A novel joint organ culture model for evaluation of the static and dynamic load on articular cartilage

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Introduction

Articular cartilage is a highly-specialised tissue that, with synovial fluid, provides almost frictionless interface between opposing bones[1]. Movement between these surfaces and throughout the tissue creates mechanical stimulation that maintains cartilage integrity through the process of 'mechanotransduction'[2, 3]. Compressive force on cartilage explants stimulates the biosynthesis of collagen, proteoglycan and fibronectin if applied in the physiological range (0.01-5 MPa) and frequency (0.01-1.0) Hz[4]. Animal studies have also demonstrated that daily physiological exercise increased proteoglycan content and the cartilage thickness, and might minimise the development of osteoarthritis[5].

A wide variety of *in vitro* (e.g. isolated chondrocytes, cartilage explants) and *in vivo* (e.g. rodent) experimental models have been utilised to understand mechanotransduction and the response of cartilage to mechanical load, however each has limitations. For example, isolated chondrocytes may change their phenotype in 2-D culture[6]. Cartilage explants might suffer from 'explantation injury', resulting from increased IL-1β levels during harvesting from the joint[7]. For *in vivo* studies, the time-consuming approval process and the significant expense and compliance with animal welfare regulations are unavoidable hurdles before live animal experiments can be performed[8]. Therefore, we considered that it may be beneficial to develop an organ culture model (*ex vivo* model) of a large synovial joint in an attempt to bridge the gap between the *in vitro* cartilage explant model and the *in vivo* animal model.

Few organ level long-term culture system of the mammalian synovial joint have, to our knowledge, been created. Nugent-Derfus *et al*.[9] described a system where a bovine stifle joint was cultured in a plastic bag for only 24hrs. However, the complicated settings of their culture system and the difficulties of maintaining aseptic conditions of the circulated culture media limited its wide reproducibility. Other connective tissue-related organ culture models have been developed but for intervertebral disc cartilage[10]. However, the differences in tissue structure, function and loading patterns suggest that these methods might not be applicable for the study of the hyaline cartilage of the articular joint. In the present study we describe an organ culture model using the bovine metatarsophalangeal joint, a relatively inexpensive, common and reliable source of articular cartilage. The static and dynamic effect of joint movement were evaluated on chondrocyte viability and matrix glycosaminoglycan content.

Materials and methods

Materials

Chemicals were purchased from Sigma-Aldrich (Dorset, UK) unless otherwise stated. The cell viability probes, 5-chloromethylfluorescein diacetate (CMFDA) and propidium iodide (PI) were prepared as described[11], and Dulbecco's Modified Eagle's Medium (DMEM; glucose 4.5g/L) were obtained from Invitrogen (Paisley, UK). The 1,9-dimethylmethylene blue (DMMB) solution was formulated as described[12] and the standard shark chondroitin sulphate (Sigma-Aldrich, UK) solution prepared at 0.1mg/ml.

Harvest of the bovine metatarsophalangeal joint

Twelve feet from separate healthy 3-year-old beef cattle were obtained from a local abattoir (Scotbeef, Bridge of Allan, UK), and processed under sterile conditions within 6hrs of slaughter. After thoroughly rinsing the feet with running water, they were securely fixed and suspended on a custom-made stand that avoided possible contamination from the working bench throughout the procedures (**Fig.1A**). The skin and hoof were removed completely, and the exposed soft tissue layer rinsed thoroughly with at least 1L of sterile phosphate buffered saline (PBS). Then, the suspended foot was moved to a laminar-flow ventilated hood for further processing.

A sterile operation field was established by wrapping sheets of sterile paper around the foot (**Fig.1B**). The metatarsophalangeal joint was opened, and all surrounding soft tissues (e.g. tendons, ligaments, joint capsules, synovia) removed. The bilateral collateral ligaments were left to reinforce joint congruency if it was to be prepared for the dynamic model. The metatarsal and the phalangeal bone were then transected using an oscillating saw to isolate the metatarsophalangeal joint from the foot. The sawing lines were approximately 1cm above and below the articular cartilage margin (**Fig.1C**). During the entire procedures, the joint was kept hydrated by frequent rinsing with PBS.

Additional steps were performed if the joint was prepared for the dynamic model. On the transected surface of the metatarsal bone, a central hole was drilled by a sterile drill bit (Ø 3.0mm). A custom-made peg, refashioned from an external fixation pin (Ø 3.5mm), was screwed into the drill hole (**Fig.1D**), and linked to a connecting bar, which was modified from the 'adjustable telescopic strut' of an Ilizarov external fixator apparatus. The connecting bar

was then linked to a driving motor for joint motion (**Fig.2**). The joint was then placed in a sterilised 1L glass beaker for subsequent culture.

Culture environment

The culture media was DMEM including penicillin (100U/ml), streptomycin (100µg/ml) and fetal bovine serum (10%v/v) (Sigma-Aldrich, UK). Typically, 300ml was sufficient for immersing a joint. The opening of the beaker was sealed with double sheets of paraffin membrane (Parafilm M®, US) and a ventilation outlet prepared for gas exchange. The joint culture system was then moved into a humidified incubator (37°C;5% CO_2) and media changed bi-weekly.

