

Optimization and Validation of a Human Ex Vivo Femoral Head Model for Preclinical Cartilage Research and Regenerative Therapies

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Abstract

Objective. Articular cartilage is incapable of effective repair following injury or during osteoarthritis. While there have been developments in cartilage repair technologies, there is a need to advance biologically relevant models for preclinical testing of biomaterial and regenerative therapies. This study describes conditions for the effective *ex vivo* culture of the whole human femoral head. **Design.** Fresh, viable femoral heads were obtained from femoral neck fractures and cultured for up to 10 weeks in (a) Dulbecco's modified Eagle's medium (DMEM); (b) DMEM + mixing; (c) DMEM + 10% human serum (HS); (d) DMEM + 10% HS + mixing. The viability, morphology, volume, and density of fluorescently labelled *in situ* chondrocytes and cartilage surface roughness were assessed by confocal microscopy. Cartilage histology was studied for glycosaminoglycan content using Alcian blue and collagen content using picrosirius red. **Results.** Chondrocyte viability remained at >95% in DMEM + 10% HS. In DMEM alone, viability remained high for ~4 weeks and then declined. For the other conditions, superficial zone chondrocyte viability fell to <35% at 10 weeks with deeper zones being relatively unaffected. In DMEM + 10% HS at 10 weeks, the number of chondrocytes possessing cytoplasmic processes increased compared with DMEM ($P = 0.017$). Alcian blue labeling decreased ($P = 0.02$) and cartilage thinned ($P \leq 0.05$); however, there was no change to surface roughness, chondrocyte density, chondrocyte volume, or picrosirius red labeling ($P > 0.05$). **Conclusions.** In this *ex vivo* model, chondrocyte viability was maintained in human femoral heads for up to 10 weeks in culture, a novel finding not previously reported. This human model could prove invaluable for the exploration, development, and assessment of preclinical cartilage repair and regenerative therapies.

Keywords

femoral head, articular cartilage, chondrocytes, disuse atrophy, phenotype

Introduction

Articular cartilage has very poor regenerative potential following injury and the repair tissue formed is mechanically weak, and has a fibro-cartilaginous, rather than the resilient load-bearing extracellular matrix (ECM) of hyaline cartilage.¹ Furthermore, the native regeneration potential of cartilage declines with age² and while fibro-cartilaginous repair can be observed to form within injured hyaline cartilage, it is more evident when the injury has penetrated the subchondral bone.^{1,3} The reasons for the production of mechanically incompetent repair tissue are not well understood, and clearly there is intense interest in developing more effective biomaterial and regenerative therapies for cartilage repair. However, current models are not optimal and usually involve preparations ranging from *in vitro*

cultures of cells, through to osteochondral explants⁴ and *ex vivo* and *in vivo* models of mainly animal (i.e., nonhuman) joints.^{5,6} The research and development of more effective cartilage repair and regenerative therapies would be enhanced by the ability to preclinically evaluate novel

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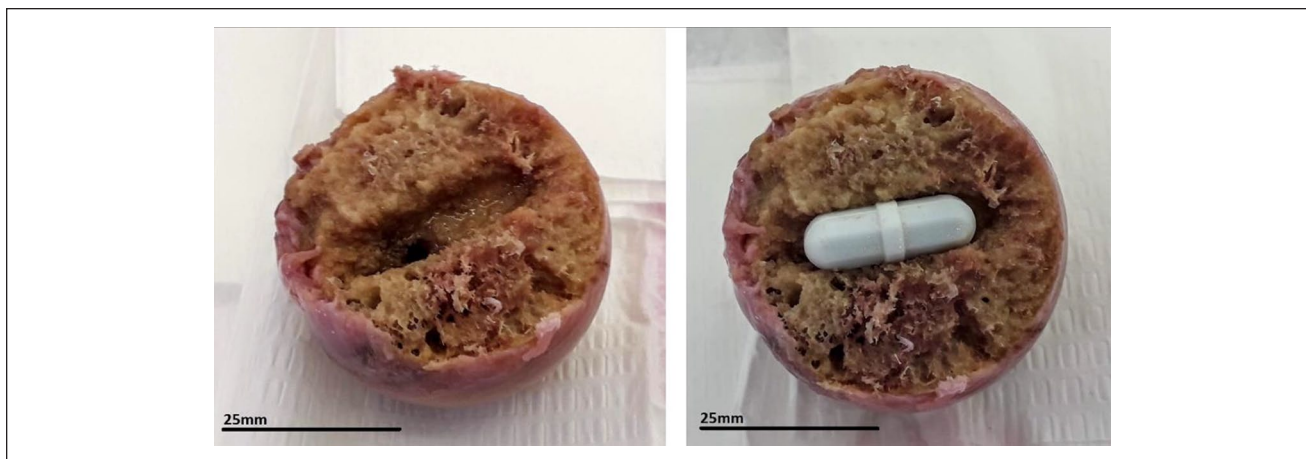


Figure 1. The cavity produced in the cancellous bone of the human femoral head to accommodate the magnetic bar for stirring culture experiments.

strategies in *ex vivo* physiological, tribological models of natural joints.

While there have been many detailed studies on explant and organ culture of animal osteochondral tissue (e.g., Lin *et al.*⁷), few studies have conducted experiments directly on human tissue. The limited studies using human cartilage for experimentation have obtained the material as discarded, and frequently degenerate osteochondral tissue (e.g., during joint replacement surgery for osteoarthritis).⁸ To test orthobiological treatments for human cartilage repair, the use of healthy cartilage is essential. However, this source of material is difficult to obtain and relies on collaboration between clinicians, theatre staff, and research scientists. While normal human cartilage may be obtained from amputations, trauma victims, or occasionally cadavers, such material is rarely available for wider adoption into experimental human cartilage research. *Ex vivo* organ culture is becoming increasingly important for basic and applied biomedical research because it is more representative of normal cellular behavior. However, obtaining a steady supply of viable human tissue, which then has to be cultured for weeks under aseptic conditions, remains a challenging research area.

Femoral neck fractures are one of the most common surgically treated injuries in elderly patients. The human femoral head is discarded during the surgery undertaken to treat femoral neck fractures and is replaced with an artificial prosthesis. While this discarded femoral head is aged, it is generally nondegenerate. We hypothesized that the human femoral head would be a viable source of normal, nondegenerate articular cartilage suitable for preclinical cartilage research and regenerative therapies. We were able to coordinate the timely, sterile collection and delivery of the discarded human femoral head from the operating theatre to the laboratory. The aim of this study was to optimize the culture conditions for fresh human femoral heads from femoral neck fractures to permit viable long-term (10 weeks) culture.

Methods

Human Femoral Heads

Femoral heads were obtained with ethical permission (Tissue Governance, National Health Service, Lothian) and patient consent from 15 patients (11 females, 4 males, mean age 75.5 years [range 56-88 years]) undergoing hemiarthroplasty or total hip replacement for femoral neck fracture (FNF). Femoral heads were carefully removed intraoperatively by a qualified orthopedic surgeon using a corkscrew device and immediately placed into a sterile container with saline (0.9% w/v; 21°C) to prevent chondrocyte death from drying.⁹ Femoral heads were then transferred to a sterile container with Dulbecco's modified Eagle's medium (DMEM) with D-glucose (25 mM), L-glutamine (4 mM), pyruvate (1 mM), 100 U/mL penicillin, 100 µg/mL streptomycin, 2.5 mg/mL amphotericin B (Sigma-Aldrich, Irvine, UK), and 10 µg/mL Fungin™ (InvivoGen, Toulouse, France) ready for transportation to the laboratory with the femoral heads being available for experiments within 1 to 2 hours.

