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1 **Introduction**

2 Articular cartilage is a highly-specialised tissue that, with synovial fluid, provides almost
3 frictionless interface between opposing bones¹. Movement between these surfaces and
4 throughout the tissue creates mechanical stimulation that maintains cartilage integrity through
5 the process of ‘mechanotransduction’^{2,3}. Compressive force on cartilage explants stimulates
6 the biosynthesis of collagen, proteoglycan and fibronectin if applied in the physiological
7 range (0.01-5 MPa) and frequency (0.01-1.0) Hz⁴. Animal studies have also demonstrated
8 that daily physiological exercise increased proteoglycan content and the cartilage thickness,
9 and might minimise the development of osteoarthritis⁵.

10 A wide variety of *in vitro* (e.g. isolated chondrocytes, cartilage explants) and *in vivo* (e.g.
11 rodent) experimental models have been utilised to understand mechanotransduction and the
12 response of cartilage to mechanical load, however each has limitations. For example, isolated
13 chondrocytes may change their phenotype in 2-D culture⁶. Cartilage explants might suffer
14 from ‘explantation injury’, resulting from increased IL-1 β levels during harvesting from the
15 joint⁷. For *in vivo* studies, the time-consuming approval process and the significant expense
16 and compliance with animal welfare regulations are unavoidable hurdles before live animal
17 experiments can be performed⁸. Therefore, we considered that it may be beneficial to develop
18 an organ culture model (*ex vivo* model) of a large synovial joint in an attempt to bridge the
19 gap between the *in vitro* cartilage explant model and the *in vivo* animal model.

20 Few organ level long-term culture system of the mammalian synovial joint have, to our
21 knowledge, been created. Nugent-Derfus *et al.*⁹ described a system where a bovine stifle joint
22 was cultured in a plastic bag for only 24hrs. However, the complicated settings of their
23 culture system and the difficulties of maintaining aseptic conditions of the circulated culture
24 media limited its wide reproducibility. Other connective tissue-related organ culture models
25 have been developed but for intervertebral disc cartilage¹⁰. However, the differences in tissue
26 structure, function and loading patterns suggest that these methods might not be applicable
27 for the study of the hyaline cartilage of the articular joint. In the present study we describe an
28 organ culture model using the bovine metatarsophalangeal joint, a relatively inexpensive,
29 common and reliable source of articular cartilage. The static and dynamic effect of joint
30 movement were evaluated on chondrocyte viability and matrix glycosaminoglycan content.

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3 31 **Materials and methods**
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6 32 *Materials*
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9 33 Chemicals were purchased from Sigma-Aldrich (Dorset, UK) unless otherwise stated. The
10 34 cell viability probes, 5-chloromethylfluorescein diacetate (CMFDA) and propidium iodide
11 35 (PI) were prepared as described¹¹, and Dulbecco's Modified Eagle's Medium (DMEM;
12 36 glucose 4.5g/L) were obtained from Invitrogen (Paisley, UK). The 1,9-dimethylmethylene
13 37 blue (DMMB) solution was formulated as described¹² and the standard shark chondroitin
14 38 sulphate (Sigma-Aldrich, UK) solution prepared at 0.1mg/ml.
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19 39 *Harvest of the bovine metatarsophalangeal joint*
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22 40 Twelve feet from separate healthy 3-year-old beef cattle were obtained from a local abattoir
23 41 (Scotbeef, Bridge of Allan, UK), and processed under sterile conditions within 6hrs of
24 42 slaughter. After thoroughly rinsing the feet with running water, they were securely fixed and
25 43 suspended on a custom-made stand that avoided possible contamination from the working
26 44 bench throughout the procedures (**Fig.1A**). The skin and hoof were removed completely, and
27 45 the exposed soft tissue layer rinsed thoroughly with at least 1L of sterile phosphate buffered
28 46 saline (PBS). Then, the suspended foot was moved to a laminar-flow ventilated hood for
29 47 further processing.
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36 48 A sterile operation field was established by wrapping sheets of sterile paper around the foot
37 49 (**Fig.1B**). The metatarsophalangeal joint was opened, and all surrounding soft tissues (e.g.
38 50 tendons, ligaments, joint capsules, synovia) removed. The bilateral collateral ligaments were
39 51 left to reinforce joint congruency if it was to be prepared for the dynamic model. The
40 52 metatarsal and the phalangeal bone were then transected using an oscillating saw to isolate
41 53 the metatarsophalangeal joint from the foot. The sawing lines were approximately 1cm above
42 54 and below the articular cartilage margin (**Fig.1C**). During the entire procedures, the joint was
43 55 kept hydrated by frequent rinsing with PBS.
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50 56 Additional steps were performed if the joint was prepared for the dynamic model. On the
51 57 transected surface of the metatarsal bone, a central hole was drilled by a sterile drill bit (\emptyset
52 58 3.0mm). A custom-made peg, refashioned from an external fixation pin (\emptyset 3.5mm), was
53 59 screwed into the drill hole (**Fig.1D**), and linked to a connecting bar, which was modified from
54 60 the 'adjustable telescopic strut' of an Ilizarov external fixator apparatus. The connecting bar
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3 61 was then linked to a driving motor for joint motion (**Fig.2**). The joint was then placed in a
4 62 sterilised 1L glass beaker for subsequent culture.