Dynamic setting

The driving motor was set at 20rpm (0.33Hz) to mimic slow human walking speed. The movement duration was controlled by an electronic timer and set to an intermittent pattern to approximate animal/human activity levels[13], i.e. 30mins continuous movement followed by 30mins of static load for 12hrs/day. Joint movement was constrained to a single plane to replicate the hinge type motion on the synovial joint. The arc of movement was from full extension to around 45° of flexion. The load applied was approximately 2.5 Newtons, which was from the weight of the upper part of the joint (metatarsus) and was sufficient to maintain firm apposition of the articulating surfaces.

Cartilage sampling

Full depth osteochondral samples were taken using fresh sterile scalpel blades (No.22)[14, 15] (**Fig.3A**). Normally, one bovine metatarsophalangeal joint could provide up to 46 sampling sites across its 8 joint facets (**Fig.1D**). Cartilage was sampled at Day 0, 7, 14, 21 and Day 28. At each time point, six cartilage explants from each joint were taken, i.e. three for assessment of chondrocyte viability and the remainder for the GAG assay.

Chondrocyte viability assessment

A custom-made double-bladed cutting tool was used to trim the cartilage explants to create two parallel straight edges so that the chondrocytes in different depths could be evaluated in coronal sections[11] (**Fig.3B**). The trimmed explant was then incubated in DMEM with CMFDA and PI (21°C; 45mins) to label living chondrocytes green and dead chondrocytes

red, respectively[14]. Explants were subsequently fixed with 10% (v/v) formalin (Fisher Scientific, Loughborough, UK) and secured to the base of a Petri dish with Blu-Tack (Bostik, Leicester, UK) (**Fig.3C**). Images were acquired using an upright confocal laser scanning microscope (Zeiss LSM510 Axioskop, Carl Zeiss, Welwyn Garden City, UK; ×10 objective) and reconstructed and analysed by ImageJ (Ver1.47, NIH, USA). Articular cartilage was divided into three regions on the basis of depth from the articular surface to subchondral bone: the first quartile was defined as the superficial quarter, followed by the central half as the middle 50%, and the deep quarter as the last quartile[11] (**Fig.3D**). Chondrocyte viability within each region was quantified as: % viable cells = (number of CMFDA-labeled live cells/number of CMFDA and PI labeled cells)×100%.

Matrix glycosaminoglycan assessment

The spectrophotometric microassay[12] was used to measure the sulphated glycosaminoglycan (GAG) content of cartilage. The central full-thickness area of the specimen was obtained using a skin biopsy punch (Ø2.5mm; Kai Industries, Japan) and the 'before-digested' wet weight determined, which included the weight of cartilage and subchondral bone. After cartilage digestion by papain solution (300μg in 1ml of 1mM EDTA, 2mM dithiothreitol, and 20mM sodium phosphate; pH 6.8; 60°C for ~4hrs), the undigested material (i.e. subchondral bone) was weighed again to obtain the 'after-digested' wet weight. The difference was the cartilage wet weight, which was used to normalise the result to allow for any variation in the size of the cartilage specimen. The absorbance of the digested solution was measured immediately after the DMMB solution was added, and the result compared with the standard solution to obtain the equivalent GAG weight of the cartilage sample. GAG content was determined as the GAG mass (in μg) per cartilage mass (in mg), and shown in the Figures as 'GAG (μg/mg cartilage)'.

Statistical analysis

Statistical analyses were performed using Minitab 16 (Minitab Inc., USA). All data were tested for normality (Kolmogorov-Smirnov test). Thereafter, parametric data were analysed using paired or unpaired t-tests if two sets of data were compared, or one-way ANOVA with *post hoc* Tukey's tests for more than two data sets. For non-parametric data, the Mann-Whitney U test was used for comparison between two independent data sets, while the

Kruskal-Wallis test was used for ≥3 data sets. Data are presented as means ± standard deviation (SD) with the significance level set at $p<0.05$.

Results

Chondrocyte viability

Six static models and 6 dynamic models were evaluated over 28 days. The samples from the fresh joint (Day 0) were taken as the control (**Fig.4A, Fig.5**). In the static model, the chondrocyte viability at Day 0 was 89.9±2.5%, 94.7±1.1% and 80.1±3.0% in the superficial quarter, central half and deep quarter, respectively, which was not significantly different to the dynamic model (p=0.381, 0.111 and 0.059, respectively; unpaired t test). After culturing, the number of dead cells increased progressively and the chondrocyte viability decreased significantly to 66.5±13.1%, 80. 9±5.8% and 46.9±8.5% in the superficial quarter, central half and deep quarter, respectively, at the end of the 4th week (p<0.001 in each zone; one-way ANOVA) (**Fig.4B&4C, Fig.5**). However, in the dynamic model, chondrocyte viability was maintained without significant change after 4wks of culture, i.e. the change of chondrocyte viability of the superficial quarter was from 92.0±4.0% (Day 0) to 89.9±0.2% (Day 28), the middle half 93.1±1.5% (Day 0) to 93.8±0.9% (Day 28) and the deep quarter 85.6±0.8% (Day 0) to 84.0±2.9% (Day28) (p=0.449, 0.312, 0.170, respectively; one-way ANOVA) (**Fig.4D&4E, Fig.6**). Further comparison between the chondrocyte viability of the static and the dynamic model revealed that there were significant differences between each region during the 4wks culture (p=0.007 in the superficial quarter, p<0.001 in both the central half and deep quarter; two-way ANOVA). Therefore, in the dynamic model, chondrocyte viability was maintained at the initial level throughout the 4wk culture period, but in contrast it decreased progressively in the static model.