Culture Conditions

Femoral heads were maintained in culture (37°C) in sealed single use sterile containers and media changed every 3 days and the container replaced with every media change. Media volume for each femoral head was ~50 to 60 mL. Male femoral heads were larger than those of females (diameter typically 6 cm vs. 4.5 cm) and required larger culture containers. The femoral heads were cultured for up to 10 weeks under the following conditions: (a) static culture in DMEM, (b) culture in DMEM with movement (DMEM + mixing), (c) as for (a) above + 10% normal human serum (HS; Merck, Feltham, UK), and (d) as for (b) above + 10% HS. A cavity was carved in the cancellous bone of the femoral head for a magnetic stirring bar using bone trimmers (**Fig. 1**). Containers were then placed on a magnetic mixer inside the

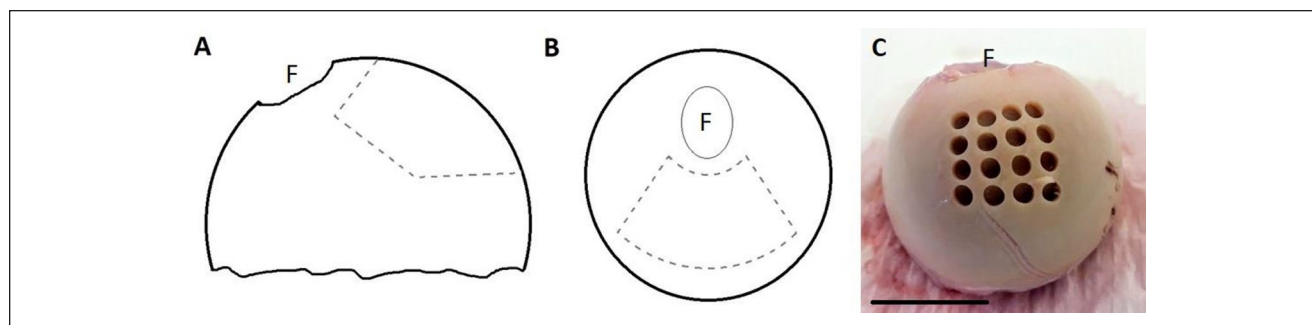


Figure 2. Localisation of the cartilage area on human femoral heads used for sampling. (A) Lateral view of the femoral head, fovea (F) on the upper left, (B) Top view of the femoral head, (C) Femoral head with biopsy explants taken from within the specified zone. Scale bar represents 25mm.

incubator and stirring was programmed for 1 hour, twice daily at ~ 1 Hz. The rationale for studying the effects of movement was based on our proof of concept bovine model where joint movement promoted chondrocyte viability.⁷

Cartilage Sampling, Fluorescent Labeling of In Situ Chondrocytes and Confocal Microscopy

Full depth cartilage explants were harvested using 3- or 5-mm diameter biopsy punches (Kai Medical, Solingen, Germany) for weekly chondrocyte viability measurements. To minimize the variability, explants were taken from random areas within the load-bearing parafoveal superior region¹⁰ (**Fig. 2**). Cartilage samples were then incubated (1.5 hours; 21°C) with CMFDA (5-chloromethylfluorescein diacetate) Cell Tracker green and propidium iodide (PI) (12.5 and 10 μM , respectively; Invitrogen, Paisley, UK) to label living (green) and dead (red) cells respectively.¹¹ Explants were washed in phosphate-buffered saline (PBS; Invitrogen, Paisley, UK), fixed (formaldehyde 4%v/v; 30 minutes; Fisher, Leicestershire, UK) and imaged in 3 dimensions by confocal laser scanning microscopy (CLSM) using established methods.¹²

Measurements of Chondrocyte Viability, Density, Volume, and Morphology

Confocal projected axial views were analyzed using ImageJ/FIJI (National Institutes of Health) and IMARIS software (Zurich, Switzerland) as described.¹² Chondrocyte viability (% live cells) was calculated as: the number of CMFDA-labeled cells/(number of CMFDA-labeled cells + number of PI-labeled cells) in a given region of interest (ROI) volume. For chondrocyte density, the total number of cells (CMFDA-labeled and PI-labeled) in the ROI volume were counted in IMARIS, and results given as cells/ μm^3 . Chondrocyte volumes were obtained using the IMARIS “Surfaces” algorithm. Volume calibration was performed

using fluorescent microspheres (Polysciences, Warrington, PA, USA). Chondrocyte morphology was considered “normal” if cells were visualized as having a “smooth” surface and elliptical/rounded shape. “Abnormal” chondrocytes exhibited at least one CMFDA-labeled cytoplasmic process ≥ 2 μm long. Abnormal cells were counted manually and divided by the total number of live cells in the field of view with results presented as the % abnormal cells in the whole cell population within the ROI.¹²

Histology, Cartilage Thickness, and Surface Roughness

Explants were frozen (-80°C) in a freezing medium (1:1 optimal temperature cutting compound with 30% w/v sucrose in PBS for histology.^{13,14} The plugs were then cut into 40 μm sections and stained with Alcian blue (Vector Laboratories Ltd, Peterborough, UK) to label cartilage glycosaminoglycans (GAGs) according to manufacturer’s instructions. For picrosirius red staining of collagens, sections of 10 μm thickness were first stained with hematoxylin for 8 minutes, then washed 3 to 4 times in distilled water. This was followed by 1-hour incubation in 0.1% picrosirius red (Direct Red 80, Sigma-Aldrich, Irvine, UK) in picric acid (VWR International, Lutterworth, UK). Sections were washed briefly 2 times in 0.5% acetic acid, dehydrated (2 washes in ethanol absolute followed by 1 wash in xylene) and mounted in resinous mounting medium. Histological sections were imaged on a Leica bright field microscope and analyzed with ImageJ/FIJI software.¹⁵ After converting the images to greyscale 8-bit pixel depth, the same intensity threshold was set for each pair of sections (week 0 vs. week 10). The stained area above the threshold was measured and presented as a % of the total sectional area. Cartilage thickness measurements were performed on the coronal sections of cartilage using ImageJ/FIJI. For the surface roughness measurements, the ImageJ/FIJI Analyse Stripes macro was used on the images of histologically stained sections.

Calculation of cartilage surface roughness was based on the deviation from an idealized smooth cartilage surface and expressed as Rq (the root mean square [RMS] deviation) in micrometers (μm).

Data Presentation and Statistical Analysis

Statistical analyses were performed using Graphpad Prism version 8.2.1 (GraphPad Software, La Jolla, CA, USA). Data were presented as $(N(n))$, with N representing the number of independent femoral heads and n the total number of replicates. Each data point presented on graphs and used for statistical analyses was an average (\pm SD or SEM as indicated) of the replicates taken from each femoral head. Unless otherwise stated, paired Student's t tests were used to compare differences within pairs of treatment groups or time points, and analysis of variance (ANOVA) used to compare differences across several groups. A significant difference was accepted when $P < 0.05$.

Results

Cartilage Grading and Chondrocyte Viability

Femoral heads were macroscopically assessed using an established system.¹⁶ For all femoral heads considered suitable, the cartilage was grade 0 over $>75\%$ of the surface, with small isolated grade 1 lesions in the parafoveal area (which were $<25\%$ of the surface area). The isolated areas of grade 1 cartilage (mild surface fibrillation) were not studied and only cartilage of grade 0 was used.

Chondrocyte viability was assessed on day 1 and any femoral heads with a viability of $<80\%$ were excluded. Out of a total of 24 femoral heads received, 5 were excluded due to low initial chondrocyte viability and a further 4 were also excluded as they developed infection during subsequent culture. In the remaining 15 femoral heads, the cell viability was $95.3\% \pm 5.3\%$ ($N(n) = 15(60)$) on day 1. Chondrocyte viability in femoral heads cultured under static conditions in DMEM remained high ($>90\%$) for ~ 4 weeks but decreased to $44.0\% \pm 22.3\%$ by week 10 ($N(n) = 4(16)$, one-way ANOVA, posttest for trend, $P = 0.045$, **Fig. 3A**). This decrease in cell viability was accelerated with movement of the femoral head and stirring of the media with virtually no viable cells by week 6 (2-way ANOVA; $P = 0.0099$, **Fig. 3A**). The addition of 10% HS to DMEM maintained chondrocyte viability at $>90\%$ to week 10 under static femoral head culture conditions (**Fig. 3B**) but did not significantly improve the viability in femoral head cultures with movement/stirring over 10 weeks.

CLSM permits 3-dimensional imaging and quantitative analyses of chondrocyte viability within the full depth of cartilage.¹⁷ To assess if chondrocytes within any zone were

more sensitive during culture, chondrocyte viability was determined as a function of depth from the articular surface. We compared the percentage cell viability within the different zones (superficial [SZ], middle [MZ], and deep [DZ]) in coronal sections of articular cartilage at week 10 compared with baseline (week 0). There was a decrease in the SZ viability in both culture conditions (DMEM only, and DMEM + mixing; [$P = 0.0425$ and $P = 0.016$, respectively; **Fig. 4A**]) with relative preservation of cell viability in deeper cartilage zones (**Fig. 4B**). This suggests that SZ chondrocytes were far more sensitive to the culture conditions compared with the cells in the deeper zones. Thus, although there were few living SZ cells remaining after 10 weeks, a substantial portion of chondrocytes in the other zones were still viable.

