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■ **CARTILAGE**

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

Y-C. Lin,
A. C. Hall,
A. H. R. W. Simpson

University of Edinburgh, Edinburgh, United Kingdom

Objectives

The purpose of this study was to create a novel *ex vivo* organ culture model for evaluating the effects of static and dynamic load on cartilage.

Methods

The metatarsophalangeal joints of 12 fresh cadaveric bovine feet were skinned and dissected aseptically, and cultured for up to four weeks. Dynamic movement was applied using a custom-made machine on six joints, with the others cultured under static conditions. Chondrocyte viability and matrix glycosaminoglycan (GAG) content were evaluated by the cell viability probes, 5-chloromethylfluorescein diacetate (CMFDA) and propidium iodide (PI), and dimethylmethylene blue (DMMB) assay, respectively.

Results

Chondrocyte viability in the static model decreased significantly from 89.9% (SD 2.5%) (Day 0) to 66.5% (SD 13.1%) (Day 28), 94.7% (SD 1.1%) to 80. 9% (SD 5.8%) and 80.1% (SD 3.0%) to 46.9% (SD 8.5%) in the superficial quarter, central half and deep quarter of cartilage, respectively ($p < 0.001$ in each zone; one-way analysis of variance). The GAG content decreased significantly from 6.01 µg/mg (SD 0.06) (Day 0) to 4.71 µg/mg (SD 0.06) (Day 28) ($p < 0.001$; one-way analysis of variance). However, with dynamic movement, chondrocyte viability and GAG content were maintained at the Day 0 level over the four-week period without a significant change (chondrocyte viability: 92.0% (SD 4.0%) (Day 0) to 89.9% (SD 0.2%) (Day 28), 93.1% (SD 1.5%) to 93.8% (SD 0.9%) and 85.6% (SD 0.8%) to 84.0% (SD 2.9%) in the three corresponding zones; GAG content: 6.18 µg/mg (SD 0.15) (Day 0) to 6.06 µg/mg (SD 0.09) (Day 28)).

Conclusion

Dynamic joint movement maintained chondrocyte viability and cartilage GAG content. This long-term whole joint culture model could be of value in providing a more natural and controlled platform for investigating the influence of joint movement on articular cartilage, and for evaluating novel therapies for cartilage repair.

■ Y-C. Lin, MD, PhD, Consultant Orthopaedic Surgeon, Department of Orthopaedic Surgery, University of Edinburgh, The Chancellor's Building, 49 Little France Crescent, Edinburgh, EH16 4SB, UK and Taipei Medical University-Shuang Ho Hospital, Zhonghe District, New Taipei City, 23561, Taiwan.

■ A. C. Hall, PhD, Reader in Physiology, Centre for Integrative Physiology, University of Edinburgh, Hugh Robson Building, George Square, Edinburgh EH8 9XD, UK.

■ A. H. R. W. Simpson, MA(Cantab), DM (Oxon), FRCS(England & Edinburgh), George Harrison Law Professor of Orthopaedic Surgery, Department of Orthopaedics, University of Edinburgh, 51 Little France Crescent, Old Dalkeith Road, Edinburgh, EH16 4SA, UK.

Correspondence should be sent to Y-C. Lin;
email: iam4290@gmail.com

Article focus

■ The creation of a novel *ex vivo* large animal joint organ culture model, which can be subjected to static or dynamic load, was described.

■ Chondrocyte viability and matrix glycosaminoglycan (GAG) content were determined to assess the validity of the model.

Key messages

■ The bovine joint could be cultured aseptically for at least four weeks.

■ Joint movement improved chondrocyte viability and GAG content, compared with static load.

Strengths and limitations

■ This large joint could be a very useful additional model for studying the effects of load on aspects of normal cartilage biology and its response to injury and subsequent repair.

■ There is a significant learning curve for the aseptic preparation of the joint.

Fig. 1a   Fig. 1b   Fig. 1c   Fig. 1d

Photographs showing preparation of the bovine metatarsophalangeal joint and the cartilage sampling sites: a) the bovine foot was suspended in the frame by inserting two metal pins at the proximal metatarsus. The incision was 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 approximately 1 cm above and below the cartilage margin; d) for the dynamic model, a specially made peg was inserted into the top of the transected metatarsal bone of the joint. Cartilage samples were taken from eight articular facets in the metatarsal part of the joint. The facets numbered 1, 4, 5 and 8 were flatter and larger than the facets numbered 2, 3, 6 and 7, which were located beside the articular ridges. In a typical experiment, facets 1 and 8 each provided five sampling sites, and the other six facets offered six sampling sites each.