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7 63 *Culture environment*

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10 64 The culture media was DMEM including penicillin (100U/ml), streptomycin (100µg/ml) and
11 65 fetal bovine serum (10%v/v) (Sigma-Aldrich, UK). Typically, 300ml was sufficient for
12 66 immersing a joint. The opening of the beaker was sealed with double sheets of paraffin
13 67 membrane (Parafilm M[®], US) and a ventilation outlet prepared for gas exchange. The joint
14 68 culture system was then moved into a humidified incubator (37°C;5% CO₂) and media
15 69 changed bi-weekly.

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21 70 *Dynamic setting*

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24 71 The driving motor was set at 20rpm (0.33Hz) to mimic slow human walking speed. The
25 72 movement duration was controlled by an electronic timer and set to an intermittent pattern to
26 73 approximate animal/human activity levels¹³, i.e. 30mins continuous movement followed by
27 74 30mins of static load for 12hrs/day. Joint movement was constrained to a single plane to
28 75 replicate the hinge type motion on the synovial joint. The arc of movement was from full
29 76 extension to around 45° of flexion. The load applied was approximately 2.5 Newtons, which
30 77 was from the weight of the upper part of the joint (metatarsus) and was sufficient to maintain
31 78 firm apposition of the articulating surfaces.

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38 79 *Cartilage sampling*

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40 80 Full depth osteochondral samples were taken using fresh sterile scalpel blades (No.22)^{14, 15}
41 81 (**Fig.3A**). Normally, one bovine metatarsophalangeal joint could provide up to 46 sampling
42 82 sites across its 8 joint facets (**Fig.1D**). Cartilage was sampled at Day 0, 7, 14, 21 and Day 28.
43 83 At each time point, six cartilage explants from each joint were taken, i.e. three for assessment
44 84 of chondrocyte viability and the remainder for the GAG assay.

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49 85 *Chondrocyte viability assessment*

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52 86 A custom-made double-bladed cutting tool was used to trim the cartilage explants to create
53 87 two parallel straight edges so that the chondrocytes in different depths could be evaluated in
54 88 coronal sections¹¹ (**Fig.3B**). The trimmed explant was then incubated in DMEM with
55 89 CMFDA and PI (21°C; 45mins) to label living chondrocytes green and dead chondrocytes

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3 90 red, respectively¹⁴. Explants were subsequently fixed with 10% (v/v) formalin (Fisher
4 Scientific, Loughborough, UK) and secured to the base of a Petri dish with Blu-Tack (Bostik,
5 Leicester, UK) (**Fig.3C**). Images were acquired using an upright confocal laser scanning
6 microscope (Zeiss LSM510 Axioskop, Carl Zeiss, Welwyn Garden City, UK; ×10 objective)
7 and reconstructed and analysed by ImageJ (Ver1.47, NIH, USA). Articular cartilage was
8 divided into three regions on the basis of depth from the articular surface to subchondral
9 bone: the first quartile was defined as the superficial quarter, followed by the central half as
10 the middle 50%, and the deep quarter as the last quartile¹¹ (**Fig.3D**). Chondrocyte viability
11 within each region was quantified as: % viable cells = (number of CMFDA-labeled live
12 cells/number of CMFDA and PI labeled cells)×100%.

13 14 15 16 17 18 19 20 21 *Matrix glycosaminoglycan assessment*

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23 101 The spectrophotometric microassay¹² was used to measure the sulphated glycosaminoglycan
24 (GAG) content of cartilage. The central full-thickness area of the specimen was obtained
25 using a skin biopsy punch (Ø2.5mm; Kai Industries, Japan) and the ‘before-digested’ wet
26 weight determined, which included the weight of cartilage and subchondral bone. After
27 cartilage digestion by papain solution (300µg in 1ml of 1mM EDTA, 2mM dithiothreitol, and
28 20mM sodium phosphate; pH 6.8; 60°C for ~4hrs), the undigested material (i.e. subchondral
29 bone) was weighed again to obtain the ‘after-digested’ wet weight. The difference was the
30 cartilage wet weight, which was used to normalise the result to allow for any variation in the
31 size of the cartilage specimen. The absorbance of the digested solution was measured
32 immediately after the DMMB solution was added, and the result compared with the standard
33 solution to obtain the equivalent GAG weight of the cartilage sample. GAG content was
34 determined as the GAG mass (in µg) per cartilage mass (in mg), and shown in the Figures as
35 ‘GAG (µg/mg cartilage)’.

36 37 38 39 40 41 42 43 44 45 46 *Statistical analysis*

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48 115 Statistical analyses were performed using Minitab 16 (Minitab Inc., USA). All data were
49 tested for normality (Kolmogorov-Smirnov test). Thereafter, parametric data were analysed
50 using paired or unpaired t-tests if two sets of data were compared, or one-way ANOVA with
51 *post hoc* Tukey’s tests for more than two data sets. For non-parametric data, the Mann-
52 Whitney U test was used for comparison between two independent data sets, while the
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120 Kruskal-Wallis test was used for ≥ 3 data sets. Data are presented as means \pm standard
121 deviation (SD) with the significance level set at $p < 0.05$.

