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Differential response to compressive loads of zones of canine hyaline articular cartilage: micromechanical, light and electron microscopic studies

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SUMMARY Thin (100 μm) perpendicular slices of canine femoral condylar cartilage were placed horizontally on the stage of a Nachet microscope and viewed by transmitted light in the differential interference contrast mode. Each slice was held on the microscope stage by a loading rig and tested mechanically in compression. Measured loads to a maximum of ~2–3 MN/m^2 were applied to the end of the slice corresponding to the articular surface. Photographs were taken of the cartilage before and during loading, and the distance by which selected chondrocytes were displaced was used as an index of mechanical strain, i.e., of change in length/original length. Maximum strains were observed in the superficial cartilage zone. Minimum strains were recorded in the mid-zone, at a depth corresponding to ~75% of the total cartilage thickness. The relative concentrations of cartilage collagen (COL) and proteoglycan (PG) were assessed by the light and electron microscopic histochemical study of cartilage sections taken from contiguous blocks. Superficial cartilage, which deformed most, had high concentrations of orientated COL fibres, low concentrations of PG. Mid-zone cartilage, which deformed least, had lower concentrations of randomly arrayed COL fibres but relatively high concentrations of PG.

Hyaline articular cartilage appears uniform and homogeneous and has unique mechanical properties that include high compression resistance. This property is determined by the retention of water within the domains of PG aggregates arranged within a gel reinforced with COL fibres. The physical properties of cartilage have been examined by many classical mechanical procedures.[1] Experiments have been made on whole joints,[2 3] parts of disarticulated joints,[4] and pieces of cartilage.[5–8] Tests have been made in tension and in compression, but most have not distinguished between the properties of different cartilage zones.

The apparent homogeneity of articular cartilage conceals remarkable molecular heterogeneity. It is useful to consider cartilage as a series of different zones, each with a distinct microscopic structure and varied composition. In the most simple analysis, articular cartilage can be said to comprise superficial, mid-, and deep zones.[9] In a more sophisticated approach, the number and morphology of the chondrocytes, the concentration of stainable PG, and the distribution of COL can be used to designate five zones[10] (Table).

It has always appeared probable that the mechanical properties of the different zones of articular cartilage would reflect this structural heterogeneity. The aim of the present study was to examine this proposal, taking advantage of apparatus that permits the simultaneous microscopic observation and mechanical testing of articular cartilage samples.[11 12]

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Materials and methods

COLLECTION OF CARTILAGE

Six young mature male and female beagle dogs from a closed colony were killed by exsanguination under thiobarbitone anaesthesia. Each hind limb in turn was dissected in a closed chamber fitted with glove ports. The atmosphere inside the chamber was maintained at ~95% relative humidity to minimise water loss from small tissue blocks during collection. The stifle (knee) joint was approached by an anterior incision through the patellar ligament. The patella was reflected proximally and the joint

Table *Zonal structure of dog femoral non-calcified condylar cartilage (Reprinted by permission of John Wiley & Sons, Ltd from Lock D J, Jasani M K, Birdwood G F B, eds. Studies in osteoarthritis: pathogenesis, intervention and assessment. Copyright (1987) John Wiley & Sons, Ltd)*

Zone	*Cells (H&E)*	*Collagen (PSR & TEM)*	*PG (ATB & CB)*	*Arbitrary layer*
Superficial				
I	Single, ellipsoidal	++++ Tangential	+	1
Mid				
II	Single, ovoid larger than I	++ Random	++	2,3,4
III	Single or occasional pair, larger than II	+ Random	+++	
IVA	Single or paired larger than III	+ Random	++++	
Deep				
IVB	Short columns of 2–4 cells	+++	++	5
Tide mark		Perpendicular		

H&E=haematoxylin and eosin; PSR=picro-Sirius red; ATB=alcoholic toluidine blue; CB=cupromeronic blue; TEM=transmission electron microscopy.

capsule opened. The cruciate and collateral ligaments were divided to allow separation of the femoral and tibial condyles. Using a scalpel blade, three parallel, perpendicular cuts were made through the articulating surface. The cuts were in a latero-medial direction across the load bearing region, at the most distal part of the medial femoral condyle. A fourth, tangential cut was made in the region of the tidemark in a plane parallel to the surface. This procedure provided two blocks each of ~6·0×2·0×1·5 mm; they were immediately frozen and subsequently stored in liquid nitrogen at −196°C.