Introduction

Articular cartilage is a highly specialized tissue which, in combination with synovial fluid, provides an almost friction-less 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 of 0.01 MPa to 5 MPa, and at a frequency of 0.01 to 1.0 Hz.[4] Animal studies have also demonstrated that daily physiological exercise increases both proteoglycan content and cartilage thickness, and may minimize 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 used to understand mechanotransduction and the response of cartilage to mechanical load, however, each model has its limitations. For example, isolated chondrocytes may change their phenotype in 2D culture.[6] Cartilage explants might suffer from 'explantation injury', resulting from increased interleukin-1 beta (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 (*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 systems 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 24 hours. However, the complicated settings of their culture system, and the difficulties of maintaining the 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 may 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 effects of joint movement were evaluated on chondrocyte viability and matrix glycosaminoglycan (GAG) content.

Materials and Methods

Materials. Chemicals were purchased from Sigma-Aldrich Co. (Dorset, United Kingdom) 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) was obtained from Invitrogen Ltd (Paisley, United Kingdom). The 1,9-dimethylmethylene blue (DMMB) solution was formulated as described,[12] and the standard shark chondroitin sulphate solution (Sigma-Aldrich) was prepared at 0.1 mg/ml.

Harvest of the bovine metatarsophalangeal joint. A total of 12 feet from separate healthy three-year-old beef cattle were obtained from a local abattoir (Scotbeef Ltd, Bridge of Allan, United Kingdom), and processed under sterile conditions within six hours 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 one litre of sterile phosphate-buffered saline (PBS). The suspended foot was then moved to a laminar-flow ventilated hood for further processing.
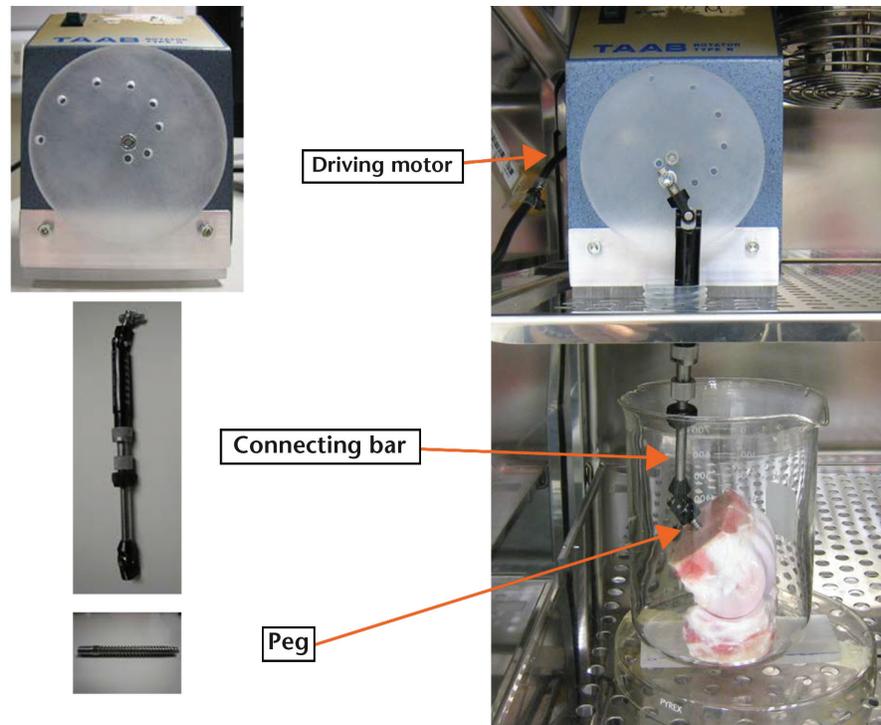
Fig. 2

Images showing construction of the motion machine for the dynamic model, 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 centre, were drilled to adjust to the different height of the joint. The adjustable telescopic strut of an Ilizarov TSF external fixator was used as the connecting bar to link the tube rotator with the joint. The external fixation pin was cut short to create the peg to fix to the top of the transected metatarsal bone of the joint. As soon as the motor rotated, the connecting bar moved upwards and downwards, and the joint model was passively moved by this oscillating motion. The equipment was designed to be as small as possible, so that it would fit in a standard temperature- and CO_2–controlled incubator for joint culture (the culture medium (see below) and the sealing Parafilm have been removed for clear demonstration).

