 %w/w and core= 1.64 ± 0.28 %w/w) (Fig 2A), although this was not found to be statistically significant ($p > 0.05$). Similar differences were also observed in core-annulus sGAG accumulation when normalised by DNA content (Fig. 2B), and were found to be statistically significant ($p < 0.01$). Interestingly, collagen content was higher in the annular region of IFP constructs compared to the core region when normalised by wet weight (annulus= 1.61 ± 0.24 %w/w, core= 1.17 ± 0.18 %w/w, $p < 0.05$), although no differences were observed when normalised by DNA content (annulus = 82.88 ± 14.32 $\mu\text{g}/\mu\text{g}$, core = 87.73 ± 15.00 $\mu\text{g}/\mu\text{g}$, $p > 0.05$), (Fig. 2 C&D).

The DNA content increased with time in the annular regions from 94.36 ng/mg w/w (± 7.63) at day 0 to 195.31 ng/mg w/w (± 5.40) at day 42 (~2.1 fold increase) indicating extensive proliferation. The IFP core region DNA content also increased from 77.92 ng/mg w/w (± 7.42) at day 0 to 133.59 ng/mg w/w (± 5.68) at day 42 (~1.7 fold increase), (Fig. 2E).

Histological evaluation revealed that constructs stained positively for sulphated proteoglycan and collagen accumulation (Fig. 3 A&B). Immunohistochemical results revealed that constructs stained weakly for collagen I, with more pronounced staining observed for collagen type II after 42 days of culture (Fig. 3 C&D).

The removal of TGF- β 3 from culture after 21 days was shown to have a significant effect on both the mechanical properties and biochemical content of IFP

constructs after 42 days (Fig. 4). No statistically significant increases occurred in any mechanical or biochemical measurement from day 21 to day 42 without the continued supplementation of TGF- β 3 ($p > 0.05$). Both the equilibrium (TGF+ = 26.26 ± 1.10 kPa, TGF- = 12.30 ± 0.74 kPa, $p < 0.001$) and 1Hz dynamic moduli (TGF+ = 272.85 ± 46.81 kPa, TGF- = 74.35 ± 10.14 kPa, $p < 0.001$) were higher for continued supplementation with TGF- β 3 from day 21 to 42 compared to withdrawal of TGF- β 3 (Fig 4A). Significantly higher levels of sGAG accumulation were observed after 42 days for constructs with continued supplementation of TGF- β 3 (TGF+ = 1.47 ± 0.22 %w/w; TGF- = 0.42 ± 0.08 %w/w, $p < 0.01$), with similar differences for collagen accumulation (TGF+ = 1.44 ± 0.22 %w/w, TGF- = 0.58 ± 0.06 %w/w, $p < 0.01$) (Fig 4B). These results were also reflected when normalised by DNA content (Fig 4C). For DNA content (Fig 4D), continued supplementation of TGF- β 3 also enhanced cellular proliferation (TGF+ = 171.65 ± 3.17 ng/mg w/w; TGF- = 129.93 ± 11.82 ng/mg, $p < 0.01$).

Discussion

Alternative cell sources for articular cartilage repair strategies have received significant attention with the identification of chondro-progenitor populations within bone marrow and in joint tissues such as synovium, infrapatellar fat pad and within the synovial fluid itself. In this study we have demonstrated that IFP derived MSCs encapsulated in agarose hydrogels undergo chondrogenesis when exposed to TGF- β 3. The IFP of the knee is a heterogeneous tissue and is composed of a thin layer of synovium that covers a relatively large subsynovium adipose-like tissue. It has previously been demonstrated that the phenotype and chondrogenic potential of IFP-derived cells is more similar to fibrous

synovium-derived cells than to subcutaneous fat-derived cells (Mochizuki et al., 2006). Synovial-membrane derived MSCs have already been shown to possess significant promise as a cell source for cartilage tissue engineering (Sakaguchi et al., 2005; Yokoyama et al., 2005; Ando et al., 2007; Ando et al., 2008; Pei et al., 2008a; Pei et al., 2008b). The findings, in the present study, therefore extend observations in relationship to MSCs isolated from within the joint space (Mochizuki et al., 2006; Yoshimura et al., 2007) and suggest that IFP derived MSCs proliferate and undergo robust chondrogenesis within agarose hydrogels.