GAG analysis

Evaluation of the cartilage matrix of the day 0 control samples revealed that the GAG content was 6.01±0.06μg/mg and 6.18±0.15μg/mg in the static and dynamic models, respectively, which were not significantly different (p=0.640; unpaired t test). The GAG content in the dynamic model was maintained at a consistent level without change throughout the culture period (p=0.887; one-way ANOVA). However, for the static model, it decreased to 4.87±0.15μg/mg at the 1st week and dropped further to 3.93±0.07μg/mg at the 3rd week. Even though at the end of the culture the GAG content recovered slightly to 4.71±0.06μg/mg, it was still significantly less compared to the dynamic model (p<0.001; two-way ANOVA). Further point-to-point comparison revealed that the difference became significant after Day

14 (**Fig.7**). Therefore, over this time period, the GAG content of cartilage matrix was also maintained in the dynamic model but not in the static model.

Discussion

This report describes a novel large *ex vivo* joint culture model assessed by chondrocyte viability and matrix GAG content in the presence or absence of joint movement. Although cell viability in the static model decreased gradually during culture, there were still >80% alive in the central half region at the end of week 4 (**Fig.5**). The zonal heterogeneity of chondrocyte viability was marked, i.e. the chondrocytes in the central half region exhibited the highest, followed by the superficial quarter, whereas the viability of the deep quarter was the lowest at all time points. This zonal heterogeneity, to our knowledge, has not been described in detail but is apparent in images in previous studies[14-17]. It is possible that the scalpel cut damaged the chondrocytes in a depth-dependent manner, however this is unavoidable in order to assess zonal viability[14]. In addition, the limitation of the chondrocytes to obtain nutrients in the deep quarter, which probably diffuse mainly from the culture media[18], may play a role in the greater decrease in the chondrocyte viability of the deep quarter.

However, with joint movement, chondrocyte viability was greatly improved over the whole culture period as viability was maintained at the initial level without significant decrease during the 4wks of culture (**Fig.6**). It is possible that mechanical stimulation directly from joint movement was important as both *in vitro* and *in vivo* studies demonstrate that loading in the physiological range maintain cartilage integrity. This is achieved through the down regulation of matrix metalloproteinases (MMPs) and aggrecanases (ADAMTS)[2, 19], and the preservation of chondrocyte viability by reduced levels of nitric oxide (NO) and reactive oxygen species (ROS)[5, 19]. Alternatively the fluid flow created by joint movement could increase the exchange of nutrients and waste products between cartilage and culture medium[20], supporting chondrocyte viability.

Matrix GAG content decreased after the first week (**Fig.7**) and similar observations have been reported in *in vitro* studies using bovine cartilage explants[21-23]. Previous work demonstrated that early matrix GAG loss occurred within the first 4hrs of culture, and most of the released GAG was not newly synthesised but previously produced and already stored in the matrix[23]. There are some *in vivo* studies with similar results. The cartilage GAG concentration decreased significantly by 20-23% if canine knee joints were fixed rigidly using an external fixator for 11wks[22]. The lack of joint movement was thought to be the main reason because it reduced the rate of chondrocyte proteoglycan synthesis[24-26] but elevated

metalloproteinase (MMP-2) production leading to accelerated loss of matrix components[27]. These results indicated that in unloaded cartilage catabolic events predominated over anabolic processes and the extracellular matrix would contain less GAG potentially reducing its resilience. In addition, the matrix porosity of the cartilage surface may affect the release of the matrix proteoglycan[28]. Only molecules smaller than the matrix pore size would pass the cartilage surface because the hydrodynamic size of the proteoglycans released in the culture media was smaller than matrix proteoglycans[29]. Thus, the observed loss of the matrix GAG in the static model (**Fig.7**) might be the outcome resulting from the reduction of the chondrocyte GAG synthesis and the acceleration of matrix proteolysis.