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 bones were then transected using an oscillating saw to isolate the metatarsophalangeal joint from the foot. The sawing lines were approximately 1 cm above and below the articular cartilage margin (Fig. 1c). During the entire procedure, the joint was kept hydrated by frequent rinsing with PBS.

Additional steps were performed if the joint was to be prepared for the dynamic model. On the transected surface of the metatarsal bone, a central hole was drilled by a sterile drill bit (3.0 mm diameter). A custom-made peg, refashioned from an external fixation pin (3.5 mm diameter), 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. The connecting bar was then linked to a driving motor for joint motion (Fig. 2). The joint was then placed in a sterilized one litre glass beaker for subsequent culture.

Culture environment. The culture medium was DMEM including penicillin (100 U/ml), streptomycin (100 µg/ml) and foetal bovine serum (10% v/v). Typically, 300 ml was sufficient for immersing a joint. The opening of the beaker was sealed with double sheets of paraffin membrane (Parafilm M, Bemis, Neenah, Wisconsin) 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 the medium changed bi-weekly.
Dynamic setting. The driving motor was set at 20 rpm (0.33 Hz) 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., 30 minutes of continuous movement, followed by 30 minutes of static load for 12 hours per 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

Fig. 3b

Fig. 3c

Fig. 3d
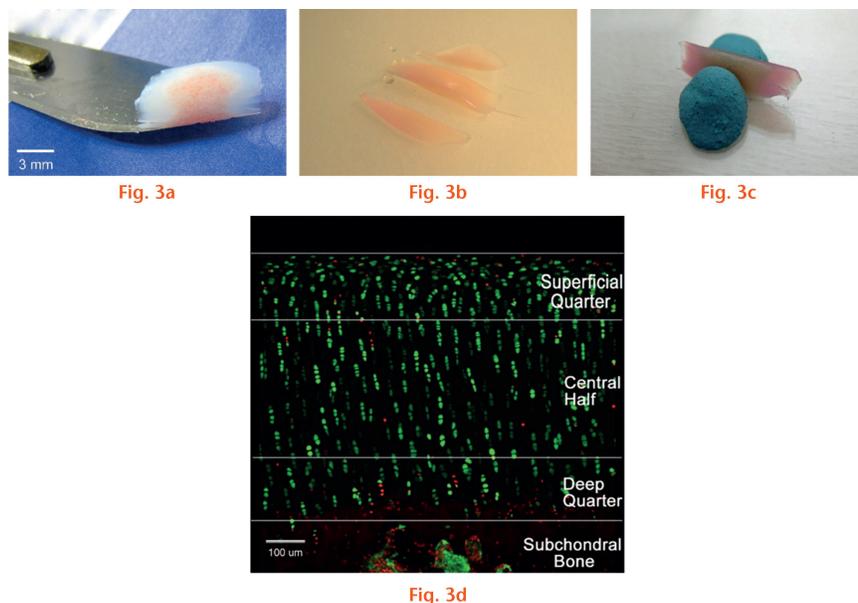
Images showing cartilage explant preparation and visualization of fluorescently-labelled *in situ* chondrocytes by confocal laser scanning microscopy: a) Explants, which included a small amount of subchondral bone attached in the centre, were taken to confirm that these were full-thickness osteochondral samples; b) the sample was cut into three pieces in which the two cut lines were parallel; c) the middle part was chosen and secured on a Petri dish with two small pieces of Blu Tack (Bostik, Leicester, United Kingdom). d) A coronal image illustrated the zonal distribution and viability of chondrocytes throughout the full cartilage thickness. Living chondrocytes were stained green by 5-chloromethylfluorescein diacetate (CMFDA) and dead chondrocytes stained red by propidium iodide (PI). The region of interest in the image was set according to the cartilage thickness. The top 25% thickness of cartilage 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.