The spatial distribution of sGAG within the agarose hydrogels was generally different regionally, with greater amounts of sGAG accumulation in the core of constructs compared to the annulus. Higher levels of core sGAG content has previously been reported by our laboratory for agarose encapsulated porcine bone marrow derived MSCs (Thorpe et al., 2008). This may simply be due to diffusion of sGAG from the periphery of the hydrogel into the media, or alternatively spatial gradients in the concentration of nutrients, growth factors, oxygen *etc.* could be leading to the development of a more chondrogenic environment within the core of the constructs. Interestingly, greater proliferation and collagen accumulation was observed in the annulus, suggesting an environment in this region that promotes a more proliferative phenotype compared to the construct core.

The mechanical properties of constructs increased significantly between day 21 and day 42 of culture. The reported mechanical properties of cartilaginous tissues engineered using chondrocytes from skeletal immature bovines embedded in agarose are generally higher than that reported here for porcine IFP derived MSCs (Mauck et al., 2006; Byers

et al., 2008). However differences due to age, species and culture conditions mean that future studies comparing donor matched chondrocytes and IFP MSCs will be critical. In addition, longer term studies will be required to assess if the mechanical properties of cartilaginous tissue generated using IFP MSCs plateau like that observed with bone marrow MSCs after extended culture periods (Mauck et al., 2006).

The motivation behind the removal of TGF- β 3 from culture was based upon the published findings of Byers et al. (2008) for chondrocyte seeded agarose hydrogels. The authors demonstrated that two week transient exposure to TGF- β 3 in a chemically defined medium elicited a superior response to that observed after continuous growth factor supplementation, with certain mechanical and biochemical levels achieved comparable to those of native cartilage. In the present study, the removal of TGF- β 3 from culture after 21 days did not significantly enhance either the mechanical properties or biochemical content of constructs at 42 days compared to constructs exposed to continued TGF- β 3 supplementation. Removal of TGF- β has also been shown to be sub-optimal for bone marrow derived MSCs in agarose hydrogels seeded at a similar density (Huang et al., in press), although a pronounced positive effect on the equilibrium modulus and sGAG accumulation was observed in that study for higher seeding densities (60 million/ml). Further studies to determine the optimal cell seeding density in order to engineer more functional cartilaginous tissues from IFP derived MSCs are required.

An obvious limitation of the study is the use of MSCs isolated from skeletally immature pigs. Characteristics of MSCs such as the ability to rapidly proliferate is known to change with age (Tsuji et al., 1990; Egrise et al., 1992). Pigs share an underlying genetic and physiological similarity to humans (Vacanti et al., 2005), and due to this it

has been suggested that they may provide a useful animal model system to evaluate tissue engineering strategies (Ringe et al., 2002). Regardless it is unclear if the results presented here would be comparable when using IFP MSCs derived from aged or diseased human tissue, although studies investigating the chondrogenic potential of such cells are promising^{32,56}.

Given its anatomical location and ease of accessibility for harvesting biopsies of tissue, the IFP may provide an attractive source of cells for articular cartilage repair. In the present work we add to this emerging concept by showing good *in vitro* functionality of cartilaginous tissues engineered using IFP derived MSCs. A critical next step is to assess *in vivo* the ability of cartilaginous tissues engineered using IFP MSC-agarose constructs to repair large chondral or osteochondral defects. Clinical studies have already been undertaken by other groups investigating agarose-alginate hydrogels seeded with chondrocytes for cartilage defect repair (Selmi et al., 2007; Selmi et al., 2008). In this clinical context, the IFP may also possess other benefits as a source of cells for cartilage repair. Although this work utilised expanded cells, future work will also investigate the feasibility and practicalities of utilising unexpanded cells from freshly digested IFP tissue, since the yield of putative MSCs may be higher in such tissues. If successful, such advantages would significantly accelerate translation into a clinical setting by avoiding the time and cost hindrances associated with large scale cell expansion. The present porcine studies therefore provide a rationale for the exploration of IFP in man as a source of cells for cartilage regeneration.

