OsteoArthritis and Cartilage (2005) **13,** 1084–1091

doi:10.1016/j.joca.2005.07.002

Osteoarthritis and Cartilage

International Cartilage Repair Society

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The response of elderly human articular cartilage to mechanical stimuli *in vitro*[1]

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Summary

Objective: To investigate the biosynthetic response of elderly human femoral head articular cartilage to mechanical stimulation *in vitro* and its variation with site.

Method: Full-depth cartilage biopsies of articular cartilage were removed from defined sites on 10 femoral heads from patients aged 68–95 years. Cartilage explants were subjected to either static or cyclic (2 s on/2 s off) loading in unconfined compression at a stress of 1 MPa for 24 h, or no load. Metabolic activity was assessed by adding medium containing ^{35}S-sulphate and ^{3}H-leucine during the last 4 h of loading and measuring the incorporated radioisotope. Matrix composition was measured in terms of the amounts of collagen, sulphated glycosaminoglycans (GAG) and water content.

Results: Loading of elderly human articular cartilage at 1 MPa significantly inhibited incorporation of ^{35}S-sulphate ($P = 0.023$) into cartilage explants. Pairwise comparisons showed that the difference in incorporation was only for static loading (43% decrease compared to unloaded) ($P < 0.05$). ^{3}H-leucine incorporation appeared to follow the same trends but neither static nor cyclic load was significantly different from control ($P = 0.31$). Significant topographical variation was found for % GAG wet and GAG:collagen but not water content, % GAG dry or collagen. Isotope incorporation rates were in the order anterior > superior > posterior.

Conclusion: Static loading inhibits matrix biosynthesis in elderly human cartilage, and cyclic loading is not stimulatory. This is in contrast to previous studies on young bovine tissue where cyclic loading is stimulatory.

Key words: Articular cartilage, Matrix biosynthesis, Mechanical stimulation, Ageing.

Introduction

Articular cartilage is essential to normal diarthrodial joint function; it reduces stresses on the subchondral bone, prevents abrasion between articulating bone extremities and provides a low-friction bearing surface. The proper mechanical functioning of articular cartilage depends on the composition and ultrastructure of the extracellular matrix[1] but the maintenance and gradual turnover of the matrix depend on the biosynthetic activity of the chondrocytes[2,3]. Studies have shown the composition and thickness of articular cartilage vary from joint to joint, and topographically within a joint, as a function of age and among species[4]. In the human hip joint the loading intensity, elastic modulus and cartilage thickness decrease from the superior aspect of the femoral head to the inferior[5].

Ageing sees an increase in senescent chondrocytes but, in the absence of pathologies such as osteoarthritis (OA), the matrix integrity is maintained[6]. Sensitivity to cytokines and growth factors, which primarily control matrix biosynthesis, decreases with age and it is presumed that matrix turnover will be slower[7]. One of the major proteoglycans, aggrecan, is modified with age with a decrease in its chondroitin sulphate content, but no appreciable change in chondroitin sulphate chain length. As the chondroitin sulphate chains play a major role in determining the cartilage elasticity, this reduction seems to relate to the reduced elasticity seen in aged cartilage[8]. Coupled with this is an increase in the number of smaller proteoglycans such as biglycan and decorin[9]. A significant decrease in glycosaminoglycan (GAG) synthesis has also been reported regionally in human femoral head cartilage with increasing age[5]. GAG synthesis was inversely related to cartilage thickness[5] and the greatest decrease in was found in the highly loaded superior region, which has the thickest cartilage and highest GAG content, whilst there was hardly any variation in the posterior and anterior regions[5].

It is generally acknowledged that physiological loading of articular cartilage is necessary to maintain normal joint function. Cyclic mechanical loading is important in development, remodelling and disease of many tissues including articular cartilage[10]. *In vivo*, joint loads are determined by body weight and activity and it has been suggested that cartilage adapts its modulus to control local strains[11]. That strain is an important factor was shown by studies of chondrocytes in agarose in which the larger strains experienced in unconfined compression resulted in greater GAG synthesis[12]. *In-vitro* studies have shown biosynthetic activity and matrix turnover are also sensitive to frequency and amplitude of stimulation. Static compression consistently shows a dose-dependant decrease in

[1]This work was supported by the Aberdeen Wishbone Studentship to MSP and the Medical Research Council.