(Fig. 3a). Normally, one bovine metatarsophalangeal joint could provide up to 46 sampling sites across its eight joint facets (Fig. 1d). Cartilage was sampled on days 0, 7, 14, 21 and 28. At each timepoint, 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, 45 minutes) to label living chondrocytes green and dead chondrocytes red, respectively.[14] Explants were subsequently fixed with 10% (v/v) formalin (Fisher Scientific UK, Loughborough, United Kingdom) and secured to the base of a Petri dish with Blu Tack (Fig. 3c). Images were acquired using an upright confocal laser scanning microscope x 10 objective (Zeiss LSM 510 Axioskop; Carl Zeiss Ltd, Cambridge, United Kingdom) and reconstructed and analyzed by ImageJ software (Version 1.47; National Institute of Health, Bethesda, Maryland). Articular cartilage was divided into three regions based on the depth from the articular surface to the subchondral bone: the first quartile was defined as the superficial quarter, followed by the central half as the middle 50%, and the last quartile as the deep quarter[11] (Fig. 3d). Chondrocyte viability within each region was quantified as: % viable

cells = (number of CMFDA-labelled live cells/number of CMFDA- and PI-labelled cells) $\times$ 100%.

Matrix glycosaminoglycan assessment. The spectrophotometric microassay[12] was used to measure the sulphated GAG content of cartilage. The central full-thickness area of the specimen was obtained using a skin biopsy punch (2.5 mm diameter; Kai Industries Co. Ltd, Tokyo, Japan) and the 'before-digested' wet weight determined, which included the weight of cartilage and subchondral bone. After cartilage digestion by papain solution (300 µg in 1 ml of 1 mM ethylenediaminetetraacetic acid, 2 mM dithiothreitol, and 20 mM sodium phosphate; pH 6.8; 60°C for ~four hours), 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 normalize 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. The GAG content was defined as the GAG mass (in µg) per cartilage mass (in mg), and is shown in the figures as 'GAG (µg/mg cartilage)'.

Statistical analysis. Statistical analyses were performed using Minitab 16 (Minitab Inc., State College, Pennsylvania). All data were tested for normality (Kolmogorov-Smirnov test). Thereafter, parametric data

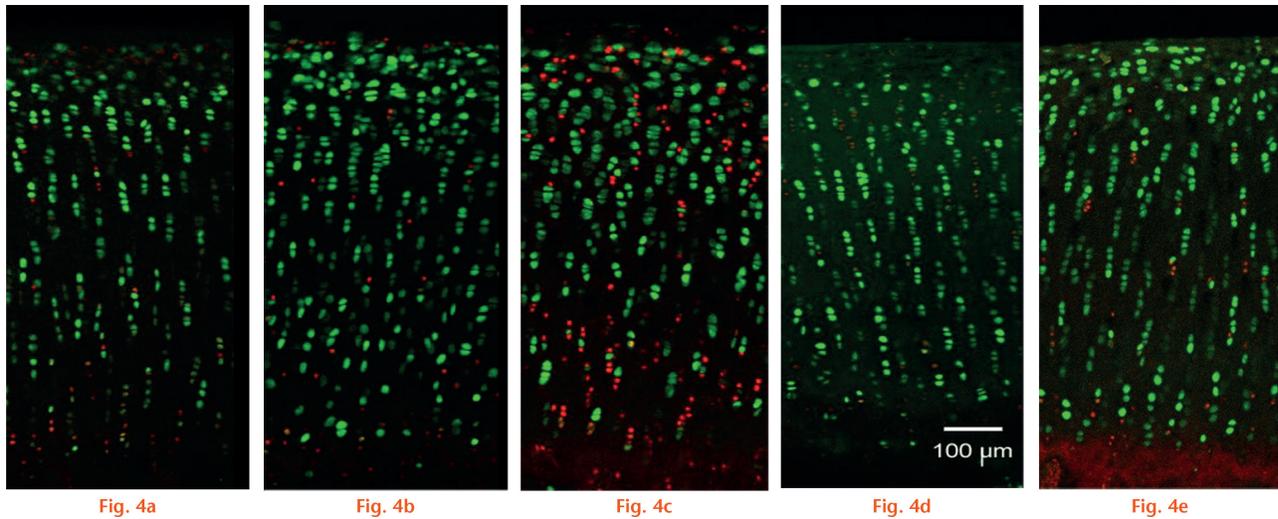| Fig. 4a | Fig. 4b | Fig. 4c | Fig. 4d | Fig. 4e |