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Figure Legends

Fig 1: Mechanical and total biochemical properties of IFP constructs at days 0, 21 and 42. **(A)** Equilibrium modulus and 1Hz dynamic modulus **(B)** Total DNA content (ng/mg w/w) **(C)** Total sGAG and collagen content (% w/w) **(D)** Total sGAG and collagen content normalised to DNA ($\mu\text{g}/\mu\text{g}$). (NS) represents no significance, (*) $p < 0.05$, (***) $p < 0.001$ represents significance compared to earlier time point(s), $n=3$ for each group.

Fig 2: Annulus-core biochemical analysis of IFP constructs at days 0, 21 and 42. **(A)** sGAG content (% w/w) **(B)** sGAG/DNA content ($\mu\text{g}/\mu\text{g}$) **(C)** Collagen content (% w/w) **(D)** Collagen/DNA content ($\mu\text{g}/\mu\text{g}$) and **(E)** DNA content (ng/mg w/w). (NS) represents no significance, (*) $p < 0.05$, (***) $p < 0.001$ represents significance compared to earlier time point(s), (\$) $p < 0.01$ compared to corresponding core at the same time point, $n=3$ for each group.

Fig 3: Histological evaluation of constructs after 42 days of culture **(A)** Alcian blue staining for sGAG **(B)** Picrosirius Red staining for collagen, **(C)** Immunohistochemical staining for type I collagen **(D)** Immunohistochemical staining for type II collagen. Scale bar is 1mm.

Fig 4: The effect of removal of TGF- β 3 from culture after 21 days. Data represents mechanical properties and total biochemical analysis of constructs at day 42. **(A)** Equilibrium modulus and 1Hz dynamic modulus **(B)** Total sGAG and collagen content (% w/w) **(C)** Total sGAG and collagen content normalised to DNA ($\mu\text{g}/\mu\text{g}$) and **(D)** DNA content (ng/mg w/w), $n=3$ for each group. (**) $p < 0.01$, (***) $p < 0.001$ represents significance of continued supplementation with TGF- β 3 (TGF +) compared to removal after 21 days (TGF -), $n=3$ for each group.