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Received 17 February 2004; revision accepted 1 July 2005.

biosynthetic activity compared with unloaded tissue[13–17], and is thought to be mediated through changes in pericellular pH and ion concentration, fixed charge density and osmotic pressure[18]. Cyclic compression, on the other hand, has been found to either stimulate, inhibit or have no effect on biosynthetic activity depending on the loading frequency and amplitude[14–16,19–23]. Generally loads applied at physiological frequencies seem to stimulate matrix biosynthesis[14,15,19,24], an exception being a recent study by Torzilli *et al.*[25] which showed a linear increase in inhibition with increasing stress on cyclic loading at 1 Hz in unconfined compression. All these studies used animal tissues.

A number of studies have investigated the composition and biosynthetic activity of elderly human cartilage[6,8,26–29] but only a few have investigated the effects of mechanical load[30–32]. All indicate a different response in elderly cartilage to that found in young bovine tissue that is widely used as a model. Schneiderman *et al.*[30] showed that both uniaxial unconfined load (3–8 atm (0.3–0.8 MPa)) and increased osmotic compression on femoral head cartilage (19–88 years) reduced the incorporation of sulphate into the matrix of human articular cartilage in a reversible manner. Another study by Maroudas *et al.*[31] investigated the effects on proteoglycan synthesis of slow, cyclic compression at 0.17 Hz using bovine (5 years old), young human (~34 years old) and elderly human (63–87 years old) cartilage using pressures of 5, 10 and 30 atm (0.5, 1 and 30 MPa). Chondrocytes in bovine and young human explants showed significant increases in GAG biosynthesis, whereas the elderly human cartilage did not respond.

Recent studies have concentrated on mechanical loading and cell viability in young and mature bovine cartilage explants[33–36]. Their main observation was an increase in cell death in the superficial zone after just 1 h of loading, which continued until it reached a plateau at 6 h. Levin *et al.*[34] observed a greater depth of cell death in young bovine compared with mature bovine cartilage. At 5 MPa cell death occurred rapidly in the superficial zone and cyclic loading for 24 h saw cell death progressing to the middle zone[36,37]. Above 6 MPa in mature bovine explants, cell death increased in proportion to applied cyclic load[33]. These studies indicate that there is a threshold level of deformation and continued, repeated loading eventually leads to membrane rupture. It is also possible that excessive loading and/or high stresses could be responsible for the initiation of the degeneration process.

This study begins to extend those data by investigating in more detail the biosynthetic response of elderly human articular cartilage to defined mechanical stimuli. To understand the aetiology and pathogenesis of disorders like OA, it is important to have an understanding of how ageing cartilage responds to mechanical loading and whether this is related to the biochemical composition of the tissue. It is also important to know the extent to which animal cartilage, especially from young animals, is a good model for elderly human tissue.

Methods

EXPLANT HARVESTING AND CULTURE

Femoral heads were retrieved from patients undergoing a hemiarthroplasty for a fractured neck femur attributed to osteoporosis. Local Ethics Committee approval was obtained for this process and all patients gave informed consent for the tissue to be used. Full thickness explants of 5 mm diameter were excised without bone, using a cork borer, from the femoral heads of 10 patients (68–95 years, 9 females and 1 male). Samples were taken from three distinct areas, anterior, superior and posterior, and samples from each region were randomised for the different loading regimes. Prior to removing samples, the surface of the joint was carefully examined visually and only those areas in which the cartilage showed no fibrillation or evident damage were removed. Each explant's original position on the femoral head was recorded. The explants were gently blotted between sterile gauze pads and weighed in preweighed sterile eppendorf tubes. They were then transferred into Dulbecco's Modified Eagle's Medium (DMEM) with GlutaMAX I, 1000 mg/l D-glucose and sodium pyruvate (Invitrogen Ltd, Paisley, UK), containing 10% foetal calf serum (Globepharm Ltd, Guildford, UK) and 25 $\mu g\,ml^{-1}$ ascorbic acid (Sigma–Aldrich Company Ltd, Dorset, UK), 50 IU penicillin and 50 $\mu g\,ml^{-1}$ streptomycin (Invitrogen, Paisley, UK), and left for 48 h at 37°C in a 5% CO_2 incubator, as this has been shown to be sufficient time for synthetic activity to reach equilibrium after explantation[20].

MECHANICAL LOADING

The loading was performed using a pneumatically driven device, similar to that previously described by Larsson *et al.*[15]. This comprised two separate loading units: the first operated via a timing device, which enables controlled cyclic loads to be generated, the other unit applies a constant load of the same magnitude. Up to six samples may be loaded in each unit. The whole system was maintained at 37°C in an incubator.