Chondrocyte Morphology

In fresh explants of macroscopically nondegenerate femoral head cartilage, a small population ($8.0\% \pm 1.5\%$; $N(n)=4(8)$) of cells in the SZ ($\sim 100 \mu\text{m}$ from the surface) demonstrated one or more cytoplasmic processes (**Fig. 5A and B**). During femoral head culture in DMEM, there appeared to be an increase in the % of cells with processes (to $15\% \pm 5.3\%$ by week 6, $N(n) = 4(16)$) and ($16\% \pm 6.4\%$ by week 10, $N(n) = 4(16)$); however, these changes were not significantly different compared with week 0 (ANOVA; $P > 0.05$). In contrast, in the presence of HS, the % of chondrocytes with processes increased to $31\% \pm 9.3\%$ by week 6 ($P = 0.008$; $N(n) = 3(12)$) and $37\% \pm 7.1\%$ by week 10 ($P = 0.002$; $N(n) = 3(12)$) compared with week 0 (**Fig. 5B**). By week 10, there were significantly more (by >2 -fold; $P = 0.017$) chondrocytes with cytoplasmic processes when femoral heads were cultured in DMEM + HS compared with DMEM alone (**Fig. 5B**).

Extracellular Matrix Composition

To evaluate whether extracellular matrix composition changed during culture, two histological stainings were performed on cartilage samples (week 0 and week 10) and analyzed semiquantitatively. There was a general trend of decreasing GAG staining using Alcian blue¹⁸ under all culture conditions, but in DMEM + HS chondrocyte viability was significantly higher (**Fig. 6A**). In these cultures, $50.0\% \pm 2.7\%$ ($N(n) = 3(6)$) of the section area was stained with Alcian blue at week 0. This decreased to $17.0\% \pm 13.8\%$ by week 10 ($N(n) = 3(6)$; $P = 0.028$). The total collagen stained with picrosirius red did not show any significant difference between DMEM and DMEM + HS samples at week 0 and week 10 ($30\% \pm 5\%$ vs. $42\% \pm 22\%$, $N(n) = 11(22)$; $P = 0.09$). These results suggest a significant loss

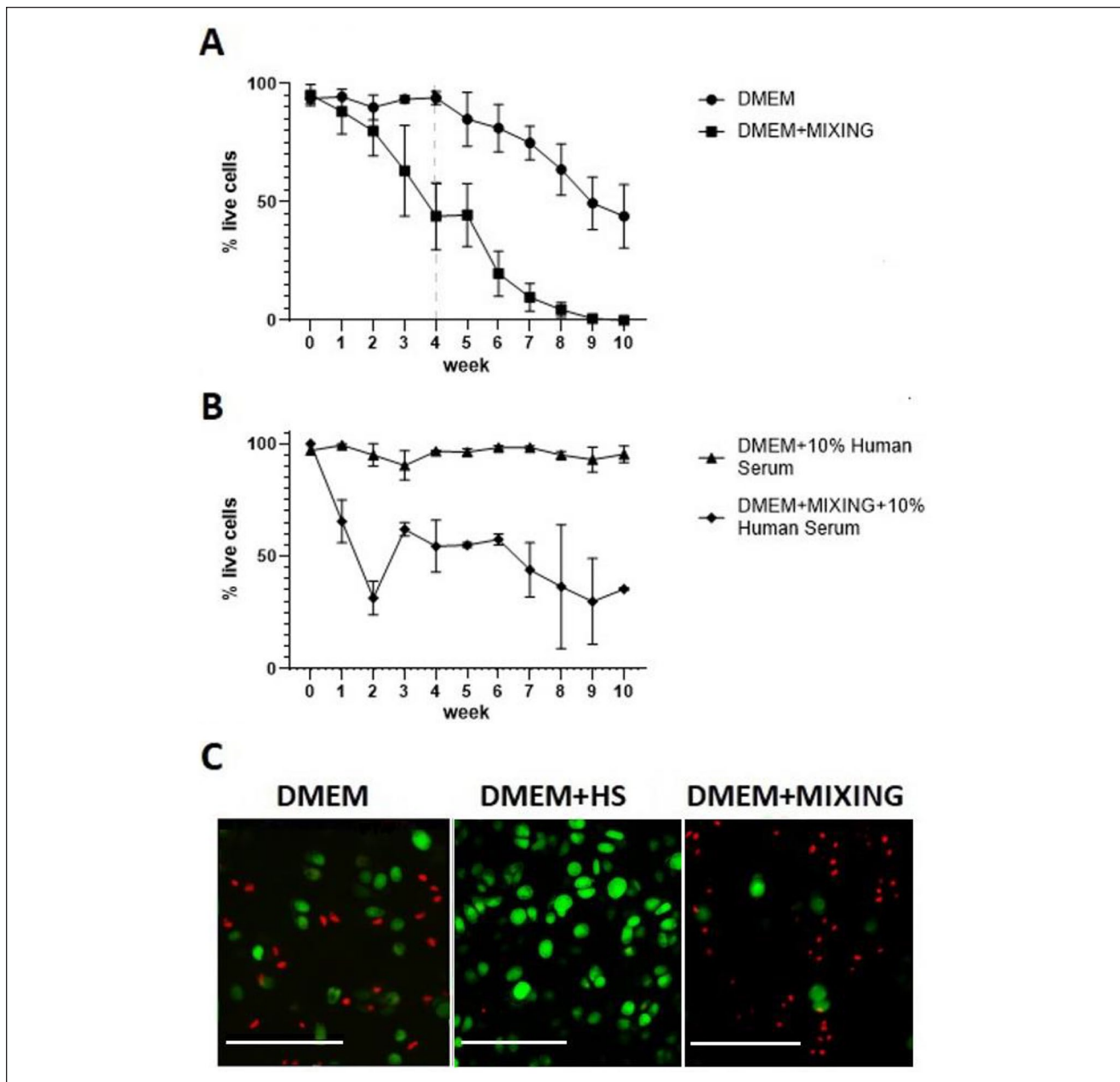


Figure 3. The viability of *in situ* human chondrocytes within femoral head cartilage cultured under various conditions. (A) DMEM $N(n) = 4(16)$; DMEM + mixing ($N(n) = 3(12)$). (B) DMEM + normal human serum (HS); ($N(n) = 3(12)$), DMEM + mixing + human serum ($N(n) = 2(8)$); error bars represent S.E.M. (or S.D. for $N = 2$). (C) Representative images of viability staining (CMFDA green – live cells, PI red – dead cells) at week 10 for DMEM, DMEM + 10% human serum and DMEM + mixing; scale bars represent $100\mu\text{m}$.

of GAGs but no change to the total collagen content of femoral head cartilage during this culture period.

Cartilage Thickness and Surface Roughness

In parallel with the loss of GAGs, cartilage thickness was reduced after 10 weeks in culture (Fig. 6B). In DMEM

cultures, it decreased from $3808 \pm 425 \mu\text{m}$ to $2828 \pm 542 \mu\text{m}$ ($P = 0.05$; $N(n) = 4(22)$). In the DMEM + mixing culture, it declined from $4226 \pm 418 \mu\text{m}$ to $2703 \pm 720 \mu\text{m}$ ($P = 0.02$; $N(n) = 4(15)$), and in DMEM + 10% HS it decreased from $3377 \pm 360 \mu\text{m}$ to $2349 \pm 160 \mu\text{m}$ ($P = 0.02$; $N(n) = 3(22)$). Articular surface roughness assessed on the same femoral heads at week 0 and week 10 was not