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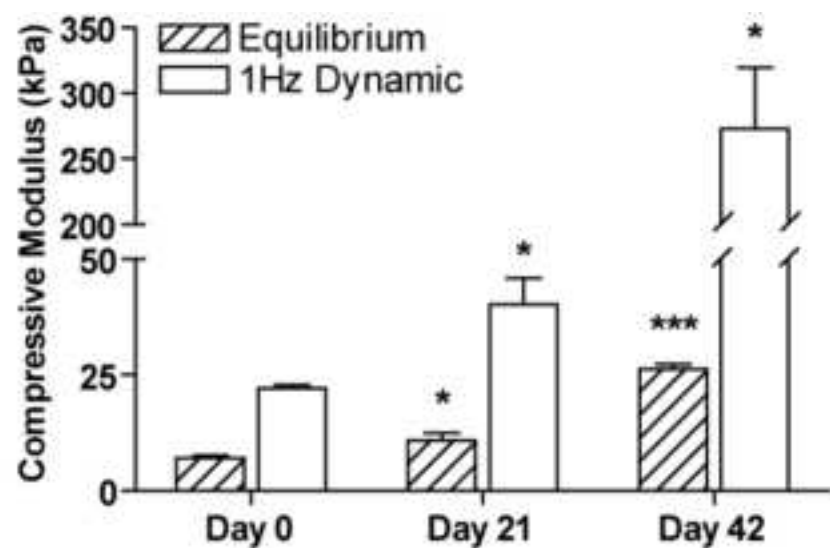
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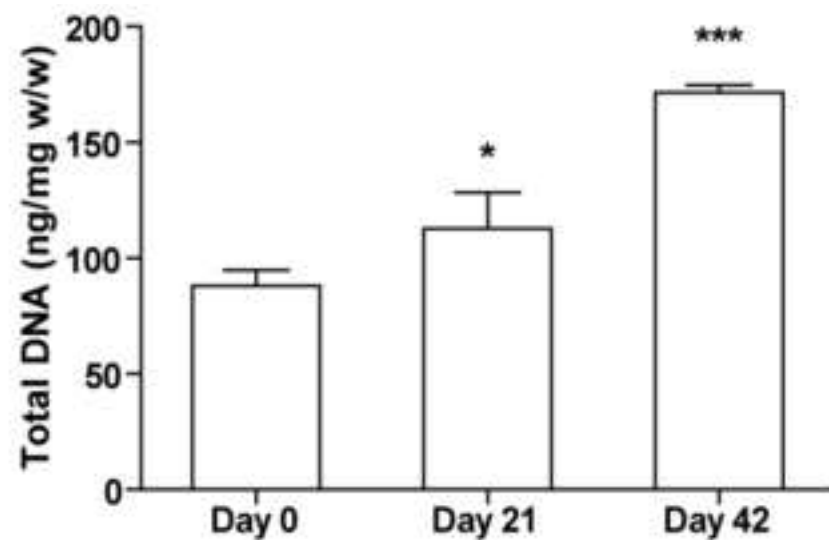
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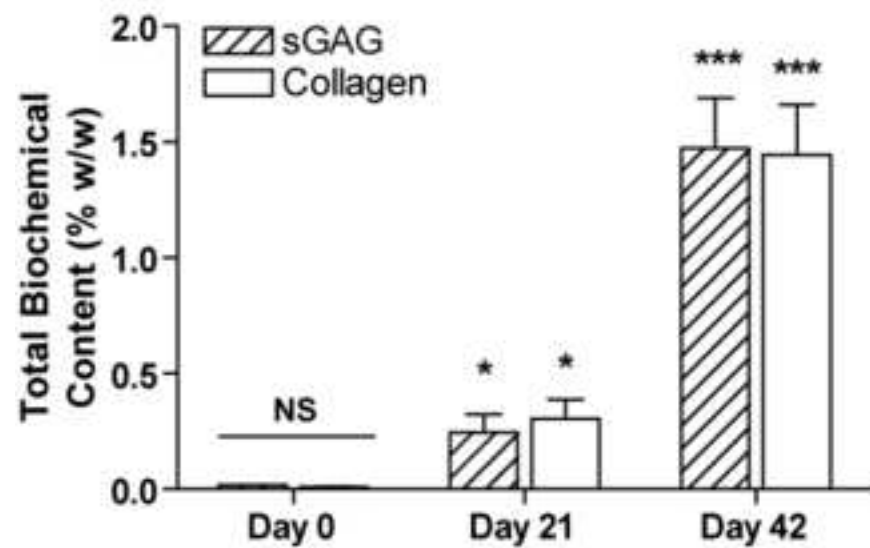
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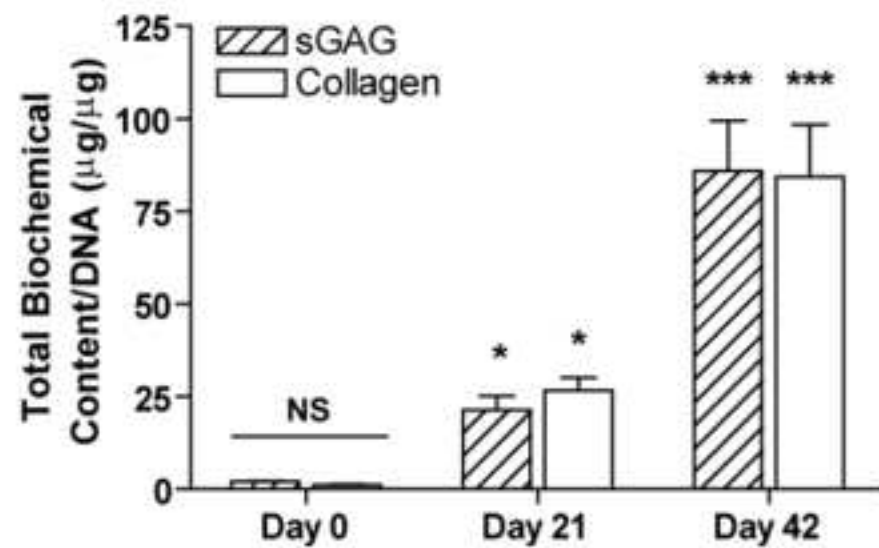