After the 48-h equilibration period each explant was weighed again and placed between 1 mm thick pre-soaked polyethylene filters in a sterile bijou containing 1 ml of DMEM. Next a polyethylene spacer, designed to transmit load from the tube lid to the tissue while allowing the medium to circulate freely, was placed on top of the upper filter and the tube lid replaced loosely. Two explants from each of the three areas were prepared for each of the loading regimes. These tubes were then placed into the loading device and loaded for a period of 24 h. Loads were applied so as to generate a stress of 1 MPa on each explant. Cyclic loads were applied for 2 s followed by 2 s of no load in a square-wave pattern with a load rise and fall time of about 90 ms. Similar tubes were subjected either to a static load of the same magnitude or to no load, which was chosen to act as a control group.

DETERMINATION OF MATRIX BIOSYNTHESIS

For the final 4 h of loading fresh medium containing radiolabelled precursors to measure matrix biosynthesis was added to each tube. Dual labelling was carried out using tritiated leucine (Amersham Biosciences, UK Limited), 0.92 MBq (25 $\mu Ci)\,ml^{-1}$ to measure general protein biosynthesis, and [^{35}S]-sulphate (Amersham Biosciences, UK Limited), 0.37 MBq (10 $\mu Ci)\,ml^{-1}$ for sulphated GAGs. Loading was paused briefly whilst fresh media containing the radiolabelled precursors were added (approximately 5 min). On completion of loading the radioactive media were removed and the explants washed in four changes of 1 ml ice-cold phosphate buffered saline (PBS) containing a protease inhibitor cocktail (Sigma–Aldrich Company Ltd, Dorset, UK). Washing was done in preference to extraction and desalting to enable accurate composition measurements to be made on the same samples. This has

previously been shown to be effective in removing non-incorporated isotope[38]. The explants were left to air dry for 48 h at 37°C and weighed, and the water content determined from the difference between wet and dry masses. Drying at 37°C is preferable to drying by vacuum dessication[39] and drying to equilibrium occurs within 48 h[40]. Explants were then digested in 20 μl × (cartilage wet mass in mg) of papain (Sigma–Aldrich Company Ltd, Dorset, UK) (135 μg ml^{-1} of papain added to a buffer of 0.1 M sodium acetate, 5 mM ethylene diamine tetra-acetic acid (EDTA) (Sigma–Aldrich Company Ltd, Dorset, UK) and 5 mM cysteine-HCL (Sigma–Aldrich Company Ltd, Dorset, UK) pH 6.0) for 24 h at 65°C.

Radioactivity was measured by taking 20 μl of the papain digest (equivalent to 1 mg wet weight of cartilage), adding 2 ml Hi-safe scintillation fluid (Wallac Scintillation Products Ltd.) and counting in a Wallac 1409 Liquid Scintillation Counter. Incorporation of the isotope into the tissue was expressed as nmol g^{-1} h^{-1} by dividing the measured disintegrations per minute by the product of the specific activity (calculated from the known concentrations of sulphate and leucine in the medium), the wet mass of the tissue and the labelling time.

LOAD-RECOVERY

Load-recovery experiments were performed to investigate whether there were any permanent changes in metabolism after 24 h loading. Explants from three femoral heads were subjected to the same loading regime described above. After 24 h the load was removed and explants were placed into fresh media and left free swelling for a further 24 h, with the addition of radiolabel for the final 4 h as described earlier. The explants were then dried, digested and matrix biosynthesis measured as before.

SHORT LOAD

The initial time period of 24 h loading was chosen to ensure a stable response would be obtained. However, this is not necessarily physiological; therefore, explants from a further five femoral heads were loaded using the same configuration for a 4-h period, with the addition of radiolabel for the whole time.

DETERMINATION OF MATRIX COMPOSITION

Matrix composition was studied using standard biochemical methods to measure gross composition in terms of amount of collagen, by hydroxyproline assay[41], and amount of GAG, by dimethlymethylene blue assay (DMMB) (Sigma–Aldrich Company Ltd, Dorset, UK)[42].

Total sulphated GAGs were measured using a DMMB method modified from that described by Stone *et al.*[43] for use in a 96-well plate. The working dye solution is unstable and care was taken to ensure that the ratio of the absorbances at 657 nm and 595 nm was between 1:1.3 and 1:1.5. Standard curves were obtained using concentrations of chondroitin 6-sulphate (Sigma–Aldrich Company Ltd, Dorset, UK) from 0 to 150 μg ml^{-1} at 10 μg ml^{-1} intervals. Triplicate 8 μl aliquots of a 20× dilution of papain digest were mixed in a 96-well plate with 200 μl of DMMB working solution and absorbance read at 525 nm using a Dynatech MR5000 plate reader 3 min after the addition of the dye.