Applying joint movement in the dynamic model significantly prevented the decrease of the matrix GAG content (**Fig.7**). However, the force between cartilages during joint movement is complex and difficult to reproduce experimentally. It is known that articular cartilage *in vivo* is subjected to both compressive and shearing force under normal physiological movement[30]. Nevertheless, most of the *in vitro* studies used mechanical compression force as a test load[4, 31]. Pure shear stress also had effects but does not appear to have been studied in detail. For example, Jin *et al.* demonstrated that using a rotational plate to produce sinusoidal shear strain increased matrix protein synthesis by ~50% and proteoglycans production by up to 25%[32]. They indicated that even though the tissue shear force caused less volumetric deformation than the compression force, its stimulatory effect was still potent. The increase in matrix protein synthesis from the stimulation of shear stress was also shown in a series of studies by Grad *et al*.[33, 34]. Their results suggested that the signal transduction pathways of the compression force and the shear stress might be different inside the cartilage tissue. Waldman *et al*. further indicated that these two forces might have a synergistic effect, which enhanced the synthesis of matrix proteins[35]. Therefore, compression with sliding movement was suggested in some studies to be a more appropriate method for loading articular cartilage[36, 37]. However, only a few *in vitro* experimental models have applied both compression and shear to cartilage. An interesting bioreactor system developed by Grad *et al*. and Wimmer *et al*. using a ceramic ball produced variable types of forces on cartilage explants[33, 34, 38]. Nevertheless, the system was relatively expensive, potentially limiting its wider utilisation. Thus, the *ex vivo* bovine joint model described in the present study had particular advantages as it tested a more natural 'cartilage-on-cartilage' joint movement, and so could be another model that produced both compression and shear force on cartilage. It should be noted that the joint model was not designed to replicate full body weight as current cartilage

regenerative medicine strategies only allow patients to bear weight minimally in the early post-operative period. However, as the results of the study revealed, only a few Newtons of load (with motion) were sufficient to maintain cartilage health.

To our knowledge, this is the first description of a long-term cultured large joint model, the validity of which was assessed by chondrocyte viability and matrix GAG content. This model may provide new directions for articular cartilage research in addition to the more commonly used *in vivo* and *in vitro* models. The relatively intact nature of the articular structure was a significant benefit of this model which has the benefit of comparing responses to static or dynamic mechanical stimulation. The relatively large volume of cartilage tissue available for sampling was another advantage as sufficient sampling areas for multiple assessments in the same joint, especially for long-term culture experiments were possible. However, a significant learning curve for the aseptic preparation of the joint and the techniques to maintain the culture sterile had to be mastered for the successful use of this model.

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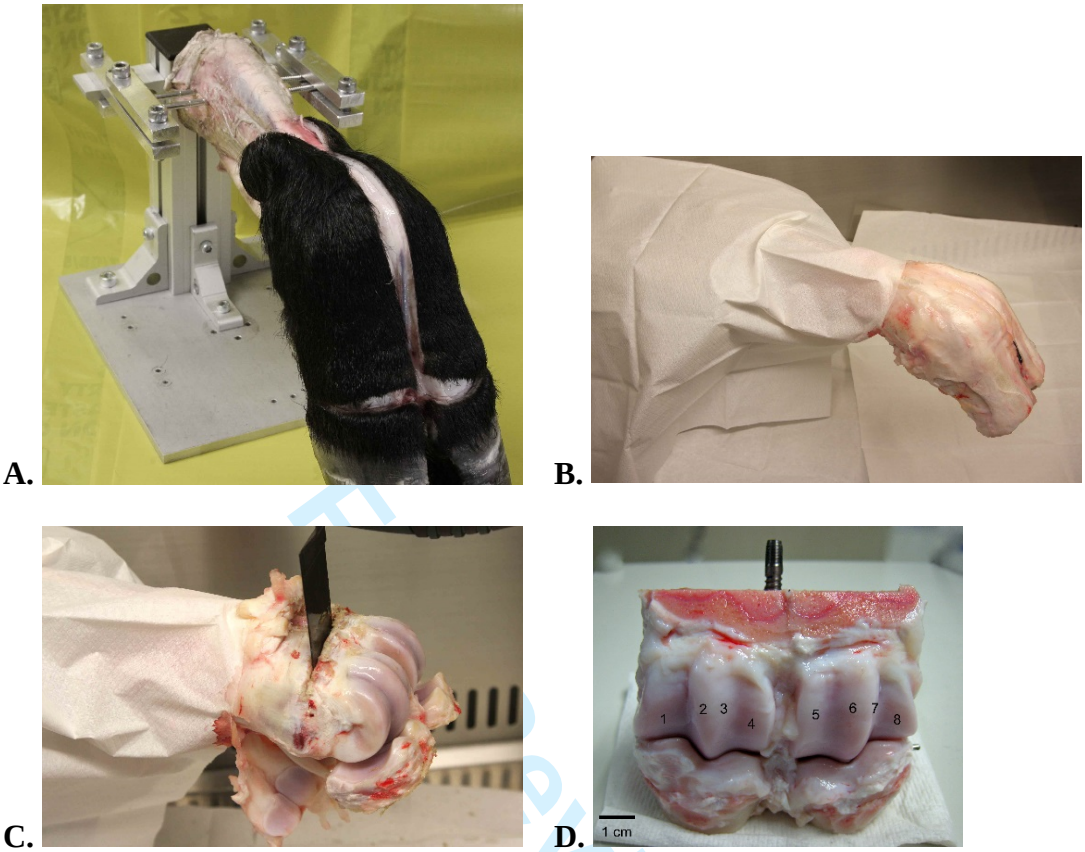


Figure 1. Preparation of the bovine metatarsophalangeal joint and the cartilage sampling sites

A. The bovine foot was suspended in the frame by insertion of 2 metal pins at the proximal metatarsus. The incision was started from the midline of the metatarsus down to the proximal interphalangeal joint with a transverse circumferential cut so that the hide and hoof could be removed. **B.** The skinned foot was wrapped with sterilized paper to establish a sterile safety zone. **C.** After removal of the surrounding soft tissues, the metatarsophalangeal joint was then exposed and cut off at a distance of approximately one centimeter above and below the cartilage margin. **D.** A specially-made peg was inserted onto the top of the transected metatarsal bone of the joint if the joint was prepared for dynamic model. Cartilage samples were taken from 8 articular facets in the metatarsal part of the joint. The facets numbered 1, 4, 5, 8 were flatter and larger than facets numbered 2, 3, 6, 7 which were located beside the articular ridges. In a typical experiment, facet-1 and 8 each provided 5 sampling sites, and the other 6 facets offered 6 sampling sites in each facet.