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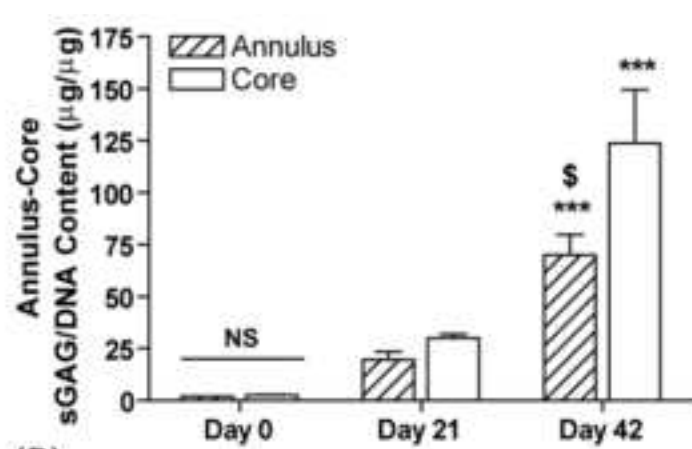
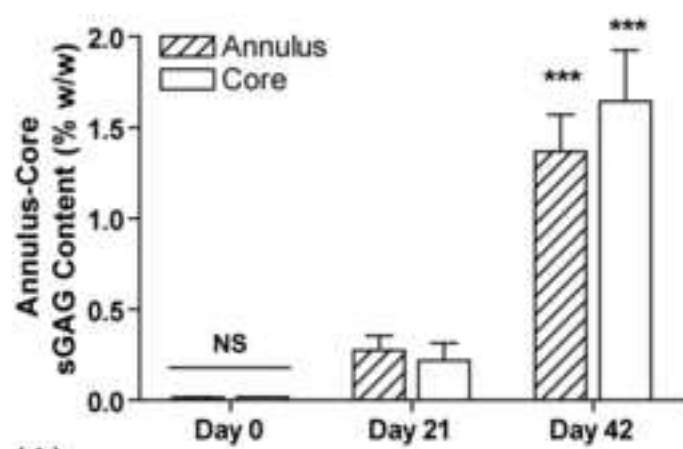
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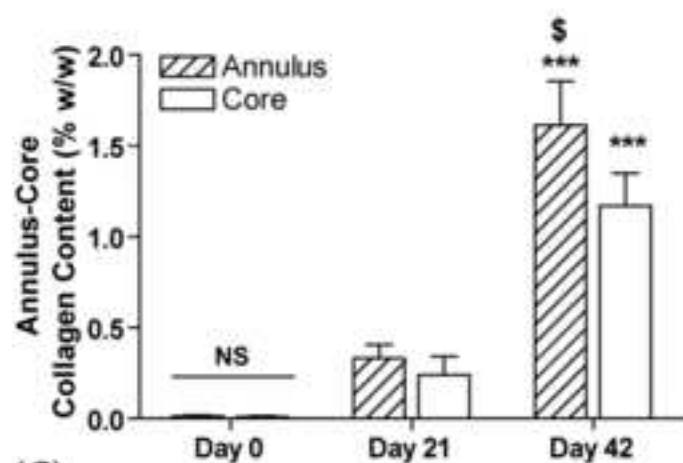
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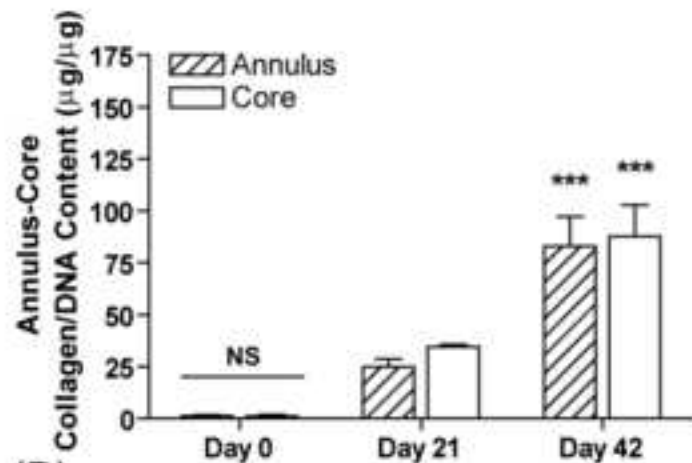