Collagen content was measured colorimetrically[44] based on the "direct acid" method of Firschein and Schill[45] and modified for use in a 96-well plate. Samples were hydrolysed by adding 180 μl of HCl to 100 μl of 10× dilution of papain digest in glass tubes which were sealed and heated at 110°C for 18 h, to liberate hydroxyproline. Standard curves were obtained using concentrations of hydroxyproline (Sigma–Aldrich Company Ltd, Dorset, UK) from 1 to 16 μg ml^{-1}. Triplicate 100 μl aliquots of hydrolysed papain digest and duplicate 100 μl of standards were placed in a 96-well plate. To all wells were added 100 μl of methyl cellosolve followed by 50 μl of chloramine T solution (Sigma–Aldrich Company Ltd, Dorset, UK) and finally 60 μl of Ehrlichs reagent (Sigma–Aldrich Company Ltd, Dorset, UK) and mixed thoroughly by back pipetting. They were then put into an oven at 80°C for 50 min, allowed to cool and the absorbance read at 570 nm on a plate reader (Dynatech MR5000, Dynatech laboratories, USA). Collagen content was calculated assuming 14 g hydroxyproline per 100 g collagen[46]. Collagen and GAG concentrations were expressed in terms of the wet and dry weight of tissue. To remove possible inaccuracies of measurement of wet mass, the ratio of GAG:collagen was also calculated.

DNA CONTENT

The DNA content of the cartilage samples was determined as a measure of the cellularity of the cartilage. Standard curves were obtained using concentrations of salmon/herring testis DNA (Sigma–Aldrich Company Ltd, Dorset), from 2 to 14 μg ml^{-1} in a 96-well plate assay. Standards (duplicates of 50 μl) and test samples (triplicates of 10 μl of the original papain digest) were added to separate wells in a 96-well plate. All wells were then made up to a volume of 100 μl by the addition of papain buffer (not containing papain). Finally, 100 μl of working Hoechst 33258 solution (0.2 μg ml^{-1}) (Sigma–Aldrich Company Ltd, Dorset) was added to all wells. The plates were read on a fluorimeter (Cytofluor Series 4000, Perspective Biosystems) at a wavelength of 455 nm using an excitation wavelength of 350 nm. The DNA content of the cartilage samples was expressed as mg DNA normalised to the wet weight of the cartilage (mg g^{-1}).

CHONDROCYTE VIABILITY AND NUMBER

Chondrocytes were isolated from explants taken from three femoral heads after being subjected to the same loading described above (12 randomised samples per loading regime) following a slightly modified protocol of Wang *et al.*[47]. In brief, after loading the samples were chopped up and treated with 0.25% hyaluronidase in DMEM on an orbital shaker for 2 h at 37°C. Then the hyaluronidase was removed and replaced with pre-warmed 0.25% pronase in DMEM for 90 min at 37°C on an orbital shaker. After this the samples were washed twice in DMEM and stored overnight in DMEM at 37°C. The following day samples were solubilized by an overnight digestion in 0.25% collagenase solution at 37°C on an orbital shaker, centrifuged at 980 *g* for 10 min, and the pellet of cells resuspended in 0.5 ml of DMEM. Chondrocyte viability was determined using the trypan blue (Sigma–Aldrich Company Ltd, Dorset, UK) exclusion test, using 50 μl of cell solution and a haemocytometer.

To confirm the chondrocyte viability and ascertain where cell death was occurring the viability was also measured in tissue sections using fluorescein diacetate (FDA) and propidium iodide (PI) (both Sigma–Aldrich Company Ltd, Dorset, UK) on samples from two femoral heads. The

membrane-permeable FDA is cleaved by esterases in live cells to yield cytoplasmic green fluorescence, whereas the membrane-impermeable PI labels nucleic acids of membrane-compromised cells with red fluorescence. An FDA stock solution was prepared by adding 40.1 mg of FDA to 10 ml of acetone, and a PI stock of 10 mg ml^{-1} in distilled water. Immediately prior to staining a working solution was made up of 0.01% v/v (FDA and PI) and 2.5% w/v EDTA in PBS. After loading, explants were washed in sterile PBS for 5 min and 1 mm sections cut perpendicular to the articular surface using a razor blade. After staining for 10 min at room temperature in the dark, and washing twice (5 min each) in PBS to remove free dye from the tissue matrix, the samples were placed on a microscope slide, covered with PBS and a coverslip and observed using a Zeiss Axioskop fitted with Filter 24 (Excitation DBP485/20 + 578/14, Emission BP515-540+LP610) to visualize non-viable (red) and viable (green) chondrocytes simultaneously.