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122 **Results**

123 **Chondrocyte viability**

124 Six static models and 6 dynamic models were evaluated over 28 days. The samples from the
125 fresh joint (Day 0) were taken as the control (**Fig.4A, Fig.5**). In the static model, the
126 chondrocyte viability at Day 0 was $89.9\pm 2.5\%$, $94.7\pm 1.1\%$ and $80.1\pm 3.0\%$ in the superficial
127 quarter, central half and deep quarter, respectively, which was not significantly different to
128 the dynamic model ($p=0.381$, 0.111 and 0.059 , respectively; unpaired t test). After culturing,
129 the number of dead cells increased progressively and the chondrocyte viability decreased
130 significantly to $66.5\pm 13.1\%$, $80.9\pm 5.8\%$ and $46.9\pm 8.5\%$ in the superficial quarter, central
131 half and deep quarter, respectively, at the end of the 4th week ($p<0.001$ in each zone; one-way
132 ANOVA) (**Fig.4B&4C, Fig.5**). However, in the dynamic model, chondrocyte viability was
133 maintained without significant change after 4wks of culture, i.e. the change of chondrocyte
134 viability of the superficial quarter was from $92.0\pm 4.0\%$ (Day 0) to $89.9\pm 0.2\%$ (Day 28), the
135 middle half $93.1\pm 1.5\%$ (Day 0) to $93.8\pm 0.9\%$ (Day 28) and the deep quarter $85.6\pm 0.8\%$ (Day
136 0) to $84.0\pm 2.9\%$ (Day28) ($p=0.449$, 0.312 , 0.170 , respectively; one-way ANOVA)
137 (**Fig.4D&4E, Fig.6**). Further comparison between the chondrocyte viability of the static and
138 the dynamic model revealed that there were significant differences between each region
139 during the 4wks culture ($p=0.007$ in the superficial quarter, $p<0.001$ in both the central half
140 and deep quarter; two-way ANOVA). Therefore, in the dynamic model, chondrocyte viability
141 was maintained at the initial level throughout the 4wk culture period, but in contrast it
142 decreased progressively in the static model.

143 **GAG analysis**

144 Evaluation of the cartilage matrix of the day 0 control samples revealed that the GAG content
145 was $6.01\pm 0.06\mu\text{g}/\text{mg}$ and $6.18\pm 0.15\mu\text{g}/\text{mg}$ in the static and dynamic models, respectively,
146 which were not significantly different ($p=0.640$; unpaired t test). The GAG content in the
147 dynamic model was maintained at a consistent level without change throughout the culture
148 period ($p=0.887$; one-way ANOVA). However, for the static model, it decreased to
149 $4.87\pm 0.15\mu\text{g}/\text{mg}$ at the 1st week and dropped further to $3.93\pm 0.07\mu\text{g}/\text{mg}$ at the 3rd week. Even
150 though at the end of the culture the GAG content recovered slightly to $4.71\pm 0.06\mu\text{g}/\text{mg}$, it
151 was still significantly less compared to the dynamic model ($p<0.001$; two-way ANOVA).
152 Further point-to-point comparison revealed that the difference became significant after Day

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3 153 14 (Fig.7). Therefore, over this time period, the GAG content of cartilage matrix was also
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5 154 maintained in the dynamic model but not in the static model.
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3 155 **Discussion**
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6 156 This report describes a novel large *ex vivo* joint culture model assessed by chondrocyte
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8 157 viability and matrix GAG content in the presence or absence of joint movement. Although
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10 158 cell viability in the static model decreased gradually during culture, there were still >80%
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12 159 alive in the central half region at the end of week 4 (**Fig.5**). The zonal heterogeneity of
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14 160 chondrocyte viability was marked, i.e. the chondrocytes in the central half region exhibited
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16 161 the highest, followed by the superficial quarter, whereas the viability of the deep quarter was
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18 162 the lowest at all time points. This zonal heterogeneity, to our knowledge, has not been
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20 163 described in detail but is apparent in images in previous studies¹⁴⁻¹⁷. It is possible that the
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22 164 scalpel cut damaged the chondrocytes in a depth-dependent manner, however this is
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24 165 unavoidable in order to assess zonal viability¹⁴. In addition, the limitation of the chondrocytes
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26 166 to obtain nutrients in the deep quarter, which probably diffuse mainly from the culture
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28 167 media¹⁸, may play a role in the greater decrease in the chondrocyte viability of the deep
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30 168 quarter.

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32 169 However, with joint movement, chondrocyte viability was greatly improved over the whole
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34 170 culture period as viability was maintained at the initial level without significant decrease
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36 171 during the 4wks of culture (**Fig.6**). It is possible that mechanical stimulation directly from
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38 172 joint movement was important as both *in vitro* and *in vivo* studies demonstrate that loading in
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40 173 the physiological range maintain cartilage integrity. This is achieved through the down
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42 174 regulation of matrix metalloproteinases (MMPs) and aggrecanases (ADAMTS)^{2, 19}, and the
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44 175 preservation of chondrocyte viability by reduced levels of nitric oxide (NO) and reactive
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46 176 oxygen species (ROS)^{5, 19}. Alternatively the fluid flow created by joint movement could
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48 177 increase the exchange of nutrients and waste products between cartilage and culture
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50 178 medium²⁰, supporting chondrocyte viability.