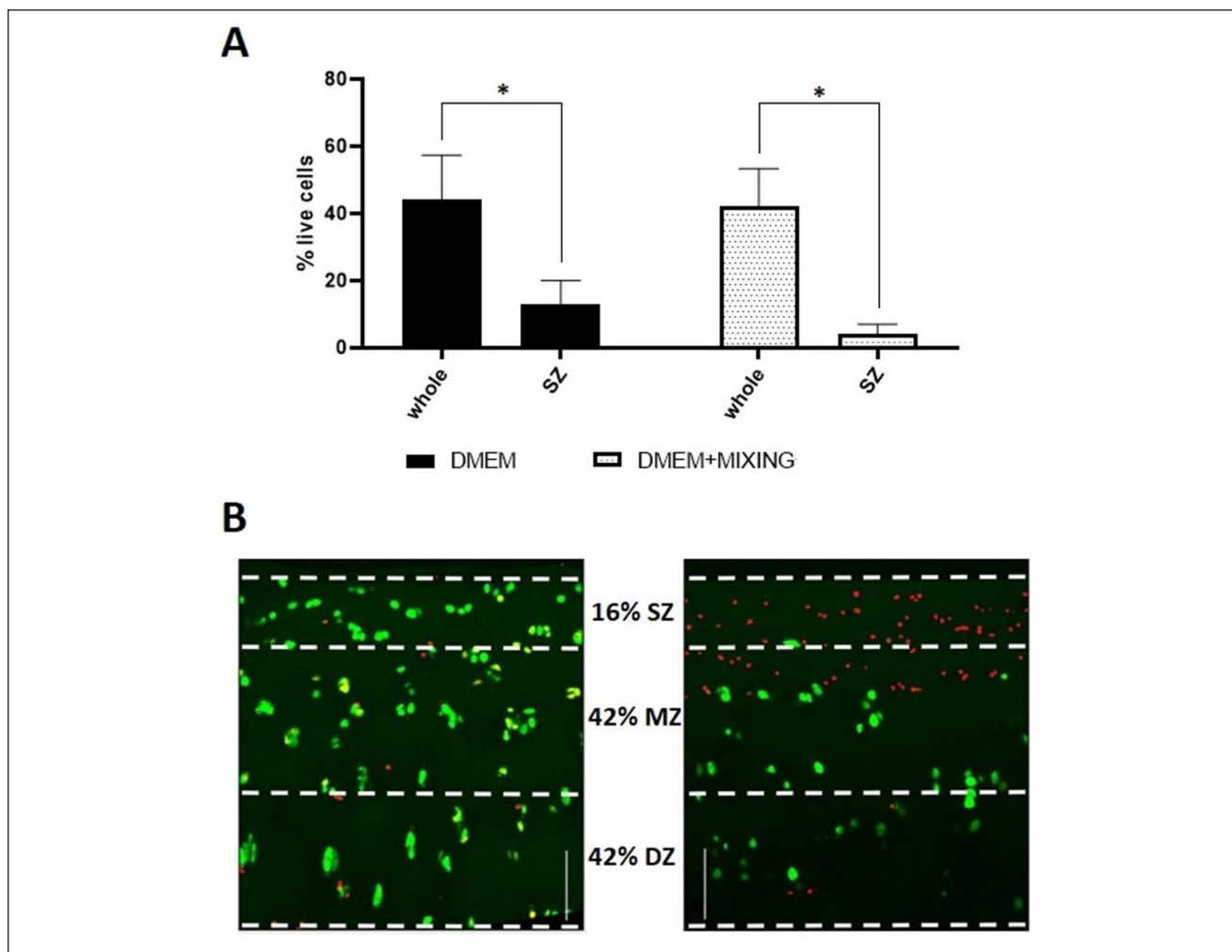


Figure 4. Viability of chondrocytes in various zones after 10 weeks of femoral head culture (A) Comparison of chondrocyte viability in the whole thickness vs superficial zone at week 10 of culture. DMEM $N(n) = 4(16)$, $*p = 0.0425$, DMEM + mixing, $N(n) = 3(12)$, $*p = 0.016$ (data shown as mean \pm S.E.M.). (B) Representative coronal images of labelled chondrocytes within cartilage sections cultured in DMEM on day 0 (left panel), and after 10 weeks of culture in DMEM + mixing + HS on week 10 (right panel). The scale bars represent 100 μ m.

significantly different ($13 \pm 0.6 \mu\text{m}$ and $15 \pm 0.7 \mu\text{m}$, respectively ($P = 0.55$; $N(n) = 9(9)$; **Fig. 7A**)).

Chondrocyte Density and Volume

There was no difference in cell density for all the samples at week 0 ($9971 \pm 2389 \text{ cells/mm}^3$, $N(n) = 15(60)$) compared with week 10 ($11256 \pm 3305 \text{ cells/mm}^3$; $P = 0.01$; $N(n) = 15(60)$; **Fig 7B**). *In situ* chondrocyte volume was also analyzed as it correlates with the progression of cartilage degeneration; however, there was no difference ($P = 0.22$) between the cell volumes of chondrocytes on day 0 ($423 \pm 49 \mu\text{m}^3$, $N(n) = 15(60)$) and week 10 ($441 \pm 48 \mu\text{m}^3$, $N(n) = 15(60)$; **Fig. 7C**).

Discussion

We have established culture conditions that maintain chondrocyte viability during *ex vivo* culture of human femoral heads for 10 weeks by supplementing standard culture medium with 10% normal HS. We have identified an excellent and reliable source of viable, nondegenerate human articular cartilage ideal for *ex vivo* experimentation. Previously, we have investigated the microscopic effects of mechanical and other forms of injury on human articular cartilage.^{9,19} However material was obtained from tissue discarded during knee replacement for osteoarthritis and the yield of nondegenerate tissue was often low. This was because the majority of the tissue was osteoarthritic with loss of superficial zone cells, even if macroscopically the