DETERMINATION OF ENDOGENOUS AND NEWLY SYNTHESIZED MATRIX COMPONENTS RELEASED TO MEDIUM

Endogenous GAG released to the medium was measured (as μg GAG per mg wet weight of cartilage) using the DMMB assay at various points after the start of loading: 20 h (load), 24 h (load + label) and 44 h (load-recovery). Newly synthesized GAG and protein, containing radiolabel, were measured at the following time points: 4 h (short load), 24 h (load) and 48 h (load-recovery). Media were desalted using a gel-filtration column (PD-10, Pharmacia Biotech, containing Sephadex G-25) to separate molecules according to size. Incorporated isotope was quantified by scintillation counting. Samples of medium corresponding to 1 mg of tissue (calculated by dividing volume of medium by the original wet weight of each explant) were aliquoted, an equal volume of 8 M guanidine hydrochloride (GuHCl) added, then made up to 0.25 ml with 4 M GuHCl desalting buffer (4 M GuHCl, 50 mM sodium sulphate, 50 mM sodium acetate and 0.1% Triton X-100). The column was equilibrated with desalting buffer, then the sample plus 0.5 ml desalting buffer were applied to the column and the fraction collected in a scintillation vial. A further five separate 0.75 ml aliquots were applied to the column and the fractions collected. To all the fractions 2 ml Hi-safe scintillation fluid (Wallac Scintillation Products Ltd.) was added and then counted on a Wallac 1409 Liquid Scintillation Counter. Matrix biosynthesis of proteoglycan and protein was calculated from the total [^{35}S]-sulphate labeled GAGs and total tritiated leucine found in the media fractions, disintegrations per minute were totalled and expressed, as above, as newly synthesized GAG or protein in nmol g^{-1} h^{-1}.

STATISTICAL ANALYSIS

Results are quoted as mean ± standard deviation, unless stated otherwise. Significant differences in radioisotope incorporation were determined between loading regimes using one-way analysis of variance (ANOVA) with the null hypothesis of no difference in incorporation rates between loading. If normality failed then a Kruskal–Wallis ANOVA on ranks was performed. When significant differences were detected a Tukey pairwise multiple comparison test was performed to identify which groups differed. To investigate incorporation in relation to load and site it was necessary to analyse each loading regime separately and ascertain site variation as a function of load. The data were not independent of each other so, a non-parametric *K*-related samples test (Freidman RM AOV) was needed and pairwise comparisons were not possible. A Freidman RM AOV test was also performed for % GAG wet and dry weight, % collagen wet and dry weight, % water and mg DNA at each site. Correlation between parameters was calculated using Pearson Product Moment Correlation. Regressions were performed with age as an independent variable to ascertain if age could predict % GAG wet and dry weight, % collagen wet and dry weight, % water and mg DNA. All analyses were done using SigmaStat 2.0 or SPSS (SPSS Science, Chicago, USA).

Results

MECHANICAL LOADING

Loading of elderly human articular cartilage at 1 MPa significantly inhibited incorporation of sulphate into cartilage explants ($P = 0.023$) (Fig. 1). Pairwise comparisons showed that the difference in incorporation was only significant for static load ($P \leq 0.05$) (4.1 ± 1.9 nmol g^{-1} h^{-1}). Cyclic load (5.7 ± 2.4 nmol g^{-1} h^{-1}) was not significantly different from the unloaded samples (7.3 ± 2.8 nmol g^{-1} h^{-1}). Leucine incorporation appeared to follow the same trends (Fig. 1) (static 13.0 ± 6.6 nmol g^{-1} h^{-1}, cyclic 15.0 ± 5.3 nmol g^{-1} h^{-1}) but neither were significantly different ($P = 0.31$) from the unloaded samples (17.0 ± 4.9 nmol g^{-1} h^{-1}) though the power was low (0.08).

Comparing the ratio of sulphate:leucine incorporation, a significant difference was observed with loading ($P = 0.0063$), with a reduction in the incorporation of sulphate relative to leucine in the statically loaded samples (0.33 ± 0.08) and cyclically loaded (0.38 ± 0.08) compared with the unloaded samples (0.45 ± 0.14). Following further analysis, a weak correlation was found between sulphate and leucine incorporation rates for the unloaded samples