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52 179 Matrix GAG content decreased after the first week (**Fig.7**) and similar observations have
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54 180 been reported in *in vitro* studies using bovine cartilage explants²¹⁻²³. Previous work
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56 181 demonstrated that early matrix GAG loss occurred within the first 4hrs of culture, and most
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58 182 of the released GAG was not newly synthesised but previously produced and already stored
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60 183 in the matrix²³. There are some *in vivo* studies with similar results. The cartilage GAG
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185 184 concentration decreased significantly by 20-23% if canine knee joints were fixed rigidly
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187 185 using an external fixator for 11wks²². The lack of joint movement was thought to be the main
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189 186 reason because it reduced the rate of chondrocyte proteoglycan synthesis²⁴⁻²⁶ but elevated

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3 187 metalloproteinase (MMP-2) production leading to accelerated loss of matrix components²⁷.
4 188 These results indicated that in unloaded cartilage catabolic events predominated over
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6 189 anabolic processes and the extracellular matrix would contain less GAG potentially reducing
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8 190 its resilience. In addition, the matrix porosity of the cartilage surface may affect the release of
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10 191 the matrix proteoglycan²⁸. Only molecules smaller than the matrix pore size would pass the
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12 192 cartilage surface because the hydrodynamic size of the proteoglycans released in the culture
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14 193 media was smaller than matrix proteoglycans²⁹. Thus, the observed loss of the matrix GAG in
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16 194 the static model (**Fig.7**) might be the outcome resulting from the reduction of the chondrocyte
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18 195 GAG synthesis and the acceleration of matrix proteolysis.

19 196 Applying joint movement in the dynamic model significantly prevented the decrease of the
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21 197 matrix GAG content (**Fig.7**). However, the force between cartilages during joint movement is
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23 198 complex and difficult to reproduce experimentally. It is known that articular cartilage *in vivo*
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25 199 is subjected to both compressive and shearing force under normal physiological movement³⁰.
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27 200 Nevertheless, most of the *in vitro* studies used mechanical compression force as a test load⁴,
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29 201 ³¹. Pure shear stress also had effects but does not appear to have been studied in detail. For
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31 202 example, Jin *et al.* demonstrated that using a rotational plate to produce sinusoidal shear
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33 203 strain increased matrix protein synthesis by ~50% and proteoglycans production by up to
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35 204 25%³². They indicated that even though the tissue shear force caused less volumetric
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37 205 deformation than the compression force, its stimulatory effect was still potent. The increase
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39 206 in matrix protein synthesis from the stimulation of shear stress was also shown in a series of
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41 207 studies by Grad *et al.*^{33,34}. Their results suggested that the signal transduction pathways of the
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43 208 compression force and the shear stress might be different inside the cartilage tissue. Waldman
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45 209 *et al.* further indicated that these two forces might have a synergistic effect, which enhanced
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47 210 the synthesis of matrix proteins³⁵. Therefore, compression with sliding movement was
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49 211 suggested in some studies to be a more appropriate method for loading articular cartilage^{36,37}.
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51 212 However, only a few *in vitro* experimental models have applied both compression and shear
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53 213 to cartilage. An interesting bioreactor system developed by Grad *et al.* and Wimmer *et al.*
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55 214 using a ceramic ball produced variable types of forces on cartilage explants^{33,34,38}.
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57 215 Nevertheless, the system was relatively expensive, potentially limiting its wider utilisation.
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59 216 Thus, the *ex vivo* bovine joint model described in the present study had particular advantages
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217 as it tested a more natural 'cartilage-on-cartilage' joint movement, and so could be another
218 model that produced both compression and shear force on cartilage. It should be noted that
219 the joint model was not designed to replicate full body weight as current cartilage