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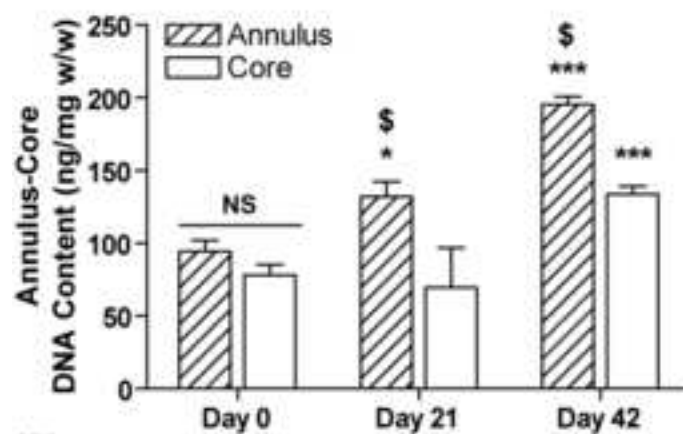
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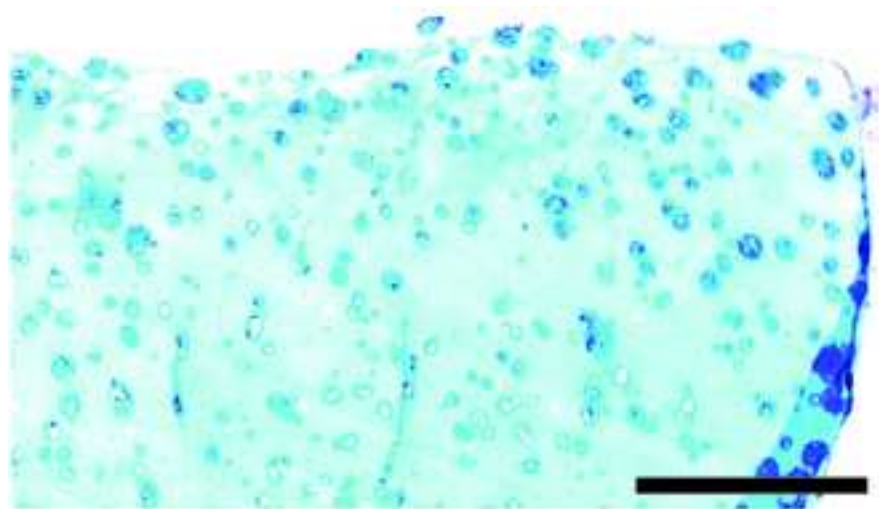