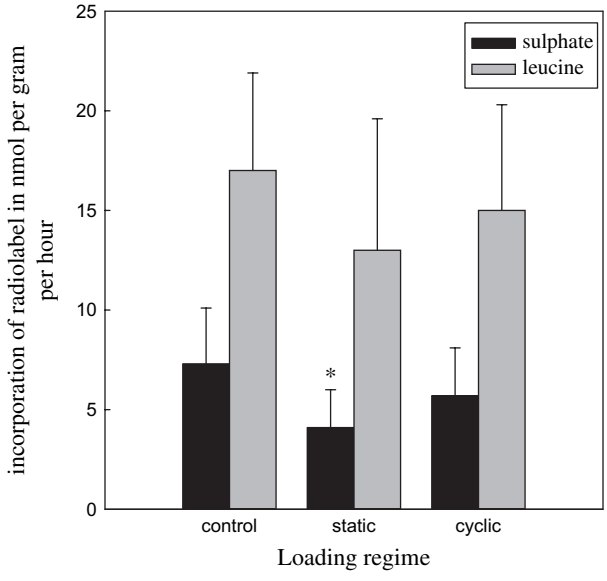

Fig. 1. Total incorporated radiolabelled leucine in nmol per g wet weight of cartilage per hour for control, static and cyclic loading regimes across sites. Load was applied for 24 h and radiolabelling was carried out for the final 4 h. Significance between sites was analysed using ANOVA, $N = 10$. Mean ± SD and significance values (*P*), * indicates a statistical significant Tukey pairwise comparision of static load compared to control, $P \leq 0.05$.

and almost perfect correlation in the statically loaded samples with the cyclically loaded samples intermediate to these (Table I).

Site variation of incorporation as a function of load was analysed for each loading regime using the non-parametric *K*-related samples test. This revealed no significant differences in the unloaded controls. Static loading, however, showed significant differences in both sulphate ($P = 0.011$) and leucine ($P = 0.021$) incorporation, being greatest in the anterior region, followed by the superior then the posterior. With cyclic loading, only leucine incorporation showed significant differences, with tissue from the posterior being much less active than superior or anterior sites (Tables II and III).

LOAD-RECOVERY

The load-recovery experiments showed incorporation rates for sulphate were back to 85% (static) and 88% (cyclic) of unloaded controls within 24 h. Leucine incorporation rates returned to 93% (static) and 99% (cyclic) of unloaded controls within the same time.

SHORT LOAD

The short loading period showed no statistical significance for either sulphate ($P = 0.60$) or leucine ($P = 0.50$) incorporation. However, the power was low (0.05) and this would bear further investigation.

TISSUE COMPOSITION

Significant topographical variations were seen in % GAG wet weight ($P = 0.03$) and the ratio of GAG:collagen ($P = 0.03$) with the anterior/superior region being higher than the posterior region. There were no significant topographical variations in % GAG dry, % collagen wet, dry mass of the tissue or DNA content (Table IV). Regressions with age as the independent variable (Table V) revealed one significant correlation. GAG content decreased with age (% GAG dry $R^2 = 0.52$, $P = 0.018$) (% GAG wet $R^2 = 0.383$, $P = 0.056$). All other variables showed no significant correlation.

The mean cell density was $12{,}900 \pm 3400$ cells mm^{-3} with a 4% decrease in the viability of cells from statically loaded samples and 2% in cyclically loaded samples compared with unloaded samples ($P = 0.011$) (Table VI).

Table I

Correlation coefficients and significance values for pairwise comparisons of sulphate and leucine incorporation at different loading regimes, calculated using Pearson Product Moment Correlation. In each block the figures quoted are the correlation coefficient, R; the significance, P; and the number of samples, N

	Load		Leucine
Sulphate	Control	*R*	0.54
		P	0.003
		N	28
	Cyclic	*R*	0.79
		P	<0.001
		N	28
	Static	*R*	0.99
		P	<0.001
		N	28

Table II

Total incorporated radiolabelled sulphate in nmol per g wet weight of cartilage per hour for control, static and cyclic loading regimes across sites. Load was applied for 24 h and radiolabelling was carried out for the final 4 h. Significance between sites was analysed using Freidman RM AOV (non-parametric K-related samples test, N = 8). Mean ± SD and significance values (P)

	Posterior	Superior	Anterior	*P*
Control	5.9 ± 2.4	6.7 ± 2.6	6.9 ± 2.7	0.33
Cyclic	3.6 ± 2.1	5.7 ± 1.2	6.2 ± 1.9	0.07
Static	2.8 ± 1.8	3.7 ± 2.0	5.2 ± 2.9	0.01

Fluorescence staining indicated that cell death was not confined to any specific zone within the tissue or to the cut edges.

GAG AND PROTEIN RELEASE TO MEDIUM DURING LOADING

The DMMB assay revealed a significant increase in GAGs (μg GAG per mg wet weight of cartilage) released to the medium from the cyclically loaded tissue (1.03 ± 0.27 μg mg^{-1}) after 24 h ($P = 0.003$) of loading compared to static load (0.304 ± 0.076 μg mg^{-1}) and control (0.23 ± 0.13 μg mg^{-1}). On removing the loads, GAG release returned to control levels and there were no differences between the groups ($P = 0.51$).