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3 220 regenerative medicine strategies only allow patients to bear weight minimally in the early
4 221 post-operative period. However, as the results of the study revealed, only a few Newtons of
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6 222 load (with motion) were sufficient to maintain cartilage health.
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9 223 To our knowledge, this is the first description of a long-term cultured large joint model, the
10 224 validity of which was assessed by chondrocyte viability and matrix GAG content. This model
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12 225 may provide new directions for articular cartilage research in addition to the more commonly
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14 226 used *in vivo* and *in vitro* models. The relatively intact nature of the articular structure was a
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16 227 significant benefit of this model which has the benefit of comparing responses to static or
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18 228 dynamic mechanical stimulation. The relatively large volume of cartilage tissue available for
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20 229 sampling was another advantage as sufficient sampling areas for multiple assessments in the
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22 230 same joint, especially for long-term culture experiments were possible. However, a
23
24 231 significant learning curve for the aseptic preparation of the joint and the techniques to
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26 232 maintain the culture sterile had to be mastered for the successful use of this model.
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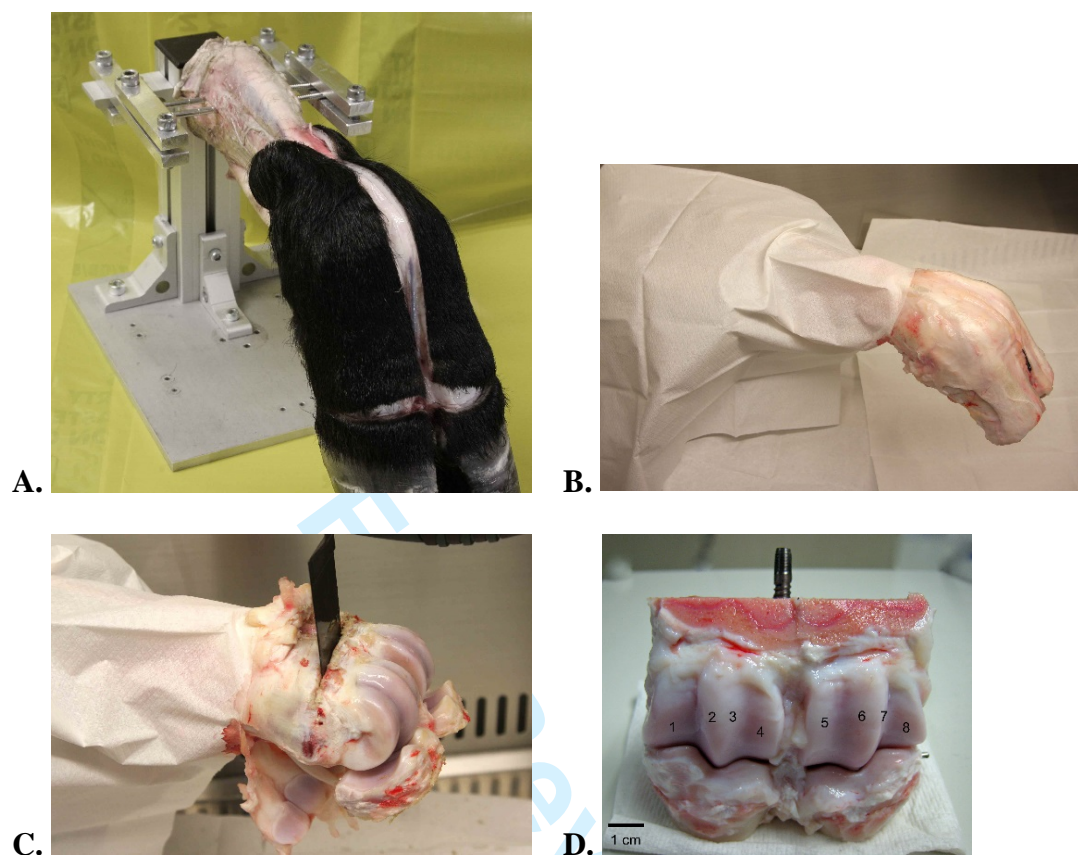


Figure 1. Preparation of the bovine metatarsophalangeal joint and the cartilage sampling sites

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.

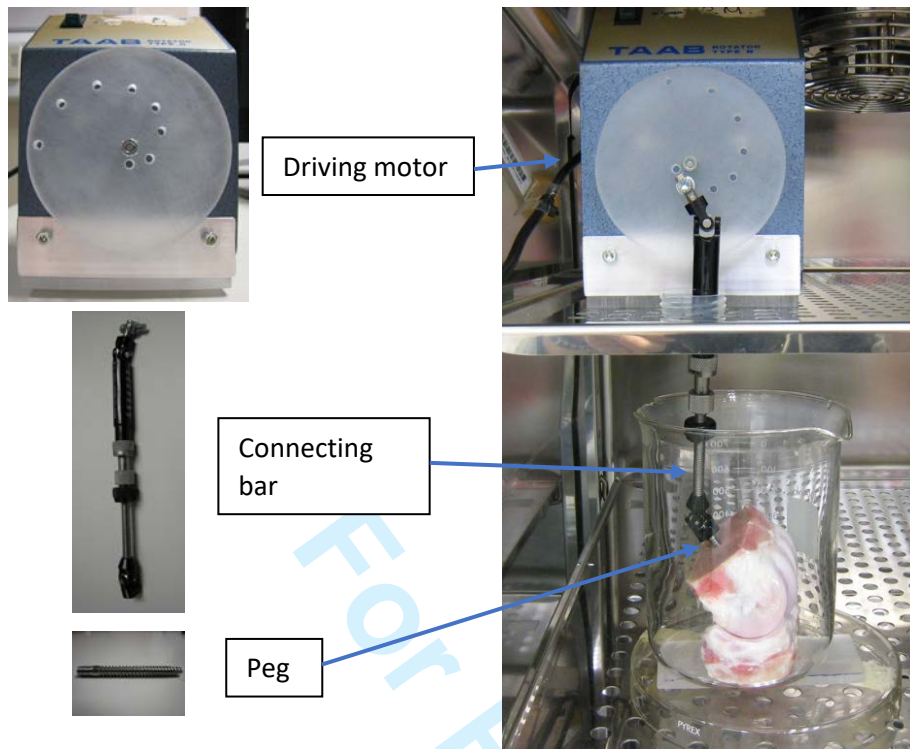


Figure 2. Construction of the motion machine for the dynamic model

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 CO₂-controlled incubator for joint culture. (The culture media and the sealing Parafilm were removed for clear demonstration).