The number of specimens collected at each time interval after surgery, four from each of three animals, meant that the cartilage had to be stored before testing. Freezing was regarded as the method that would cause the least change or damage. A comparison of the mechanical behaviour of fresh specimens and those that had been frozen in liquid nitrogen or liquid nitrogen slush suggested that freezing in liquid nitrogen was optimal for the present study. Freezing occurs most rapidly at the surfaces of a specimen: the relatively large surface area to volume ratio of the present specimens accelerates the rate of freezing. The rates and directions of freezing and thawing together with the variations in water content throughout the cartilage thickness may set up molecular concentration gradients, which in turn may cause some redistribution of water. Previous investigations of cartilage retrieved after low temperature scanning electron microscopy, however, showed cellular damage due to freezing/thawing but no visible alterations to the PG or COL (Oates K, Gardner D L, unpublished data). It is anticipated that the unchanged PG within each sample determines the distribution of free water when the tissue is returned to room temperature. Thus the mechanical properties of frozen/thawed tissue are not likely to differ significantly from those of fresh material.

When required for mechanical testing, blocks were transferred under liquid nitrogen to a Bright cryostat, where 100 μm perpendicular sections were cut by Histolab disposable blades (Histolab, PO Box 101, Hemel Hempstead, Hertfordshire).

In a second pilot study specimens were mechanically tested in a variety of solutions, including saline, trometamol (TRIS) buffered saline, Eagle's balanced salt solution, and Krebs-Ringer bicarbonate buffer. There were no detectable differences in mechanical behaviour between the specimens maintained in these different solutions. All subsequent tests were therefore performed in trometamol buffered saline. Contiguous sections were cut for light microscopy.

MECHANICAL TESTING APPARATUS (after Broom[11 12])

The loading rig (Fig. 1) consisted of an optical glass slide (G) to which four stainless steel, 100 μm thick spacers had been bonded by Glassbond resin (Locktite UK Ltd, Welwyn Garden City, Hertfordshire, England). A glass coverslip was attached to the top face of the spacers. Two 100 μm thick stainless steel plates, one ~2·0×10·0 mm (the anvil) and the other ~10·0×10·0 mm (the base plate) could be positioned between the glass slide and the coverslip. The plates were retained laterally by the spacers. When a 100 μm section of tissue was inserted into the rig the tissue was located so that the articulating surface came into contact with the smaller of the two plates, the anvil (A). The anvil was an extension of a threaded drive advanced manually by rotation of a

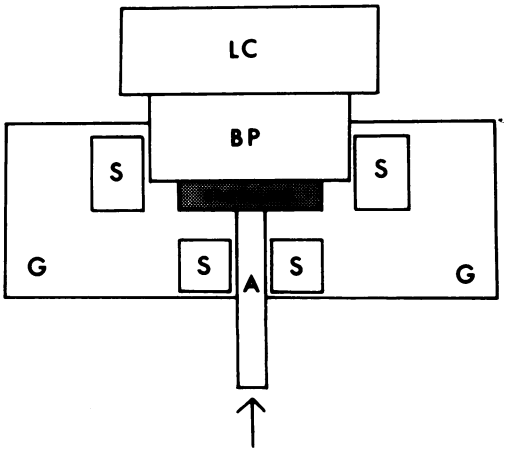

Fig. 1 *Diagrammatic representation of loading device showing anvil (A), base plate (BP), glass slide (G), load cell (LC), and spacers (S). Arrow indicates direction of loading.*

hand wheel. The base plate (BP), adjacent to the deep cut surface of the tissue, was also in contact with a Sensotec, 0–1000 g load cell, which, in turn, was connected to the Y terminal of a Linseis LY 1700 chart recorder. The specimen was confined above by the coverslip and below by the glass plate so that deformation could take place only within a single plane (plane strain compression). The loading rig was positioned on a rotating stage in the optical path of a Nachet light microscope fitted with transmitted light differential interference contrast optics (Nomarski) and an Olympus OM2N camera.

MECHANICAL TESTING

Tests were made at room temperature. A cartilage section was placed in the loading rig and trometamol buffered saline introduced between the coverslip and glass slide. The cartilage was compressed in a surface to bone direction by manual rotation of the hand wheel driving the anvil. This provided a strain rate of $\sim 10 \times 10^{-4}\ s^{-1}$. The anvil was advanced in a series of 7–10 movements, each taking ~0·4 s, to produce a total deformation of ~30%. Immediately after each advance a photomicrograph of the tissue was taken. The applied load, which was continuously monitored throughout the procedure, showed a slight but measurable decay (decrease) during photography. The maximum load after each compressive step was used to determine the value of the applied stress (force per unit area).