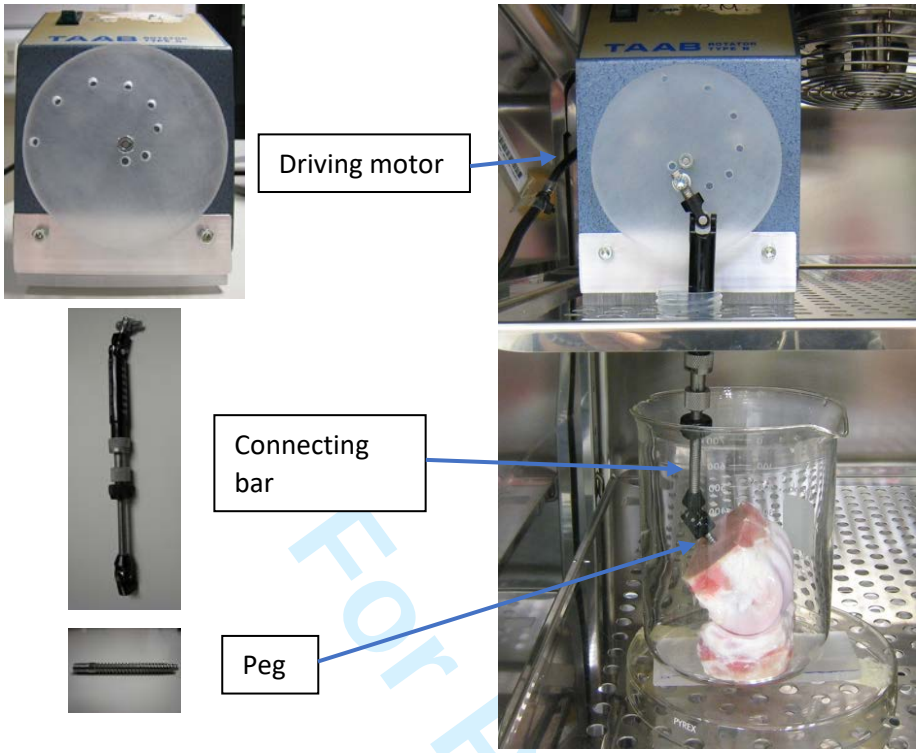


Figure 2. Construction of the motion machine for the dynamic model

The motion machine was constructed using readily-accessible components. The driving motor was converted from a tube rotator by replacing the original rotator plate with a custom-made plastic plate, in which a series of holes at different distances from the plate center were drilled in order to adjust the different height of the joint. The adjustable telescopic strut of an Ilizarov external fixator was used as the connecting bar to link the tube rotator with the joint. The external fixation pin was cut short to be the peg to fix into the top of the transected metatarsal bone of the joint. As soon as the motor rotated, the connecting bar moved upward and downward and the joint model was passively moved by this oscillating motion. The equipment was designed as small as possible so that it would fit in a standard temperature and CO_2 –controlled incubator for joint culture. (The culture media and the sealing Parafilm were removed for clear demonstration).