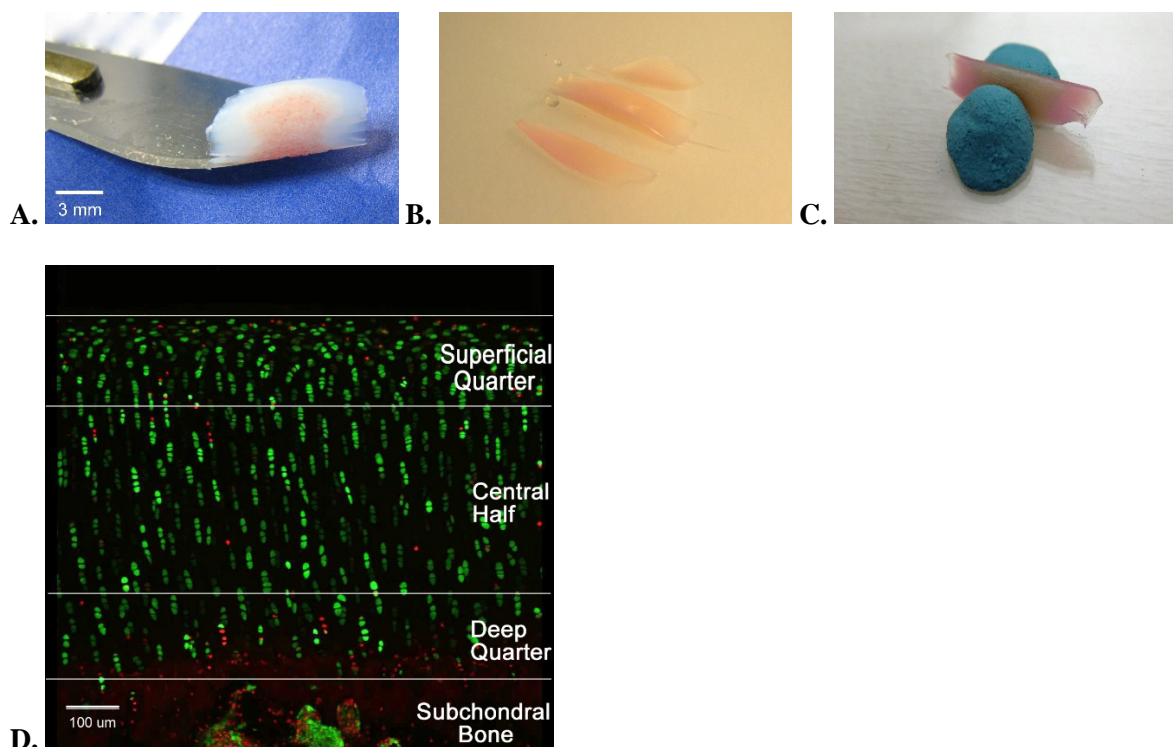


Figure 3. Cartilage explant preparation and visualization of fluorescently-labeled *in situ* chondrocytes by confocal laser scanning microscopy (CLSM)

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.