Image showing chondrocyte viability over time in the static and dynamic models. Under the confocal laser scanning microscope, images of the cartilage coronal section showed that the total cell population labelled with propidium iodide (PI) (in red) increased gradually from day 0 to day 28 in the static model (a to c), and most of the red cells were located in the superficial and deep quarter of the cartilage. In the dynamic model (d and e), only a few sporadically distributed PI-labelled chondrocytes were observed on day 28, and there were still live cells (in green) adjacent to the subchondral bone (a) day 0 control; b) day 14 static; c) day 28 static; d) day 14 dynamic; e) day 28 dynamic).

were analyzed using paired or unpaired t-tests if two sets of data were compared, or one-way analysis of variance (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 ⩾ three data sets. Data are presented as means with SD, with the significance level set at $p < 0.05$.

Results

Chondrocyte viability. Six static models and six dynamic models were evaluated over 28 days. The samples from the fresh joint (day 0) were taken as the control (Figs 4a and 5). In the static model, the chondrocyte viability at day 0 was 89.9% (SD 2.5%), 94.7% (SD 1.1%), and 80.1% (SD 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% (SD 13.1%), 80.9% (SD 5.8%), and 46.9% (SD 8.5%) in the superficial quarter, central half, and deep quarter, respectively, at the end of the fourth week ($p < 0.001$ in each zone; one-way ANOVA) (Figs 4b, 4c and 5). However, in the dynamic model, chondrocyte viability was maintained without significant change after four weeks of culture, i.e. the change of chondrocyte viability of the superficial quarter was from 92.0% (SD 4.0%) (day 0) to 89.9% (SD 0.2%) (day 28), the middle half from 93.1% (SD 1.5%) (day 0) to 93.8% (SD 0.9%) (day 28), and the deep quarter from 85.6% (SD 0.8%) (day 0) to 84.0% (SD 2.9%) (day 28) ($p = 0.449$, 0.312, 0.170,

respectively; one-way ANOVA) (Figs 4d, 4e and 6). Further comparison between the chondrocyte viability of the static and dynamic models revealed that there were significant differences between each region during the four-week culture period ($p = 0.007$ 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 four-week 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 µg/mg (SD 0.06) and 6.18 µg/mg (SD 0.15) 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 µg/mg (SD 0.15) at the end of the first week, and dropped further to 3.93 µg/mg (SD 0.07) at the end of the third week. Even though, at the end of the culture period, the GAG content recovered slightly to 4.71 µg/mg (SD 0.06), it was still significantly less than that of 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, 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
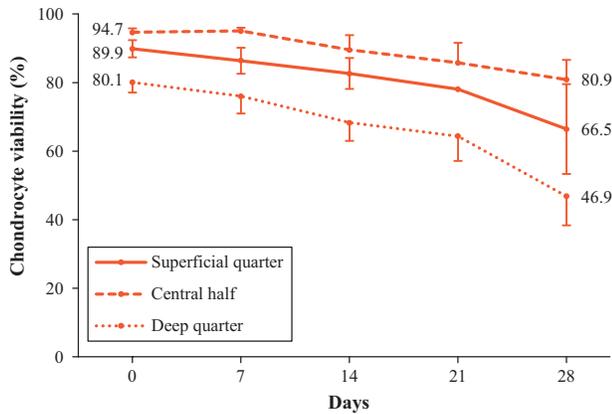
Fig. 5

Graph showing chondrocyte viability in cartilage in the static joint model. This decreased gradually in all three regions during the culture period. The viability of the central half remained the highest among these three regions in all timepoints, which only decreased by 13.8% (from 94.7% to 80.9%) in the 28 days of culture. The superficial quarter decreased by 23.4% (from 89.9% to 66.5%), and the deep quarter decreased by 33.2% (from 80.1% to 46.9%). The decreases of the three regions were statistically significant (p < 0.001 in each region; one-way analysis of variance). Data are presented as means and SD.
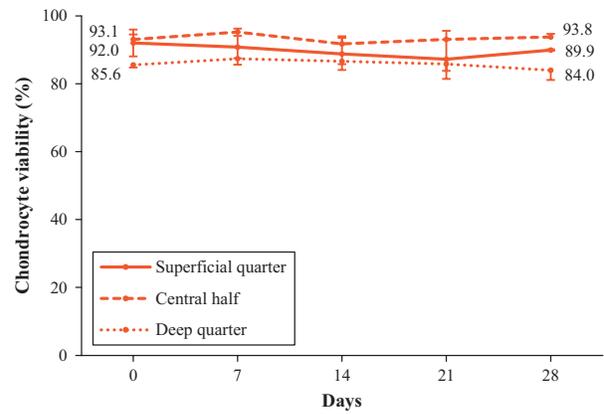