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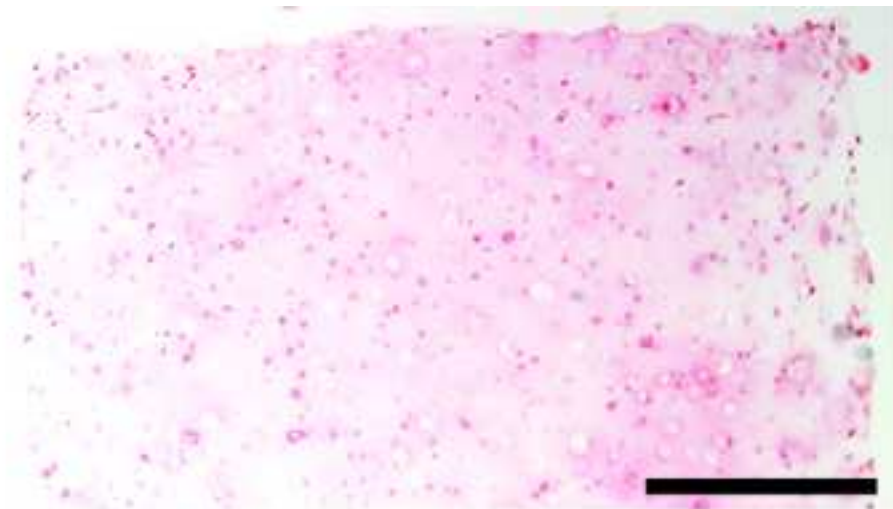