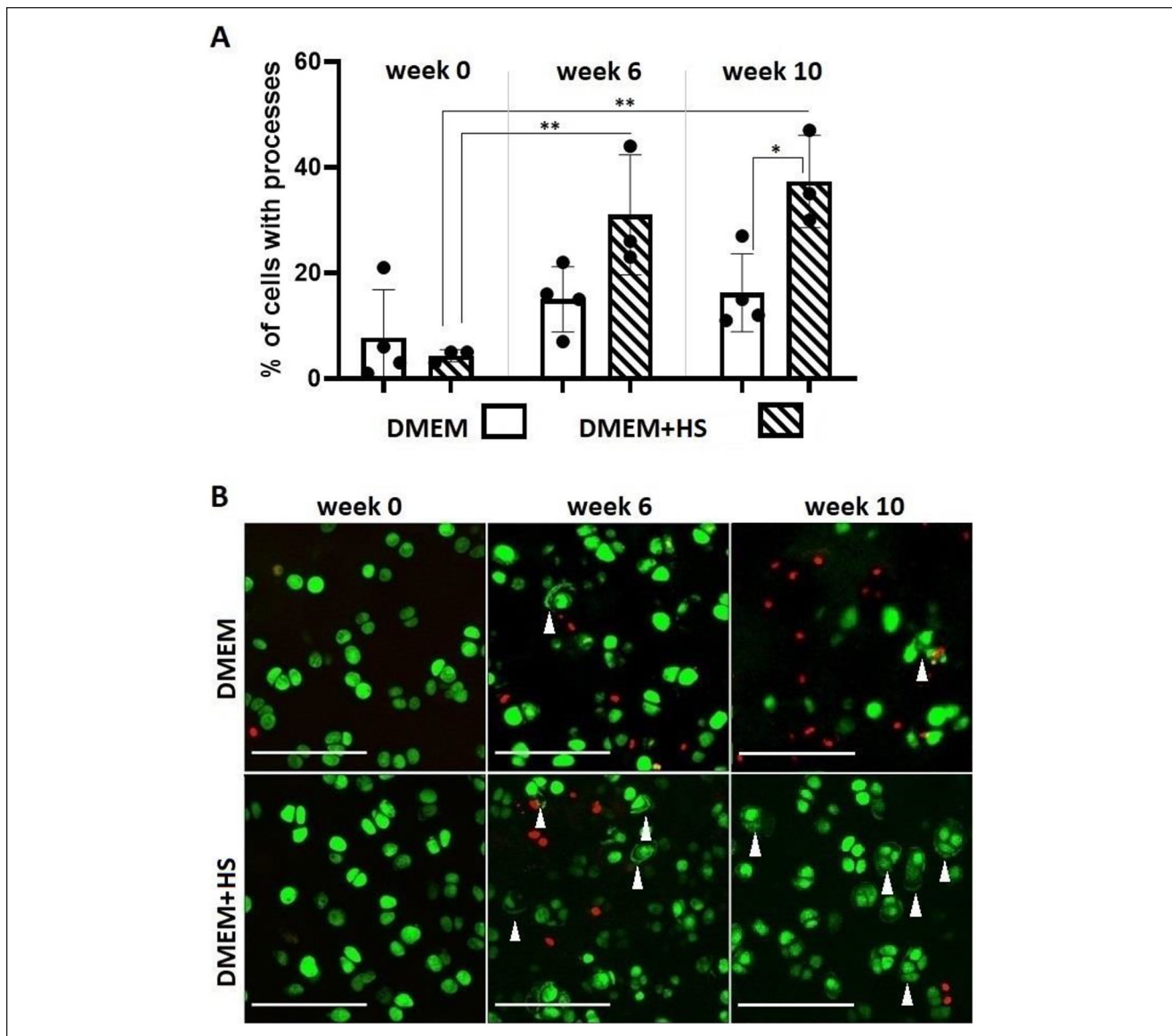


Figure 5. Changes to the morphology of *in situ* femoral head chondrocytes during culture. Panel (A) shows the % of cells with cytoplasmic processes at three time points (weeks 0, 6 and 10) in cultures with DMEM (N(n) = 4(16)) and DMEM + human normal human serum (HS) (N(n) = 3(12)). (Data shown as mean \pm S.D.). Panel (B) shows representative images of cell stained with CMFDA Cell Tracker green to visualise chondrocyte morphology, and propidium iodide (PI) red to identify dead cells. The top row of images is from DMEM cultures, (left to right panels for weeks 0, 6 and 10 respectively), the bottom row of images for DMEM + human serum (left to right panels for weeks 0, 6 and 10 respectively). Note examples of chondrocytes with cellular processes indicated by white arrowheads. The scale bar represents 100 μ m.

tissue may have appeared nondegenerate. In contrast, the cartilage of the femoral head discarded after femoral neck fracture was in most cases nondegenerate and our experiments have confirmed the presence of viable cartilage tissue that is macroscopically and microscopically ideally suited for investigating cartilage repair and regeneration. Due to the excellent chondrocyte viability throughout culture, the model may also allow *ex vivo* validation of an optimal combination of cells, growth factors, and scaffolds that

lead to the formation of repair tissue resembling the desirable hyaline articular cartilage at the microscopic level.

Current knowledge of the microscopic quality of cartilage repair tissue in humans is based on histological assessment of opportunistic biopsy specimens retrieved during “second look” arthroscopy (keyhole surgery). While these specimens have provided valuable insight into the quality of cartilage repair, with so-called “hyaline-like” composition, the information is limited by small

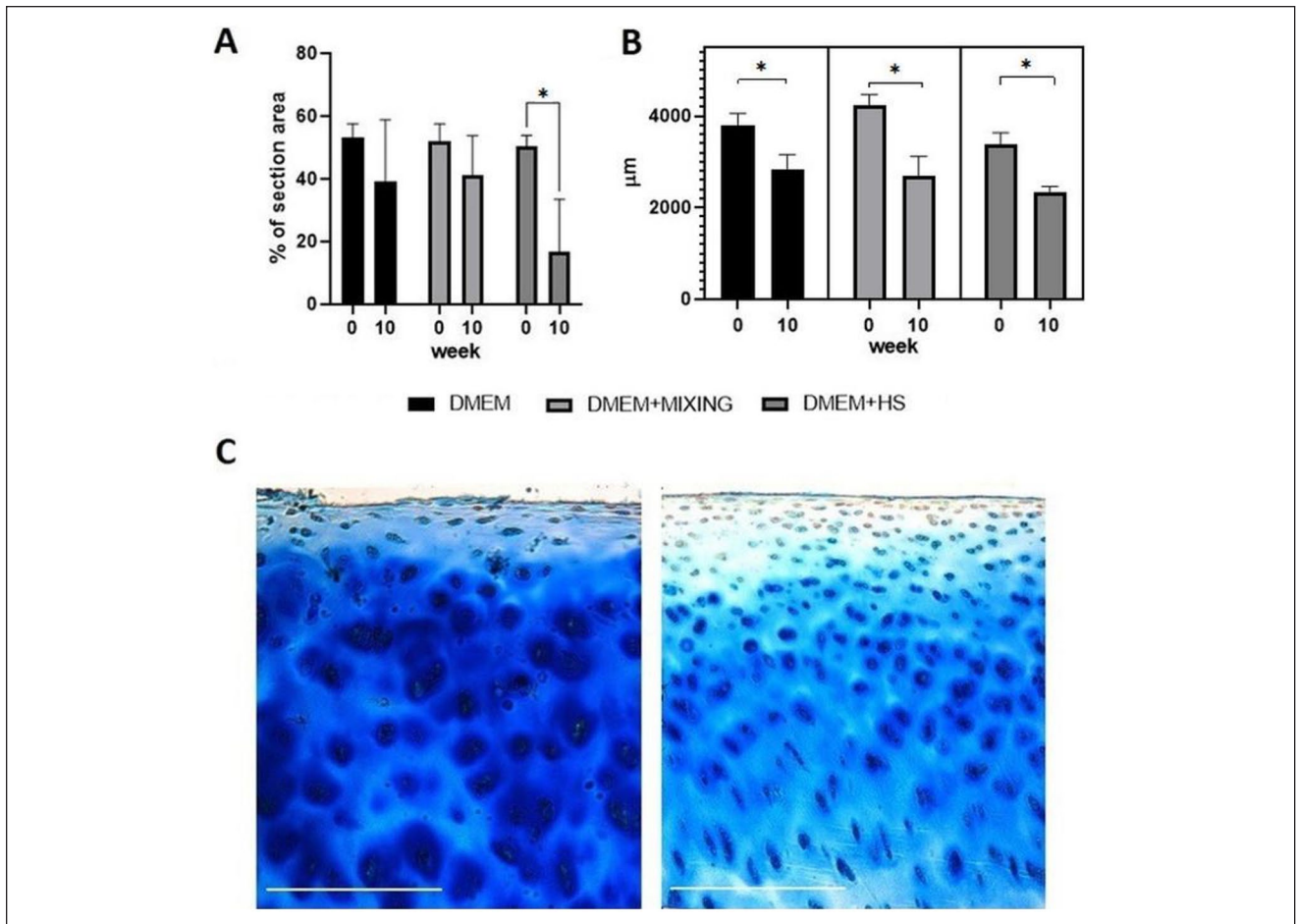


Figure 6. Histology of femoral head cartilage with time in culture. (A) GAG content in various culture conditions, (a) DMEM N(n) = 4(8), (b) DMEM + mixing N(n) = 4(8), (c) DMEM + 10% human serum N(n) = 3(6), * $p = 0.028$. (B) Cartilage thickness in various culture conditions, (a) DMEM N(n) = 4(24), (b) DMEM + mixing N(n) = 4(24), (c) DMEM + 10% human serum N(n) = 3(18), ($p = 0.05$; $p = 0.02$; $p = 0.02$ respectively). (Data shown as mean \pm S.E.M.). (C) Representative images for Alcian blue staining of DMEM + human serum on week 0 (left panel) and week 10 (right panel).

numbers of specimens, distortion of the tissue during biopsy, variability in the site/size of biopsies and heterogeneity of the study sample. The lack of a noninvasive method of evaluating the microscopic characteristics of the quality of the cartilage repair tissue has also been recognized as a major problem limiting advances in cartilage repair and regenerative techniques by the International Cartilage Repair Society (ICRS).²⁰ Modern imaging techniques (e.g., CLSM) allow microscopic examination of articular cartilage by optically sectioning the tissue. We believe that the significantly easier access to normal, human cartilage *ex vivo* will help overcome the problems associated with *in vivo* biopsy, and allow detailed quantitative microscopic assessment and optimization of the quality of cartilage repair. The proposed model will significantly enhance our ability to test a wide range of preclinical therapeutic cartilage repair and regenerative strategies directly in human

tissue so that the best candidate therapies can be identified for subsequent clinical study.