Desalting of loaded, recovery and short load 4-h radiolabelled media indicated new protein was being synthesized and released to the medium by all loading regimes (11.75 ± 8.23 nmol g^{-1} h^{-1}) but there were no differences between loading regimes and no newly synthesized GAGs could be detected in the culture media.

Discussion

These data show that both cyclic and static loading appear to inhibit matrix biosynthesis in elderly human articular cartilage, compared with unloaded tissue. The effect was statistically significant for GAG synthesis though it did not reach significance for protein synthesis. This suppression appears to start within 4 h of initiating loading, though these methods are not sensitive enough temporally or quantitatively to explore this further. The results of cyclic loading are in contrast to previous studies on young bovine and canine tissue where it has been found almost universally to be stimulatory compared with unloaded tissue[15,19,48,49]. An interesting observation was the ratio of sulphate:leucine incorporation, which in the presence of load showed a very high correlation that was absent in unloaded samples. This indicates a decoupling of GAG and protein synthesis in the absence of load with a disproportionate increase in GAG synthesis. Leucine was chosen to

Table III

Total incorporated radiolabelled leucine in nmol per g wet weight of cartilage per hour for control, static and cyclic loading regimes across sites. Load was applied for 24 h and radiolabelling was carried out for the final 4 h. Significance between sites was analysed using Freidman RM AOV (non-parametric K-related samples test, N = 8). Mean ± SD and significance values (P)

	Posterior	Superior	Anterior	*P*
Control	12.4 ± 3.3	17.9 ± 8.7	15.6 ± 4.9	0.093
Cyclic	10.8 ± 6.3	16.4 ± 5.0	15.1 ± 4.8	0.034
Static	9.6 ± 7.8	12.0 ± 7.4	16.3 ± 9.3	0.021

Table IV
*Composition of human articular cartilage at three sites on the human femoral head. Significance between sites was analysed using Freidman RM AOV (non-parametric K-related samples test, N = 8). Mean ± SD and significance values (P), * indicates a statistical difference between sites*

Composition	Posterior	Superior	Anterior	*P*
% GAG wet	3.8 ± 0.90	4.2 ± 1.0	4.3 ± 0.83	0.03*
% GAG dry	12.2 ± 2.0	13.3 ± 1.6	13.1 ± 1.7	0.22
% Collagen wet	13.5 ± 3.6	11.6 ± 4.0	12.2 ± 4.5	0.33
% Collagen dry	42.7 ± 8.8	36.7 ± 9.8	37 ± 11	0.20
% Water	72 ± 10	68.4 ± 5.5	67.5 ± 4.0	0.33
DNA (mg/g)	0.45 ± 0.11	0.44 ± 0.078	0.45 ± 0.09	0.88
GAG:collagen	0.31 ± 0.10	0.40 ± 0.13	0.39 ± 0.14	0.03*

measure protein synthesis, in preference to proline, because it constitutes a higher percentage of the composition of most of the proteins in cartilage matrix; approximately 10% of the amino acid composition, from 4% in collagen type II to 14% in biglycan, decorin and fibromodulin[50]. Proline is more specific for collagen synthesis.

There are two factors that could underlie the differences between this and previous studies: the first is the age of the tissue and the other is a species difference. To our knowledge this is the first study of different direct loading regimes on elderly human cartilage. The only other similar study of which we are aware used osmotic and static mechanical compression of human cartilage from OA, osteoporotic (op) and normal subjects aged 19–88 years but unfortunately did not distinguish between the respective groups or classify into age. They subjected cartilage plugs to steady compression at 3–8 atm (0.3–0.8 MPa) and found a reduction in sulphate uptake. This is in agreement with our results for static load, but is also consistent with all the studies using animal tissues.

Maroudas *et al.*[31] showed that with dynamic compression, significant increases in GAG biosynthesis were observed in chondrocytes from young bovine and young human explants, whilst aged human cartilage showed no difference.

For maintaining tissue homeostasis, it may be that it is not only the absolute amount of matrix components synthesized that is important, but also their relative amounts. It is increasingly clear that the simple relationships traditionally proposed between compressive and tensile properties and GAG and collagen content are a gross simplification and that interactions between these molecules, and possibly other components, are at least as important[40,51]. Some degree of loading, though, would be normal in any joint and to use an unloaded tissue as the control is not necessarily the best for comparison with loading *in vivo*. Cyclic load induces a greater metabolic response than static load, and the disproportionate increase in GAG in the unloaded tissue still suggests that absence of load variation is to be avoided.