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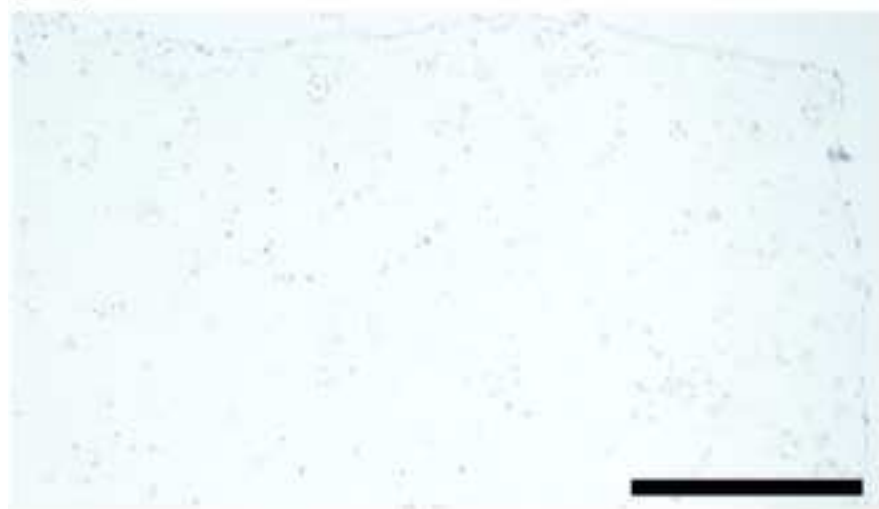
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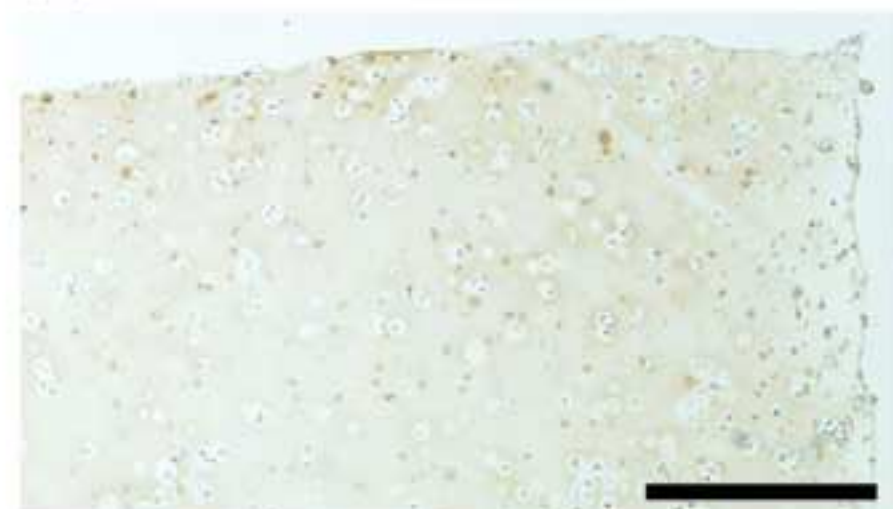
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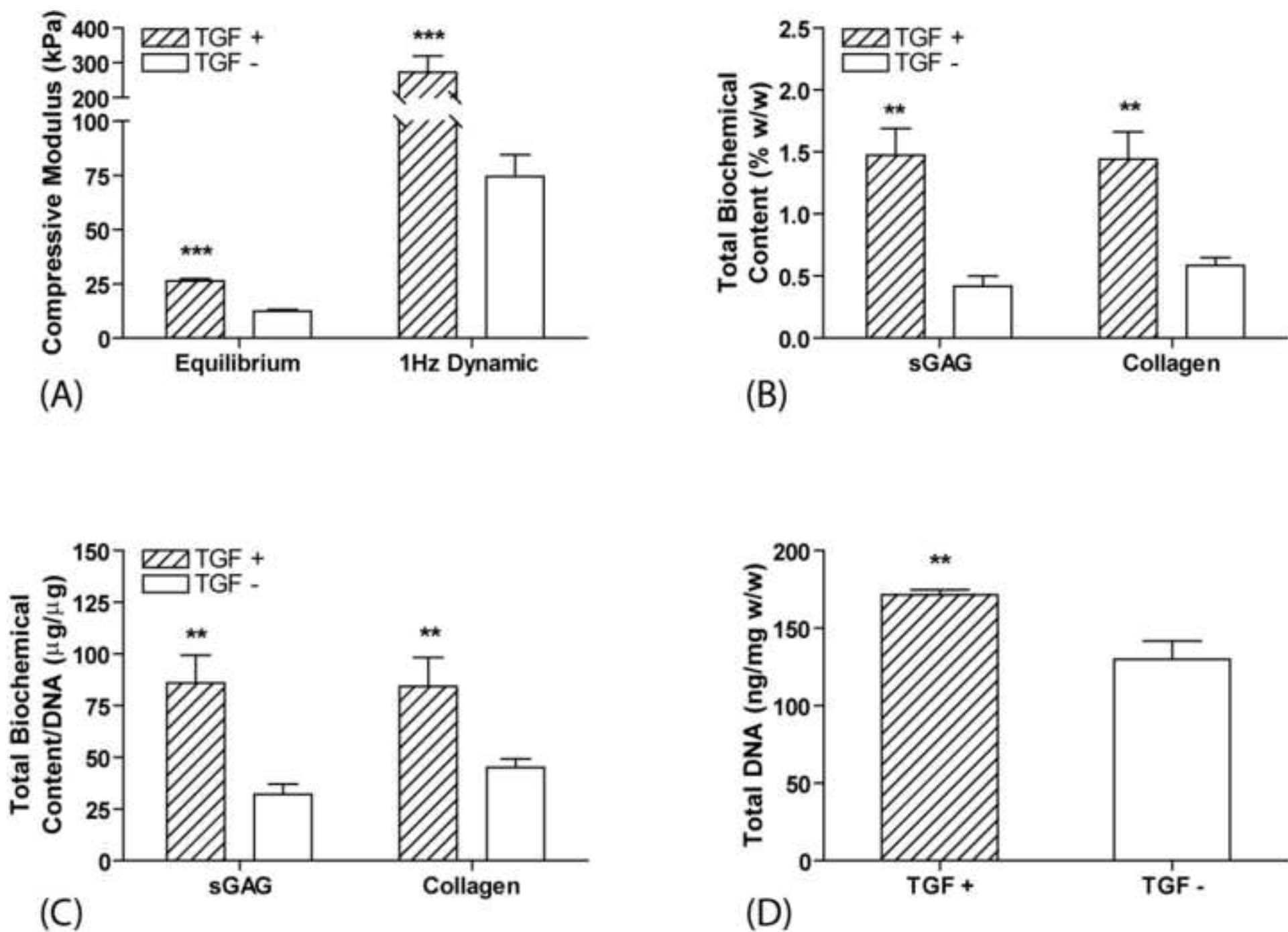


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Figure 4
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Conflict of interest statement

The authors have nothing to disclose.