In our model, the large area of grade 0 cartilage (Fig. 1) permitted multiple samples to be taken either at a single time point, or a smaller number over a longer time course. A gap between samples was retained so that cutting trauma using the biopsy punch²¹ did not influence neighboring samples. Furthermore, if cartilage wells were to contain biological models for testing, then it would be possible for the full depth sample to be “scooped” out using a fine scalpel blade. Chondrocyte viability in both axial and coronal projections was initially high for all samples (Fig. 3A). There was no change in viability during DMEM + HS culture, suggesting that taking multiple cartilage plugs over the 10-week period did not adversely affect the viability of the surrounding cartilage. It should be noted that if serum (HS) was used, then it must be heat-treated because it contains

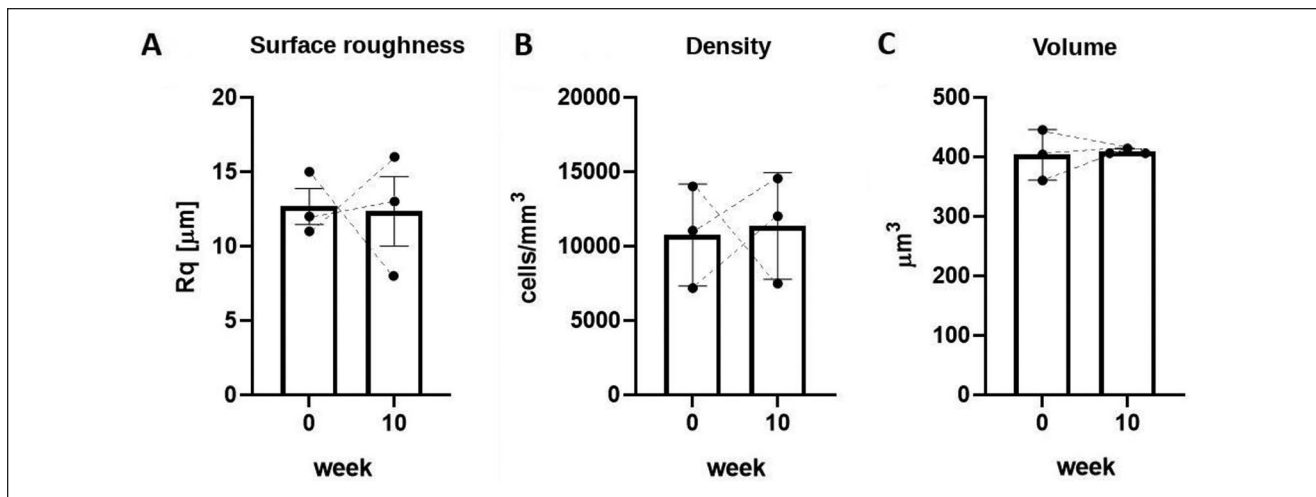


Figure 7. Surface roughness, chondrocyte density and volume of *in situ* femoral head chondrocytes with time in culture in DMEM + HS. (A) Surface roughness expressed as the root mean square (RMS) deviation (in μm) at week 0 and week 10, $N(n) = 3(9)$, $p = 0.2$. (B) Cell density (total number of chondrocytes per mm^3) at week 0 and week 10, $N(n) = 3(60)$, $p = 0.22$. (C) Chondrocyte volume at week 0 and week 10, $N(n) = 3(44)$, $p = 0.22$. Broken lines illustrate pairs of data at week 0 and week 10 for each femoral head. (Data shown as mean \pm S.D.).

enzymes that digest the DNA of dead cells leading to an underestimation of the dead cell population.^{22,23}

Femoral head culture in DMEM maintained chondrocyte viability for ~ 4 weeks after which there was an increase in chondrocyte death (Fig. 3A). With mixing, viability decreased progressively after the start of the culture, such that by week 10 there were virtually no remaining viable cells. Furthermore, cell death started in the SZ so that after 10 weeks, all these chondrocytes were dead whereas those in the deeper zones were still viable (Fig. 4). This suggests that there are factors in bone supporting chondrocyte viability in the SZ that were washed out during media changes. The importance of bone in cultures of bovine cartilage has been reported previously as SZ chondrocyte viability was maintained in cartilage cultures when bone was present either when attached to the explants or in co-culture.²⁴ This was in contrast to deep zone chondrocytes which survived, and the relative viability of these chondrocytes within the femoral head cartilage of our elderly patients (Fig. 4) parallels the long-term (~ 25 years) survival of DZ chondrocytes in osteochondral allografts used for the treatment of focal posttraumatic defects in young individuals.²⁵ It is possible that these chondrocytes are well adapted to this relatively hostile environment for example chondrocytes in the DZ utilize different membrane transport systems for the regulation of intracellular acidity compared with cells in the SZ.²⁶ The addition of HS in the mixed DMEM condition provided some protection for SZ chondrocytes as cell viability by week 10 was 35% (Fig. 3B). However, if there was no mixing and HS was present, there was complete chondrocyte protection suggesting that serum was protecting the cells

that were vulnerable during the latter stages of the culture. While the cross-talk between subchondral bone and cartilage has received considerable attention,²⁷ the factor(s) released from bone and/or present in the serum that promote chondrocyte viability are unclear, with TGF β (transforming growth factor- β), IGF-1 (insulin-like growth factor-1), and BMP (bone morphogenetic protein) being implicated.²⁸

Visualisation of fluorescently labeled *in situ* chondrocytes revealed the classical morphology of elliptical cells in the SZ with the more spheroidal forms in the deeper zones (Fig. 4).¹² In grade 0 cartilage in axial projections, a small proportion ($\sim 8\%$) of the SZ cells (within $\sim 100 \mu\text{m}$ depth) exhibited cytoplasmic processes. Of interest was that after 10 wks of culture in DMEM + HS, there was a significant (>4 -fold) increase in the percentage of cells exhibiting processes (Fig. 5A). At week 10, significantly (by >2 -fold) more chondrocytes demonstrated a cytoplasmic process when HS was present compared with culture in DMEM alone. However, in the DMEM + HS condition, chondrocyte viability and density did not change during culture (Figs. 3B and 7B, respectively), whereas in DMEM alone viability had decreased by $\sim 50\%$ (Fig. 3A) but abnormal chondrocytes were still present (Fig. 5B). The changes to chondrocyte shape might be related to the properties of the ECM. In healthy cartilage, the pore size of the proteoglycan (PG) network is $\sim 3 \text{ nm}$ whereas that for collagen is $\sim 100 \text{ nm}$ and thus the PGs will regulate cartilage permeability and solute diffusivity.²⁹ GAG loss during unloading will increase matrix permeability and thus potent growth factors, etc., in serum will start to penetrate and act particularly on SZ chondrocytes, which are normally shielded by the

tight matrix. The development of processes and subsequent abnormal morphology of chondrocytes is a feature associated with de-differentiation to a fibroblastic phenotype^{30,31} and has been observed in osteoarthritic tibial and femoral head cartilage.^{17,32,33} A change in phenotype is characterised by decreased hyaline cartilage-specific collagen type II and aggrecan production, and an increase in collagen type I production. It would be of particular interest to determine if physiological levels of loading could reverse some of these changes and protect the chondrocyte phenotype and promote the production of a hyaline cartilage.¹⁷

There was no evidence of chondrocyte clustering³⁴ under any of the experimental conditions. Nomura *et al.*³⁵ did not observe changes to chondrocyte morphology in mice subjected to hindlimb unloading when cartilage was studied by histology. This might appear to conflict with the changes to chondrocyte morphology reported here. However, the detection of the fine cytoplasmic process is not possible with their histological techniques as they involve tissue shrinkage³⁶ and high-resolution imaging of unperturbed *in situ* chondrocytes is essential for the visualization of the processes.^{17,31}