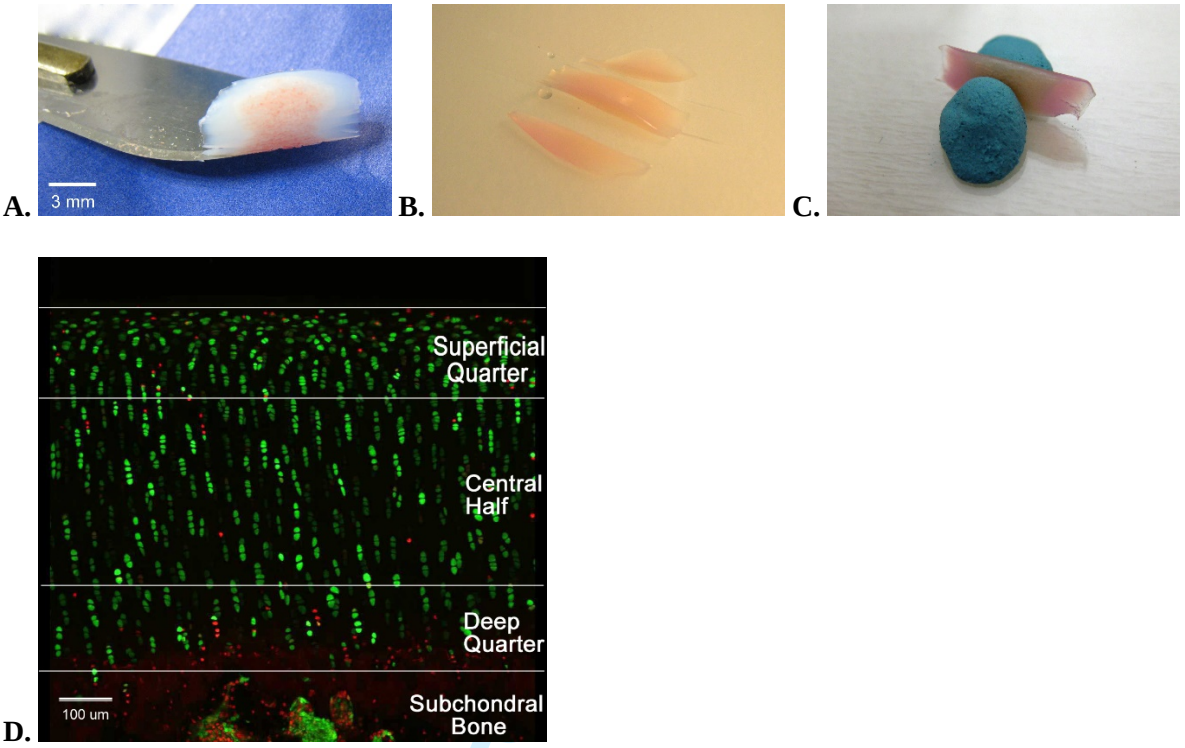
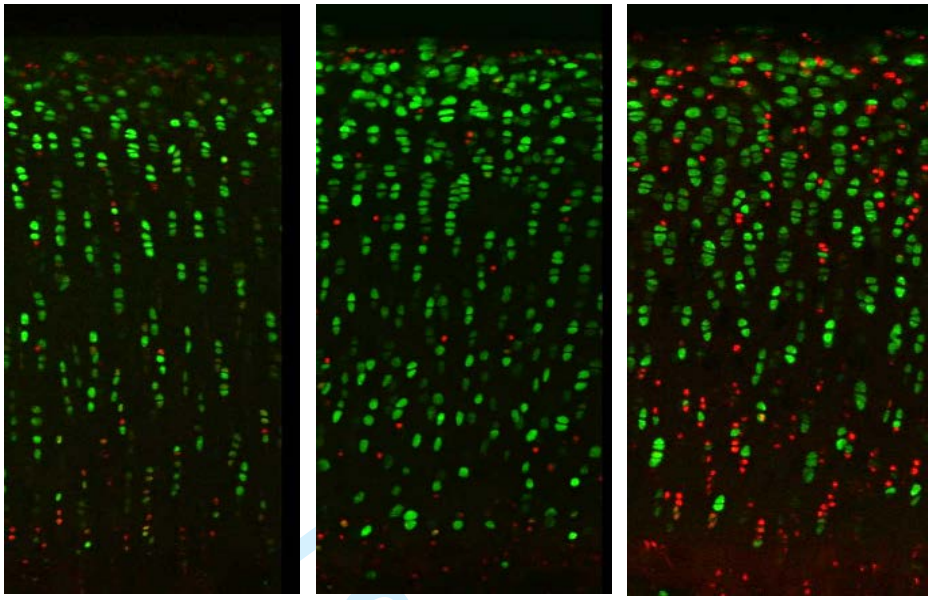
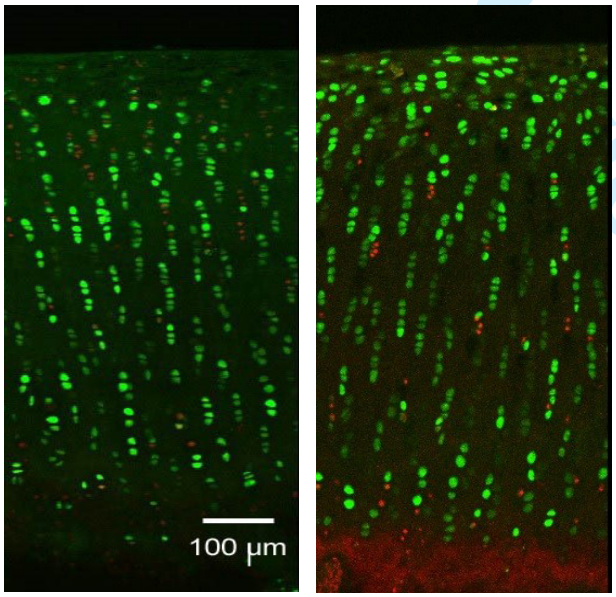


Figure 3. Cartilage explant preparation and visualization of fluorescently-labeled *in situ* chondrocytes by confocal laser scanning microscopy (CLSM)

A. Explants which included a small amount of subchondral bone attached in the center were taken to confirm that full thickness osteochondral samples were taken. **B.** The sample was cut into 3 pieces in which the two cut lines were parallel. **C.** The middle part was chosen and secured on a Petri dish with 2 small pieces of Blu-Tack (Bostik, Leicester, UK). **D.** A coronal image illustrated the zonal distribution and viability of chondrocytes throughout the full cartilage thickness. Living chondrocytes were stained green by CMFDA and dead chondrocytes red by PI. The region of interest (ROI) in the image was set according to the cartilage thickness. The first 25% thickness of cartilage from the top was considered the superficial quarter, the subsequent 50% the central half and the final 25% the deep quarter. At the bottom of the image, the subchondral bone, contained multinucleated osteoblasts and osteoclasts.