Table V
*Linear regression of composition of human articular cartilage as a function of age. The * indicates a statistical difference. The only significant relationship was a decrease in % GAG, both wet and dry, and age*

	R^2 ($N = 10$)	*P*
% GAG wet	0.38	0.056*
% GAG dry	0.52	0.018*
% Collagen wet	0.013	0.75
% Collagen dry	0.042	0.57
% Water	0.002	0.91
DNA (mg/g wet)	0.028	0.64
GAG:collagen	0.22	0.18

Table VI
Chondrocyte viability (mean ± SD) across sites in tissues subjected to the three loading regimes. ANOVA (P = 0.011) and a pairwise Tukey test showed that the difference between the control and statically loaded samples was significant (P ≤ 0.05)

	N	% Viability
Control	12	98.9 ± 1.6
Cyclic	12	96.7 ± 2.9
Static	12	94.7 ± 4.4*

Continuous loading for 24 h is not physiological and to ensure that the changes observed were not a consequence of chondrocyte death, cell viability studies were performed. Mean cell counts were consistent with previously published data of 10,000 cells mm^{-3} in adult human femoral head cartilage[26] and 14,500 ± 3500 in adult human femoral condylar cartilage[52]. There was indeed a small (4%) but significant decrease in cell viability in the statically loaded tissues compared with the control. This is not great enough, though, to account for the magnitude of the changes found in incorporation rates, which were all much larger than this. Load-recovery experiments confirmed that 24-h loading does not seriously compromise the cellular responsivity and incorporation rates were nearly back to control levels 24 h after removing the load.

Histology also showed that a small number of cells had died and that these were scattered throughout the whole depth of the tissue. This is in contrast to a recent study of mature bovine cartilage subjected to loading of 1 MPa in confined compression which found that static loading induced cell death after 3 h and cyclic loading after 6 h[35]. Cell viability was assessed in that study by fluorescence staining, but not quantified, and showed that chondrocyte death was confined to the superficial tangential zone. The difference in these results may arise from the different loading environments, i.e., confined vs unconfined compression. Recent studies seem to conclude that cell death is greater in young tissue compared to old and, at physiological loading regimes (1 MPa)[34–36], a maximum cell death is observed at 6 h and is confined to the superficial zone. At loads of 5 MPa cell death is observed rapidly and at 24 h has progressed into the middle zone. In this study, 1 MPa appeared not to induce any significant cell death and this could not be the cause of the reduction in biosynthesis observed.

Comparisons in this study cannot be made with applied strain as we used load control on full-depth biopsies rather than strain control. Tissue biopsies vary in thickness and therefore it is difficult to measure strain in intact, full-depth tissue. Strain-control studies, however, generally remove the surface zone to produce a uniform thickness of tissue. Thus, both methods have their limitations.

A question mark will always remain over such elderly tissue as to how 'normal' it is. Young human articular cartilage is almost impossible to obtain so ageing effects are difficult to determine. As far as is known, cartilage on femoral heads from patients following hemiarthroplasty operations for fractured neck of femur commonly appears visually normal, albeit thinner and slightly discoloured. It has been noted that OA and OP have little overlap and rarely are both seen in the same patient[53,54] and care was taken

to avoid fibrillated areas of tissue commonly found in the inferior region of the femoral head. Significant topographical variations were seen in % GAG wet weight which are in agreement with previous data[55] with greater levels in the more highly loaded anterior/superior regions. The ratio of GAG:collagen also showed a greater proportion of GAG with regard to collagen in these regions. There were no other significant topographical variations in composition across the femoral head, possibly suggesting a more congruent hip with ageing[56] and therefore a more even loading distribution across the joint. Regressions with age, over the limited range used, showed a significant decrease in % GAG with age in agreement with previous studies[26].

Our primary goal in these experiments was to begin to determine the response of elderly human articular cartilage to mechanical stimuli in a controlled environment. This has been little studied, animal models are most commonly used but it is not clear how well they represent the elderly human. The results suggest that elderly human articular cartilage responds differently to cyclic mechanical stimuli than animal tissue, though the reasons for this are not clear. This knowledge is important to tissue engineering approaches to repair osteoarthritic cartilage in which the source of the replacement tissue or cells needs to be considered. Though the tissue response appears different to that in young individuals, it still supports the importance of exercise by the elderly to regulate the biosynthetic activity of the tissue.

Acknowledgements

We thank the Wishbone Trust and the Medical Research Council for funding. MSP is the recipient of the Aberdeen Wishbone Studentship. We are also grateful to the Orthopaedic surgeons of Grampian Universities Hospital Trust for allowing us to use tissue donated by their patients and Mrs L.A. Bestwick for excellent technical help.

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