It might be considered that a limitation in our study was that no mechanical load was applied to the femoral heads. However, this revealed that after 10 weeks there were features of the cartilage ECM and *in situ* chondrocytes that bear similarities to the changes observed with both *in vivo* and *in vitro* cartilage disuse atrophy. Alcian blue staining reflecting GAG content was decreased (**Fig. 6A** and **C**). This was not significant in the DMEM and DMEM + mixing conditions when there was substantial chondrocyte death (~50% and 100%, respectively). However, interestingly, the decrease was significant in the DMEM + HS condition (**Fig. 6A**) when cell viability was high (>95%). This suggests that there was an *active* chondrocyte-driven process mediating reduced GAG levels.³⁷ GAG loss would probably account for the cartilage thinning (**Fig. 6B**), reported by others using animal joint immobilization models.³⁵ GAG loss did not, however, affect surface roughness (**Fig. 7A**), which is in contrast to the changes occurring in OA, where GAG levels decrease and surface roughness and cartilage fibrillation increase.²⁸ Palmoski *et al.*³⁸ noted a similar decrease in PG staining in healthy adult dogs after ~6 days of immobilization and by 8 weeks there was a 30% to 50% reduction in cartilage thickness with an almost complete loss of PG. In human joints immobilized as a result of ankle fracture, there was a 6.6% loss of cartilage thickness over 7 weeks following fracture.³⁹ The loss of GAGs is thought to be due to a reduction in synthesis as well as the stimulation of chondrocyte degradative enzyme (MMP-13, ADAMTS5) activity^{35,37} from a mechano-adaptive response to reduced load. Mechanical loading of joints is a key parameter for maintaining the differentiated, rounded, chondrocyte phenotype.^{40,41} Recent studies implicate an

essential role for the mechanosensitive ion channel Transient Receptor Potential Vanilloid 4 (TRPV4) in the signal transduction pathway. Inhibiting TRPV4 prevents loading-mediated increases in matrix synthesis, whereas activating TRPV4 in the absence of loading increases matrix synthesis in a manner analogous to loading.⁴²

While there were changes to GAG labeling, there was no change to picrosirius red labeling suggesting the cartilage collagen content remained unaltered throughout culture. While picrosirius red does not discriminate between the collagen types,⁴³ it is possible that there were changes to collagen metabolism and/or its organization. For example, while total collagen content might not have changed significantly, it is possible that there was a decrease in the collagen type II: type I ratio reflecting chondrocyte de-differentiation. In addition, changes to collagen fiber distribution/orientation could be evident, and future studies utilising polarized light microscopy would be worth pursuing. A small decrease in collagen cross-linking, which recovers after remobilization, has been reported⁴⁴ and the immobilization of rabbit knee joint leads to a partial shift in the density of collagen composition from type II to type I.⁴⁵ However, in human cartilage, there is negligible/minimal collagen turnover over the lifetime in a healthy joint.⁴⁶

There was no change to the volume of *in situ* chondrocytes following 10-weeks of culture in DMEM + HS (**Fig. 7C**). It is likely that with the loss of GAGs, interstitial osmolarity of the matrix would decrease, leading to hypo-osmolarity and cell swelling. However, *in situ* chondrocytes possess effective volume-regulatory channels and transporters^{47,48} and over a long time period, despite the change in osmolarity, these mechanisms could compensate leading to no volume change. This phenomenon (termed isovolumetric volume regulation [IVR]) has been described in various cell types, including chondrocytes.⁴⁹ The lack of chondrocyte swelling is in contrast to that observed in osteoarthritic cartilage where increased chondrocyte volume/hypertrophy has been reported.^{17,50}

This study described the first successful *ex vivo* culture of a large human joint. *In situ* chondrocyte viability remained high in DMEM + HS; however, it decreased under the other conditions tested. Although chondrocyte viability was optimal, there were changes to the matrix (cartilage thinning, GAG loss, no change to collagen or surface roughness) and chondrocytes (development of cytoplasmic processes, no change to volume or density). This preclinical model may be an invaluable addition for the assessment of human cartilage repair therapies and may replace some animal studies.

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Author Contributions

Study concept and design; Amin, Simpson, Hall. Acquisition of data; Styczynska-Soczka. Analysis and interpretation of data; Styczynska-Soczka, Amin, Simpson, Hall. Manuscript preparation; Styczynska-Soczka, Amin, Simpson, Hall.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Ethical Approval

Femoral heads were obtained with ethical permission from the Tissue Governance, National Health Service, Lothian.

Informed Consent

Informed consent was obtained from all patients.

Trial Registration

Not applicable.

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References

- Hunziker EB, Lippuner K, Keel MJB, Shintani N. An educational review of cartilage repair: precepts & practice—myths & misconceptions—progress & prospects. *Osteoarthritis Cartilage*. 2015;23:334-50. doi:10.1016/j.joca.2014.12.011
- Brittberg M, Gomoll AH, Canseco JA, Far J, Lind M, Hui J. Cartilage repair in the degenerative ageing knee. *Acta Orthop*. 2016;87(sup363):26-38. doi:10.1080/17453674.2016.1265877
- Simon TM, Aberman HM. Cartilage regeneration and repair testing in a surrogate large animal model. *Tissue Eng Part B Rev*. 2010;16:65-79. doi:10.1089/ten.TEB.2009.0304
- Maglio M, Tschon M, Sicuro L, Lolli R, Fini M. Osteochondral tissue cultures: between limits and sparks, the next step for advanced in vitro models. *J Cell Physiol*. 2018;234:5420-35. doi:10.1002/jcp.27457
- Elson KM, Fox N, Tipper JL, Kirkham J, Hall RM, Fisher J, *et al*. Non-destructive monitoring of viability in an ex vivo organ culture model of osteochondral tissue. *Europ Cells Mat*. 2015;29:356-69. doi:10.22203/eCM.v029a27
- Cope PJ, Ourradi K, Li Y, Sharif M. Models of osteoarthritis: the good, the bad and the promising. *Osteoarthritis Cartilage*. 2019;27:230-9. doi:10.1016/j.joca.2018.09.016
- Lin YC, Hall AC, Simpson AHRW. A novel joint organ culture model for evaluation of static and dynamic load on articular cartilage. *Bone Joint Res*. 2018;7(3):205-12. doi:10.1302/2046-3758.73BJR-2017-0320
- Geurts J, Juric D, Müller M, Schären S, Netzer C. Novel ex vivo human osteochondral explant model of knee and spine osteoarthritis enables assessment of inflammatory and drug treatment responses. *Int J Mol Sci*. 2018;19:1314. doi:10.3390/ijms19051314
- Paterson SI, Amin AK, Hall AC. Airflow accelerates bovine and human articular cartilage drying and chondrocyte death. *Osteoarthritis Cartilage*. 2015;23:257-65. doi:10.1016/j.joca.2014.10.004
- Greenwald AS, Haynes DW. Weight-bearing areas in the human hip joint. *J Bone Joint Surg Br*. 1972;54(1):157-63.
- Amin AK, Huntley JS, Bush PG, Simpson AHW, Hall AC. Osmolarity influences chondrocyte death in wounded articular cartilage. *J Bone Joint Surg Am*. 2008;90:1531-42. doi:10.2106/JBJS.G.00857
- Karim A, Amin AK, Hall AC. The clustering and morphology of chondrocytes in normal and mildly-degenerate human femoral head cartilage studied by confocal laser scanning microscopy. *J Anat*. 2018;232(4):686-98. doi:10.1111/joa.12768
- Hyllested JL, Veje K, Ostergaard K. Histochemical studies of the extracellular matrix of human articular cartilage—a review. *Osteoarthritis Cartilage*. 2002;10:333-43. doi:10.1053/joca.2002.0519
- Schmitz N, Lavery S, Kraus VB, Aigner T. Basic methods in histopathology of joint tissues. *Osteoarthritis Cartilage*. 2010;18(Suppl 3):S113-6. doi:10.1016/j.joca.2010.05.026
- Crowe AR, Yue W. Semi-quantitative determination of protein expression using immunohistochemistry staining and analysis: an integrated protocol. *Bio Protoc*. 2019;9(24):e3465. doi:10.21769/BioProtoc.3465
- Pritzker KPH, Gay S, Jimenez SA, Ostergaard K, Peletier JP, Revell PA, *et al*. Osteoarthritis cartilage histopathology: grading and staging. *Osteoarthritis Cartilage*. 2006;14:13-29. doi:10.1016/j.joca.2005.07.014
- Bush PG, Hall AC. The volume and morphology of chondrocytes within non-degenerate and degenerate human articular cartilage. *Osteoarthritis Cartilage*. 2003;11(4):242-51. doi:10.1016/s1063-4584(02)00369-2
- Fisher M, Solursh M. Glycosaminoglycan localization and role in maintenance of tissue spaces in the early chick embryo. *J Embryol Exp Morphol*. 1977;42:195-207.
- Amin AK, Huntley JS, Patton JT, Brenkel IJ, Simpson AHRW, Hall AC. Hyperosmolarity protects chondrocytes from mechanical injury in human articular cartilage: an experimental. *J Bone Joint Surg Br*. 2011;93:277-84. doi:10.1302/0301-620X.93B2.24977
- Hoemann C, Kandel R, Roberts S, Saris DB, Creemers L, Mainil-Varlet P, *et al*. International Cartilage Repair Society (ICRS) recommended guidelines for histological endpoints for cartilage repair studies in animal models and clinical trials. *Cartilage*. 2011;2(2):153-72. doi:10.1177/1947603510397535
- Huntley JS, McBirnie JM, Simpson AH, Hall AC. Cutting-edge design to improve cell viability in osteochondral grafts. *Osteoarthritis Cartilage*. 2005;13:665-71. doi:10.1016/j.joca.2005.04.007