An aim of the present study was to test mechanically, unfixed, unstained cartilage under microscopic observation. The mode of illumination of unstained 100 μm sections did not permit recognition of cartilage zones as described previously.[9 10] It was therefore necessary to define a further series of arbitrary layers. Four marker chondrocytes, situated at 200–300 μm intervals along an imaginary reference line drawn radially through the cartilage, were selected to divide the section into five horizontal layers (Fig. 2). The Table shows the relation between these arbitrary layers and the structured zonal divisions of cartilage. The distance between adjacent cells and thus the width of each layer, together with the total width of the specimen, were measured from the photomicrographs taken at zero compression and after each advance of the anvil. Changes in width caused by compression represented deformations and were considered as *strain* values, i.e., *change in width/original width*. Strain is a concept not expressed in units; it is therefore of value when comparing the deformation of layers of different initial width.

MICROSCOPY

For light microscopy, 5–8 μm serial cryostat sections

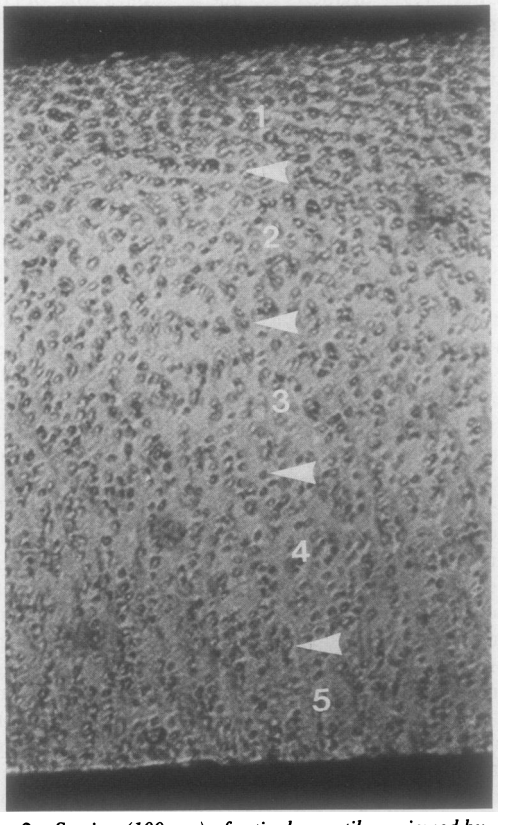

Fig. 2 *Section (100 μm) of articular cartilage viewed by differential interference contrast microscopy showing five arbitrary layers. Arrows indicate position of four marker chondrocytes. (Unfixed, unstained.)*

were stained by an alcoholic toluidine blue technique[13] and by picro-Sirius red.[14]

For transmission electron microscopy (TEM) the tissue slices, retrieved after mechanical testing, were allowed to recover at zero load in trometamol buffer for 30 minutes. The specimens were then divided into three full depth blocks, stained, and simultaneously fixed in a solution of glutaraldehyde and cupromeronic blue (0·05%)[15] in the presence of 0·3 M $MgCl_2$. Under these conditions the dye stains all sulphated glycosaminoglycans (GAG).[16] Specimens were embedded in Epon and sectioned at 1–4 μm for light microscopy and at 70 nm for TEM. Further, adjacent blocks of cartilage were fixed in 2·5% glutaraldehyde (pH 7·2) followed by 1% aqueous osmium tetroxide for TEM. Sections (70 nm) from these blocks were stained with phosphotungstic acid, uranyl acetate, and lead citrate to demonstrate COL fibres. TEM examinations were performed on an AEI 801 instrument at an accelerating voltage of 80 kV. Photographic records were made on Kodak Tri-X Pan film using a shutter speed of 0·125 s. Films were developed in Promicrol (Ilford) for 20 minutes.

Results

MECHANICAL TESTING

Results were obtained from 24 specimens. Each specimen provided data consisting of a sequence of applied stress values and a corresponding series of strain measurements. The latter were obtained from 24 series of micrographs each similar to that shown in Fig. 3. The form of the data determined that they were not suitable for statistical analysis. The results can, however, be represented graphically as stress-strain curves. For each specimen stress-strain curves were plotted for the five horizontal layers. Fig. 4 is representative of these graphs.