A. Day 0 Control | B. Day 14 Static | C. Day 28 Static



D. Day 14 Dynamic | E. Day 28 Dynamic

Figure 4. Images of chondrocyte viability with time in the static and dynamic model

Under the confocal laser scanning microscope (CLSM), images of the cartilage coronal section showed that the total cell population that labeled with PI (in red) increased gradually from Day 0 to Day 28 in the static model (**A** to **C**), and most of the red cells located in the superficial and deep quarter of the cartilage. In the dynamic model (**D** and **E**), only a few

sporadically-distributed PI-labeled chondrocytes were observed in Day 28, and there were still live cells (in green) adjacent to the subchondral bone.

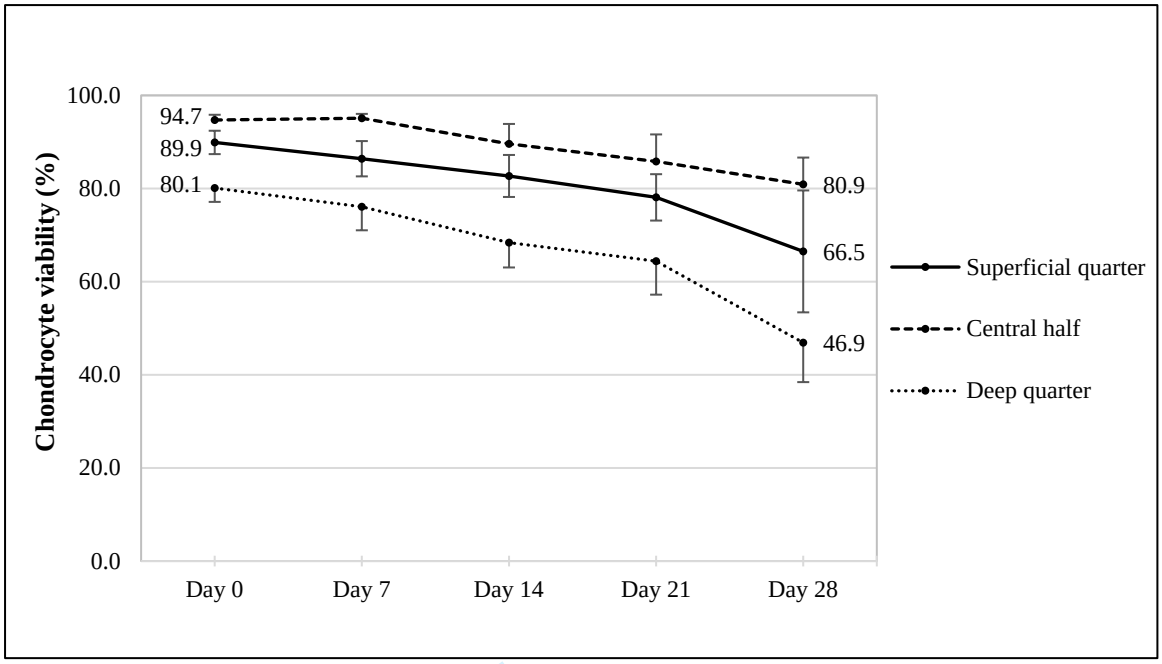


Figure 5. Chondrocyte viability in cartilage in the static joint model

Chondrocyte viability decreased gradually in all 3 regions during culture. The viability of the central half was maintained the highest amongst these 3 regions in all time points, which only decreased 13.8% (from 94.7% to 80.9%) in the 28 days of culture. The superficial quarter decreased 23.4% (from 89.9% to 66.5%) and the deep quarter decreased 33.2% (from 80.1% to 46.9%). The decreases of the 3 regions were statistically significant ($p<0.001$ in each region; one-way ANOVA). Data are presented as means ± standard deviation.