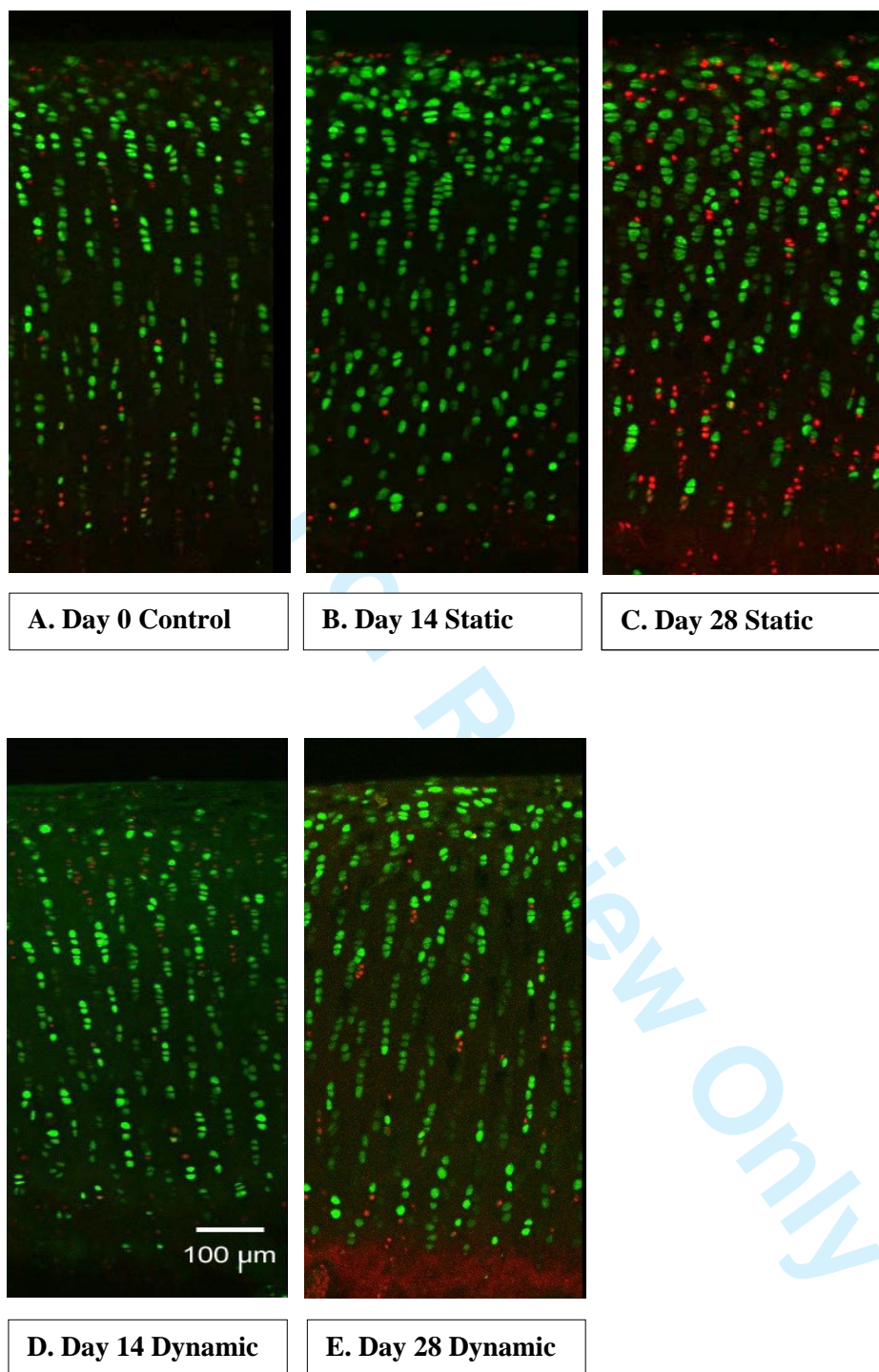


Figure 4. Images of chondrocyte viability with time in the static and dynamic model

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

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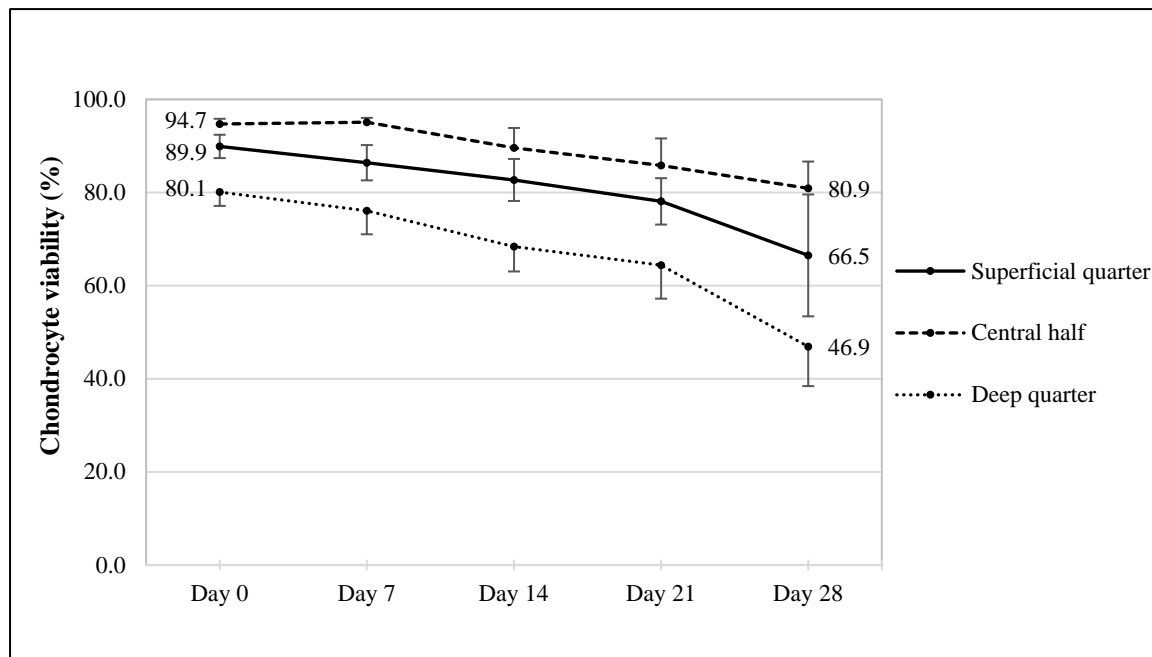


Figure 5. Chondrocyte viability in cartilage in the static joint model

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 \pm standard deviation.