For each specimen tested the curve for arbitrary layer 1, representing the most superficial 200–300 μm, was furthest to the right hand side of the graph. Layer 3 was always positioned to the left hand side of the graph. The curves for layers 2, 4, and 5 were between those for 1 and 3, with that for layer 5 always adjacent to layer 1. All the curves were non-linear with the slope increasing with increasing strain. The slope of a stress-strain curve is representative of the stiffness of a specimen. Thus, under the present conditions of testing, arbitrary layer 3 has the maximum stiffness and arbitrary layer 1 the minimum value.

MICROSCOPY

The results from toluidine blue and cupromeronic blue staining for GAG in full depth dog femoral

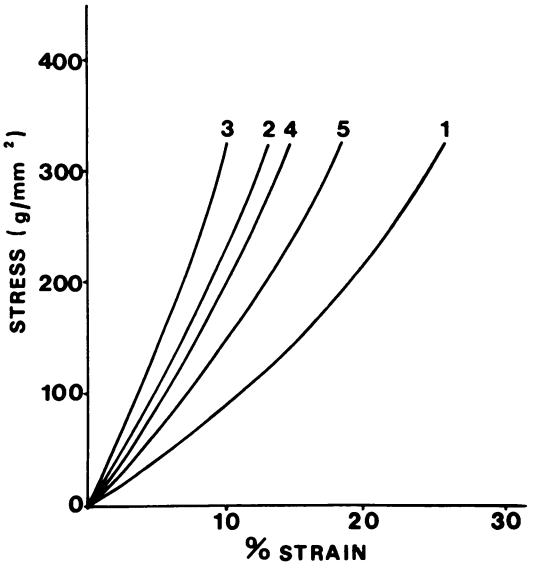

Fig. 4 *Representative stress-strain curves for arbitrary layers 1–5 at ~0·5 s after start of loading.*

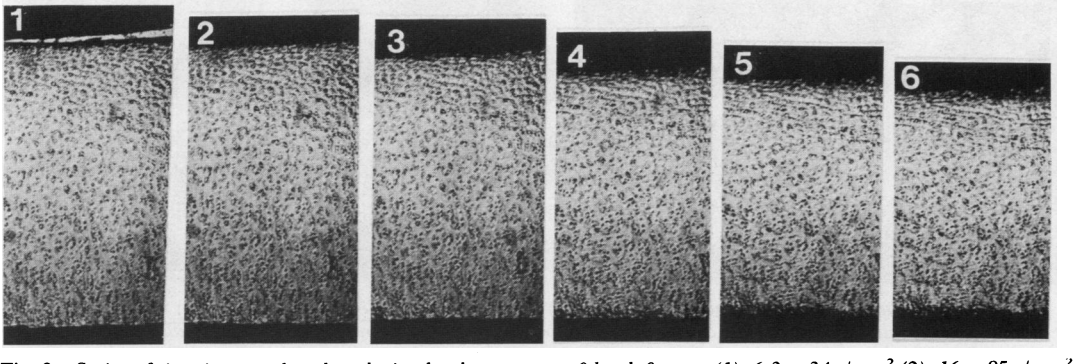

Fig. 3 *Series of six micrographs taken during load sequence at 0 load, 0 stress (1); 6·3 g, 34 g/mm² (2); 16 g, 85 g/mm² (3); 32 g, 170 g/mm² (4); 44 g, 238 g/mm² (5); 50 g, 270 g/mm² (6).*