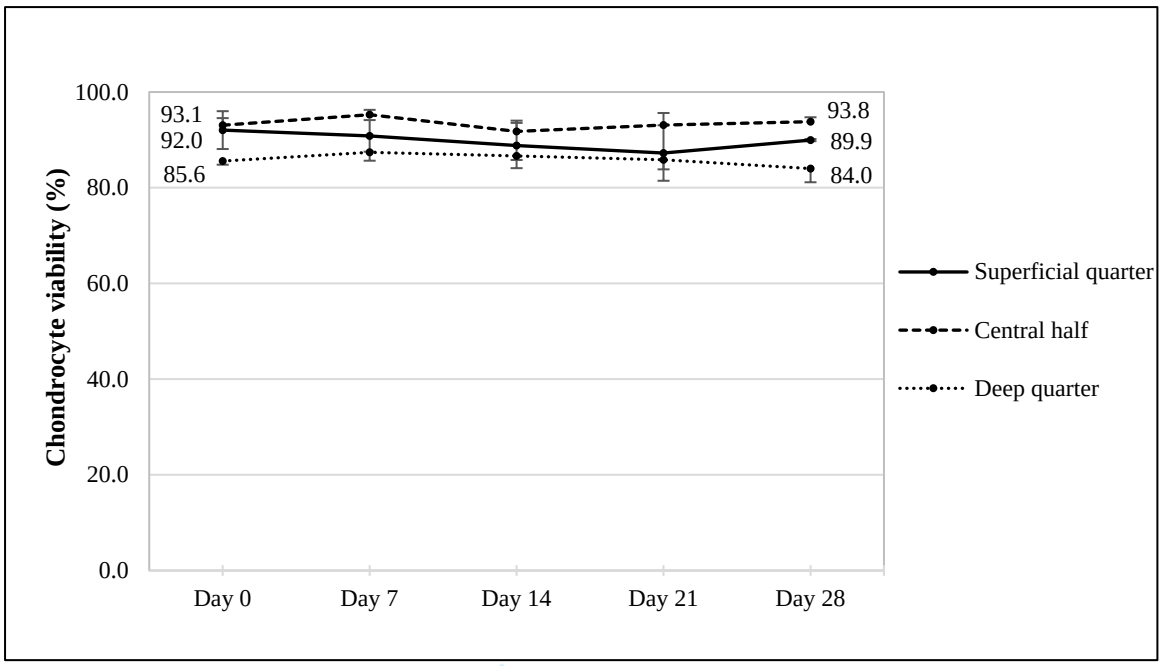


Figure 6. Chondrocyte viability in cartilage in the dynamic joint model

Chondrocyte viability was maintained similarly at the initial level throughout the culture period. The central half was also the highest region in all time points, following by the superficial quarter and the deep quarter in sequence. Although there was a little increase/decrease of the chondrocyte viability during the 4 week culture, it was no statistically significant difference in all 3 regions (p=0.449, 0.312 and 0.170 in the superficial quarter, central half and deep quarter, respectively; one-way ANOVA).

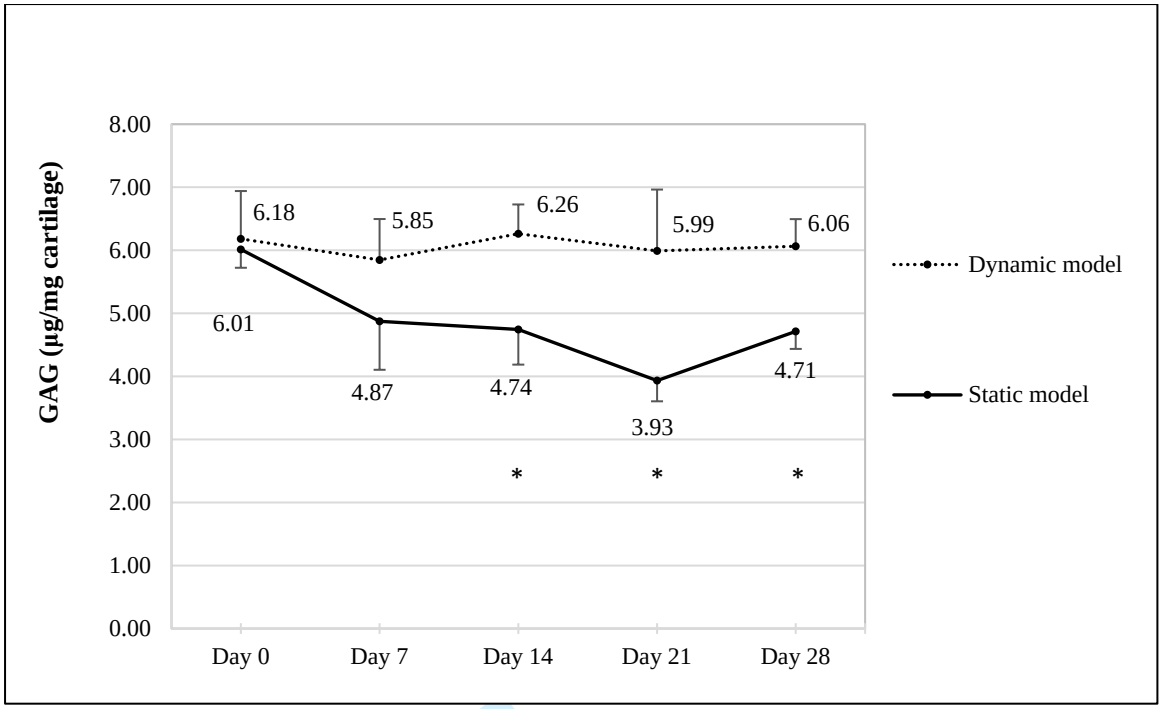


Figure 7. Cartilage GAG content in the dynamic and the static model

Quantification of the matrix GAG content showed that, in both models, the Day 0 values started at a similar level without significant difference (p=0.640, unpaired t test). However, after Day 14, the difference became significant (p=0.003, 0.004 and 0.001 at Day 14, 21 and 28, respectively; unpaired t test; indicated with an asterisk). In the dynamic model, the GAG content was maintained as similar to the initial level during the whole culture period, but in the static model, it dropped to the lowest value at Day 21 with a slight increase at Day 28.