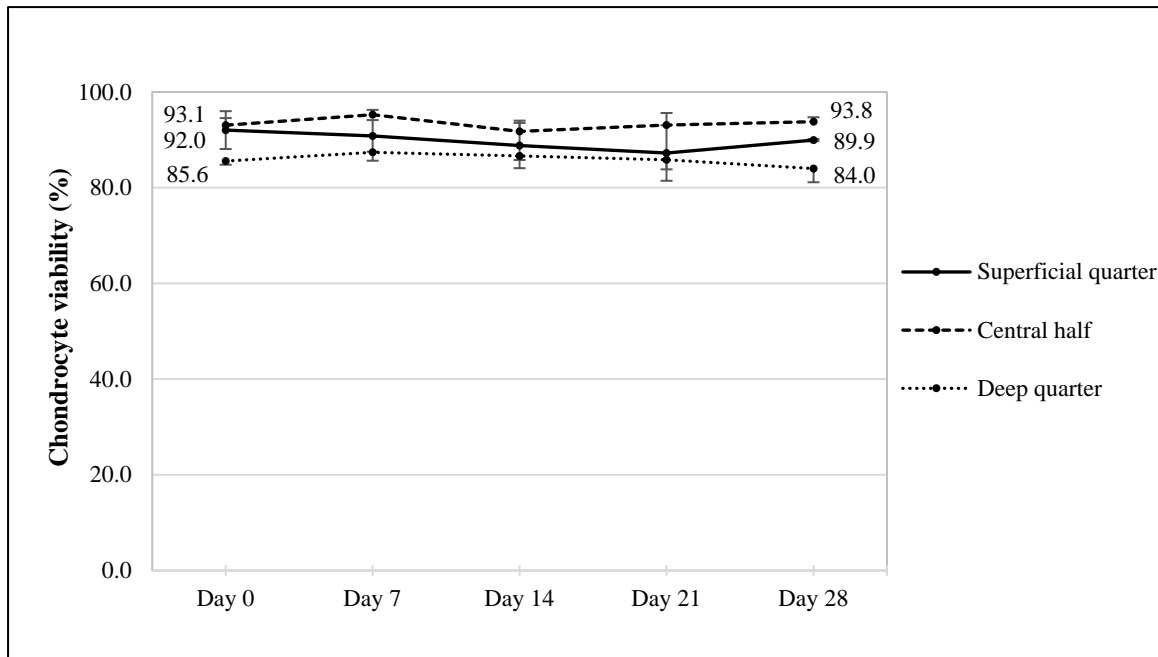


Figure 6. Chondrocyte viability in cartilage in the dynamic joint model

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).

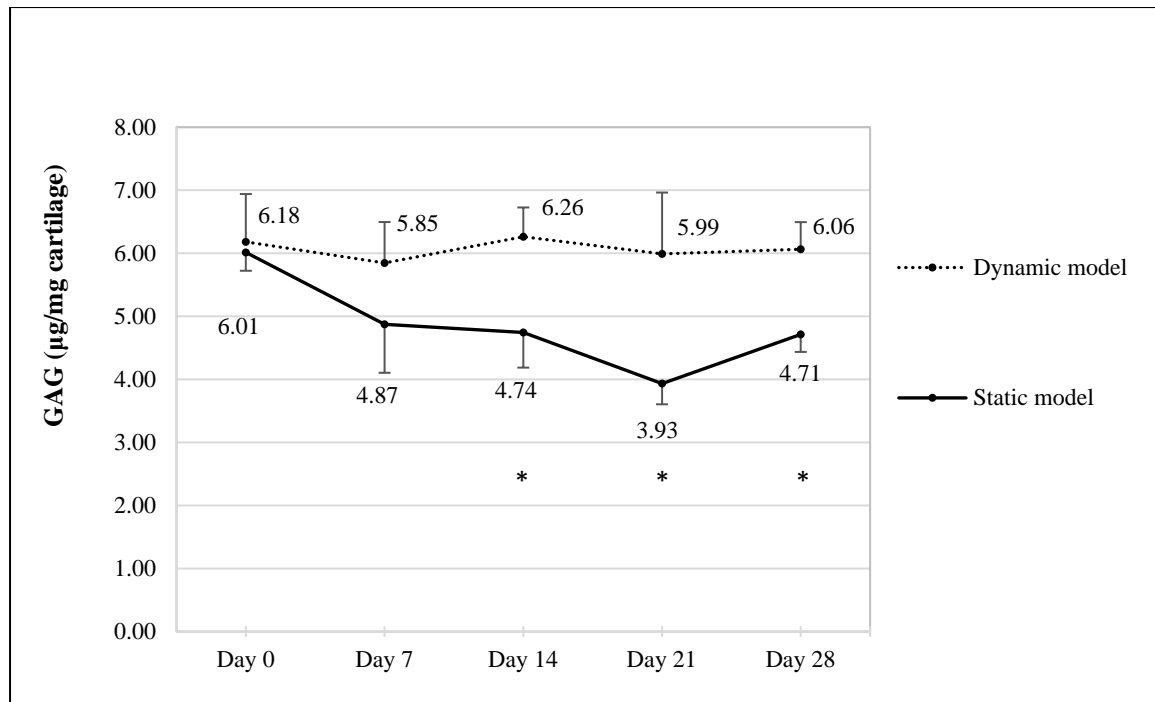


Figure 7. Cartilage GAG content in the dynamic and the static model

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.