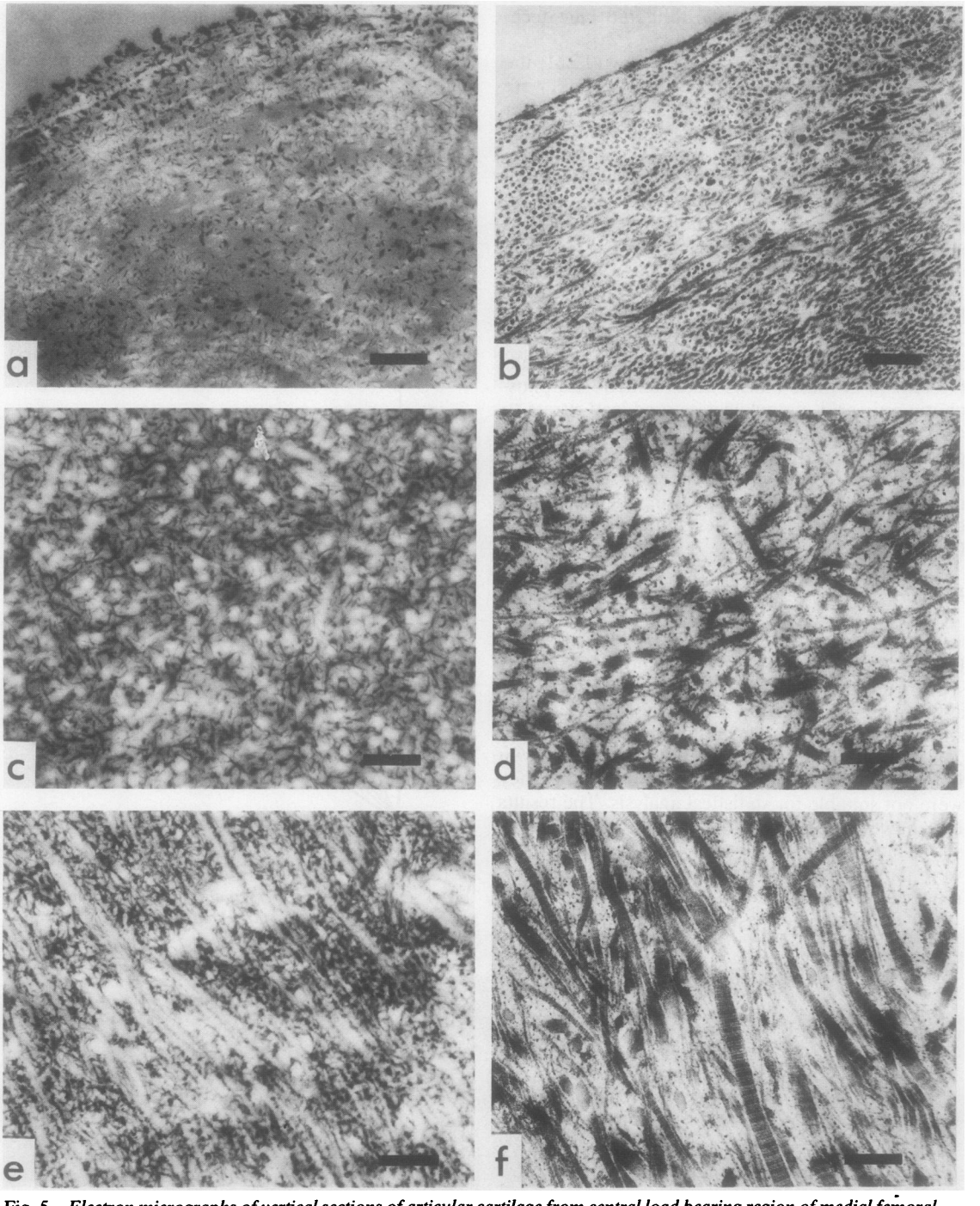

Fig. 5 *Electron micrographs of vertical sections of articular cartilage from central load bearing region of medial femoral condyle of mature beagle dog to illustrate the distribution of sulphated GAG (a, c, and e) and the distribution and arrangement of COL (b, d, and f). a and b show articular surface and superficial zone (arbitrary layer 1); c and d show mid-zone, 200 μm from surface (arbitrary layer 3); e and f show deep zone, 700 μm from surface (arbitrary layer 5). a, c, and e are stained with cupromeronic blue in the presence of 0·3 M $MgCl_2$. b, d, and f are stained with phosphotungstic acid, uranyl acetate, and lead citrate. Bar=0·4 μm.*

condylar cartilage were similar to those found in human hyaline cartilage.[10] The stain intensity was least near the surface and increased gradually with depth to reach a maximum at 50–75% thickness. Nearer the bone, the stain intensity was less constant: the pericellular matrix stained more intensely than the intercellular matrix. The distribution and orientation of COL in fresh, frozen sections of dog articular cartilage agreed with the established pattern as described by Gardner *et al.*[10]

After fixation and staining with cupromeronic blue (Fig. 5a, c, and e) electron dense products represent GAG-dye complexes.[14] The filaments correspond to partially collapsed PG monomers or PG aggregates, or both. The filaments are sparse in the cartilage matrix close to the articular surface (Fig. 5a) by comparison with deeper cartilage (Figs 5c and e). Furthermore, the stained units were smaller near the surface than in the deeper layers. Sections stained for COL (Figs 5b, d, and f) illustrate an alignment of fibres in the superficial cartilage in a direction parallel to the articular surface (Fig. 5b). A random collagenous array was seen in the mid-zones (Fig. 5d), while below this there was a tendency to a radial array, with fibres running perpendicular to the surface (Fig. 5f).