22. Zhou S, Cui Z, Urban J. Dead cell counts during serum cultivation are underestimated by the fluorescent live/dead assay. *Biotechnol J*. 2011;6(5):513-8. doi:10.1002/biot.201000254
23. Karim A, Hall AC. Hyperosmolarity normalizes serum-induced changes to chondrocyte properties in a model of cartilage injury. *Eur Cells Mater*. 2016;31:205-20. doi:10.22203/ecm.v031a14
24. Amin AK, Huntley JS, Simpson AHW, Hall AC. Chondrocyte survival in articular cartilage: the influence of subchondral bone in a bovine model. *J Bone Joint Surg Br*. 2009;91-B:691-9. doi:10.1302/0301-620X.91B5.21544
25. Gross AE, Kim W, Las Heras F, Backstein D, Safir O, Pritzker KP. Fresh osteochondral allografts for posttraumatic knee defects: long-term followup. *Clin Orthop Relat Res*. 2008;466(8):1863-70. doi:10.1007/s11999-008-0282-8
26. Simpkin VL, Murray DH, Hall AP, Hall AC. Bicarbonate-dependent pH(i) regulation by chondrocytes within the superficial zone of bovine articular cartilage. *J Cell Physiol*. 2007;212(3):600-9. doi:10.1002/jcp.21054
27. Goldring SR, Goldring MB. Changes in the osteochondral unit during osteoarthritis: structure, function and cartilage-bone crosstalk. *Nat Rev Rheumatol*. 2016;12(11):632-44. doi:10.1038/nrrheum.2016.148
28. Loeser RF, Goldring SR, Scanzello CR, Goldring MB. Osteoarthritis: a disease of the joint as an organ. *Arthritis Rheum*. 2012;64(6):1697-707. doi:10.1002/art.34453
29. Graham BT, Wright AD, Burris DL, Axe MJ, Raisis LW, Price C. Quantification of solute diffusivity in osteoarthritic human femoral cartilage using correlation spectroscopy. *J Orthop Res*. 2018;36:3256-67. doi:10.1002/jor.24138
30. Graceffa V, Vinatier C, Guicheux J, Stoddart M, Alini M, Zeugolis DI. Chasing chimeras—the elusive stable chondrogenic phenotype. *Biomaterials*. 2019;192:199-225. doi:10.1016/j.biomaterials.2018.11.014
31. Hall AC. The role of chondrocyte morphology and volume in controlling phenotype—implications for osteoarthritis, cartilage repair, and cartilage engineering. *Curr Rheumatol Rep*. 2019;21:38. doi:10.1007/s11926-019-0837-6
32. Holloway I, Kayser M, Lee DA, Bader DL, Bentley G, Knight MM. Increased presence of cells with multiple elongated processes in osteoarthritic femoral head cartilage. *Osteoarthritis Cartilage*. 2004;12:17-24. doi:10.1016/j.joca.2003.09.001
33. Tesche F, Miosge N. New aspects of the pathogenesis of osteoarthritis: the role of fibroblast-like chondrocytes in late stages of the disease. *Histol Histopathol*. 2005;20:329-37. doi:10.14670/HH-20.329
34. Lotz MK, Otsuki S, Grogan SP, Sah R, Terkeltaub R, D'Lima D. Cartilage cell clusters. *Arthritis Rheum*. 2010;62:2206-18. doi:10.1002/art.27528
35. Nomura M, Sakitani N, Iwaawa H, Kohara Y, Takano S, Wakimoto Y, *et al.* Thinning of articular cartilage after joint unloading or immobilization. An experimental investigation of the pathogenesis in mice. *Osteoarthritis Cartilage*. 2017;25:727-36. doi:10.1002/jor.1100070305
36. Loqman MY, Bush PG, Farquharson C, Hall AC. A cell shrinkage artefact in growth plate chondrocytes with common fixative solutions: importance of fixative osmolarity for maintaining morphology. *Eur Cell Mater*. 2010;19:214-27. doi:10.22203/ecm.v019a21
37. Grumbles RM, Howell DS, Howard GA, Roos BA, Setton LA, Mow VC, *et al.* Cartilage metalloproteinases in disuse atrophy. *J Rheumatol Suppl*. 1995;43:146-8.
38. Palmoski M, Perricone E, Brandt KD. Development and reversal of a proteoglycan aggregation defect in normal canine knee cartilage after immobilization. *Arthritis Rheum*. 1979;22:508-17.
39. Behrens F, Kraft EL, Oegema TR Jr. Biochemical changes in articular cartilage after joint immobilization by casting or external fixation. *J Orthop Res*. 1989;7(3):335-43. doi:10.1002/jor.1100070305
40. Sanchez-Adams J, Leddy HA, McNulty AL, O'Connor CJ, Guilak F. The mechanobiology of articular cartilage: bearing the burden of osteoarthritis. *Curr Rheumatol Rep*. 2014;16(10):451. doi:10.1007/s11926-014-0451-6
41. Wuest SL, Calio M, Wernas T, Tanner S, Giger-lange C, Wyss F, *et al.* Influence of mechanical unloading on articular chondrocyte dedifferentiation. *Int J Mol Sci*. 2018;19(5):1289. doi:10.3390/ijms19051289
42. O'Connor CJ, Leddy HA, Benefield HC, Liedtke WB, Guilak F. TRPV4-mediated mechanotransduction regulates the metabolic response of chondrocytes to dynamic loading. *Proc Natl Acad Sci U S A*. 2014;111:1316-21. doi:10.1073/pnas.1319569111
43. Lattouf R, Younes R, Lutomski D, Naaman N, Godeau G, Senni K, *et al.* Picrosirius red staining: a useful tool to appraise collagen networks in normal and pathological tissues. *J Histochem Cytochem*. 2014;62(10):751-8. doi:10.1369/0022155414545787
44. Haapala J, Arokoski JP, Hyttinen MM, Lammi M, Tammi M, Kovanen V, *et al.* Remobilization does not fully restore immobilization induced articular cartilage atrophy. *Clin Orthop Relat Res*. 1999;362:218-29.
45. Aldahmash AM, El Fouhil AF, Mohamed RA, Ahmed AM, Atteya M, Al Sharawy SA, *et al.* Collagen types I and II distribution: a relevant indicator for the functional properties of articular cartilage in immobilised and remobilised rabbit knee joints. *Folia Morphol (Warsz)*. 2015;74(2):169-75. doi:10.5603/FM.2015.0027
46. Heinemeier KM, Schjerling P, Heinemeier J, Møller MB, Krogsgaard MR, Grum-Schwensen T, *et al.* Radiocarbon dating reveals minimal collagen turnover in both healthy and osteoarthritic human cartilage. *Sci Transl Med*. 2016;8:346ra90. doi:10.1126/scitranslmed.aad8335
47. Bush PG, Hall AC. Regulatory volume decrease (RVD) by isolated and in situ bovine articular chondrocytes. *J Cell Physiol*. 2001;187:304-14. doi:10.1002/jcp.1077
48. Lewis R, Feetham CH, Barrett-Jolley R. Cell volume control in chondrocytes. *Cell Physiol Biochem*. 2011;28:1111-22. doi:10.1159/000335847
49. Wang Z, Irianto J, Kazun S, Wang W, Knight MM. The rate of hypo-osmotic challenge influences regulatory volume decrease (RVD) and mechanical properties of articular chondrocytes. *Osteoarthritis Cartilage*. 2015;23:289-99. doi:10.1016/j.joca.2014.11.003
50. Gratal P, Mediero A, Sánchez-Pernaute O, Prieto-Potin I, Lamuedra A, Herrero-Beaumont G, *et al.* Chondrocyte enlargement is a marker of osteoarthritis severity. *Osteoarthritis Cartilage*. 2019;27(8):1229-34. doi:10.1016/j.joca.2019.04.013