Fig. 6

Graph showing chondrocyte viability in cartilage in the dynamic joint model. It was maintained throughout the culture period. The central half was also the highest region in all timepoints, followed by the superficial and the deep quarter, in that order. Although there was a small increase/decrease of the chondrocyte viability during the four-week culture period, there was no statistically significant difference in any of the three regions (p = 0.449, 0.312, and 0.170 in the superficial quarter, central half, and deep quarter, respectively; one-way analysis of variance). Data are presented as means and SD.

content in the presence or absence of joint movement. Although cell viability in the static model decreased gradually during culture, over 80% of the cells were still alive in the central half region at the end of the fourth week (Fig. 5). The zonal heterogeneity of chondrocyte viability was marked, i.e. the chondrocytes in the central half region exhibited the highest viability, followed by the superficial quarter, whereas the viability of the deep quarter was the lowest at all timepoints. 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 inability 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 four weeks 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 downregulation of matrix metalloproteinases and aggrecanases,[2,19] and the preservation of chondrocyte viability by reduced levels of nitric oxide and reactive oxygen species.[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.
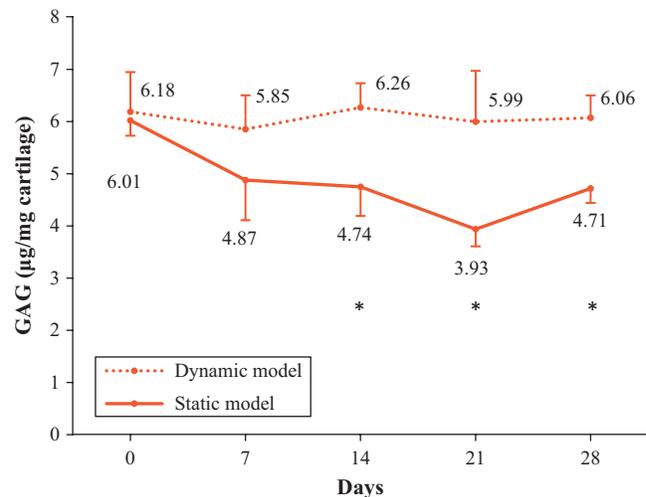

Fig. 7

Graph showing cartilage glycosaminoglycan (GAG) content in the dynamic and static models. 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 at 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.

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 four hours of culture, and most of the released GAG was not newly synthesized, 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 in canine knee joints that were fixed

rigidly using an external fixator for 11 weeks.[22] The lack of joint movement was thought to be the main reason for this because immobility reduces the rate of chondrocyte proteoglycan synthesis,[24-26] but elevates metalloproteinase production, leading to an 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 through the cartilage surface because the hydrodynamic size of the proteoglycans released in the culture medium was smaller than that of the 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 cartilage surfaces 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 forces 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[32] demonstrated that using a rotational plate to produce sinusoidal shear strain increased matrix protein synthesis by ~50% and proteoglycan production by up to 25%. The authors 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[35] further indicated that these two forces might have a synergistic effect which enhances the synthesis of matrix proteins. 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[33,34] and Wimmer et al[38] using a ceramic ball, produced variable types of forces on cartilage explants. Nevertheless, the system was relatively expensive, potentially limiting its wider use. Thus, the *ex vivo* bovine joint model described in the present study has particular advantages as it tested a more natural 'cartilage-on-cartilage' joint movement, and is therefore a potential alternative (model) which can exert 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 and may be useful for evaluating novel cartilage repair techniques such as cell therapies[39,40] and potentially reducing the amount of *in vivo* testing. The relatively intact nature of the articular structure was a significant advantage of this model in which responses with static or dynamic mechanical stimulation can be assessed. 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 techniques to maintain the sterility of the culture had to be mastered for the successful use of this model.

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