Discussion

The present results show that under the specific conditions of compressive loading used in this study, normal dog articular cartilage does not deform uniformly throughout its thickness: there is a variable response at different depths beneath the surface. It is shown that these variations in mechanical behaviour correspond to differences in composition.

In mechanical terms, articular cartilage can be considered as a fibre reinforced gel in which highly hydrated PG molecules are retained by, and interact with, a complex network of COL fibres.[17] The strong affinity of PG for water creates an internal swelling pressure, which places the COL under tension. This swelling pressure gives cartilage its compressive resistance. On application of a compressive load there is an immediate deformation due to rapid bulk movement of hydrated PG molecules, resisted by the COL fibre network. This is followed by a time dependent, viscoelastic (creep) response resulting from fluid flow away from the site of loading. Under plane strain conditions the instantaneous deformation of thin cartilage slices involves a reduction in width with a corresponding expansion laterally to maintain a constant volume. During the viscoelastic phase there is a further reduction in width together with a decrease in volume as fluid moves away from the site of loading. In the present study the load was observed to decay (fall off) during the photographic time of 0·125 s. This decay, which occurs while the section is maintained at a constant width, is known as stress relaxation, and is primarily due to fluid movement. This relaxation is the topic of a further paper (in preparation). Thus during the ~0·5 s from the start of compression to the end of each photographic exposure both instantaneous deformation and fluid flow took place, and all the present results, therefore, represent the situation after some viscoelastic deformation has occurred.

Using a sequence of compression testing it has been shown that the compressive resistance, represented by measurements of strain, varies with distance below the articulating surface. Strain is greatest, resistance least, in the superficial zone. There is a gradual increase in compressive resistance with depth to a maximum at 50–75% of the total thickness; resistance then decreases in the deep zone. Comparable variations in cartilage compliance with depth were reported by Gore *et al* for human patellar cartilage plugs using macroscopic observation during unconfined compression.[18]

Mizrahi *et al*, testing human femoral condylar or femoral head cartilage, have also shown that the compressive behaviour of cartilage varies with depth beneath the articular surface.[19] They show, however, that the superficial zone has the greatest stiffness. The results of these authors show the instantaneous deformation phase only as determined from measurements of immediate change in specimen diameter, during unconfined compression. The present studies and those of Mizrahi *et al* are not directly comparable because of differences in the source of the tissue and because, in the former, radial stiffness was measured, whereas in the latter, lateral stiffness was determined. It could be suggested that the differential behaviour of cartilage in response to compressive loading changes rapidly with time. In the immediate deformation response, strains are least in the superficial zones, greatest in the deeper zones. This is succeeded by a situation where strains become greatest superficially and least in the mid-zone tissue. The changes in the differential response with time have been investigated more fully elsewhere (O'Connor P, Gardner D L, unpublished data).

Instantaneous deformation is thought to be determined by the COL network. In turn, this is influenced by the osmotic pressure of the PG.[19] The time dependent, compressive resistance of articular cartilage has been shown to be determined by the concentration and distribution of PG.[16] In the present study the total deformation includes both an instantaneous and a viscoelastic phase so that the differences in compressive resistance among the

different layers are attributable to both PG and COL. The large difference in resistance between the superficial zone and the lower mid-zone cartilage is primarily due to variations in PG concentration. The configuration of COL may also have an influence on the instantaneous mechanical behaviour of articular cartilage as suggested by Broom[20 21] and Broom and Marra.[22] These authors proposed a model in which the apparently random appearance of COL fibres disguises a more orderly arrangement. They suggest that the fibres of the mid and deep cartilage zones are organised radially with short range deflections superimposed to form a braced structural system. The deflections are thought to be sustained by cross links. The greater the number of deflections and thus the greater the number of cross links, the less compliant the system. Thus in the present study the COL arrangement is likely to enhance the resistance of the middle layers where an apparently random arrangement of COL persists. Near the surface and in the deep layers the orderly parallel array of COL fibres implies fewer cross links and a more compliant system.

The mechanical behaviour of articular cartilage is complex. The present study shows that at a single point in time the compressive resistance varies with depth below the articulating surface. Clearly the present testing technique is applicable to abnormal as well as normal tissue. It is therefore proposed to investigate the mechanical behaviour of canine articular cartilage after surgical division of an anterior cruciate ligament. This approach should enable comparisons to be made between the mechanical behavioural changes and the structural and biochemical alterations[23 24] that occur with the development of experimental degenerative joint disease.

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