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A novel microassay for the quantitation of the sulfated glycosaminoglycan content of histological sections: its application to determine the effects of Diacerhein on cartilage in an ovine model of osteoarthritis

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Summary

Objective: A new micro-histological method of assessing the sulfated glycosaminoglycan (S-GAG) content in unstained histological sections of articular cartilage was developed and used to study the effects of orally administered Diacerhein (DIA) on joint cartilage in an ovine model of osteoarthritis (OA).

Methods: Twenty adult, age-matched Merino wethers were subjected to bilateral lateral meniscectomy, while 10 served as non-operated controls (NOC groups). Half of the operated sheep (N=10) remained untreated (MEN groups), while the other 10 animals were given DIA (25 mg/kg orally) daily for 3 months, then 50 mg/kg daily for a further 6 months (DIA groups). Five animals each of the DIA, MEN and NOC groups, respectively, were sacrificed at 3 months post-operatively, and the remainder 6 months later. For the present study only one knee joint of each animal was used for histological processing. The tissues studied were from the lateral femoral condyles (LFC) and lateral tibial plateaux (LTP). Each of these joint regions was further subdivided into inner (I), middle (M), and outer (O) zones. Unstained histological sections from these AC regions and zones were then analysed for S-GAG content using the following procedure. Images of each section of 6 μm thickness were acquired using a flatbed scanner and the area determined with an image analysis software program. The sections were then transferred to wells of a microtiter plate, digested with papain and the S-GAG content quantitated using a modification of the 1,9-dimethylmethylene blue dye binding assay. The data was represented as μg S-GAG/mm^3 of each tissue section. These data were also compared with toluidine blue stained sections from the same paraffin blocks.

Results: The results obtained showed that the area of histological sections could be very accurately determined by computer assisted image analysis using a 10 mm×10 mm calibration grid. Cartilage sections of areas ranging from 1 mm^2 up to 25 mm^2 were analysed for S-GAG content with this simple technique. There was a linear relationship between section thickness (2–10 μm) and S-GAG content per unit area (R^2=0.993). Sections of 6 μm thickness were found to be optimal. S-GAG analyses of serial sections from tibial and femoral articular cartilage (I, M and O zones) revealed an average coefficient of variation of 7.0±2.3% (range 4.9–10.2%) confirming the accuracy and reproducibility of this assay method. A separate experiment showed that no significant losses of S-GAG occurred during the histological sample processing.

The different regions and zones of the knee joint AC in the six experimental groups revealed variable levels of S-GAG which did not necessarily correlate with the histochemical distribution of toluidine blue staining. The major S-GAG changes occurred in the middle (lesion zone) and outer zones (hypertrophic zone) of both the LFC and LTP of the MEN groups. In the lesion (M) zone the S-GAG content was reduced while in the O zone levels were increased at both 3 and 6 months post-surgery. In animals receiving Diacerhein S-GAG levels in the M zone were lower than or equivalent to those of non-drug treated OA or non-operated controls for both joint regions at 3 and 6 months. While the hypertrophic response in the outer zone of the LFC, as assessed by S-GAG content, was enhanced by drug treatment, the cartilage of the outer zones of the LTP was not affected by drug treatment.

Conclusion: The results of this study have demonstrated that the S-GAG (and therefore proteoglycan [PG]) content in different cartilage zones of OA joints can be readily quantitated by direct biochemical analysis of unstained histological sections. By this means subtle changes in PG distribution in different cartilage zones, which were not evident using traditional histochemical staining methods, could be readily detected.

Key words: Osteoarthritis, Diacerhein, Cartilage, Glycosaminoglycan, Proteoglycan.

Introduction

Osteoarthritis (OA) is a multifactorial disorder in which pathological changes become manifest in articular cartilage, synovium and subchondral bone[1–3] leading to pain and loss of joint function. In the past, pharmacological intervention in OA has been largely confined to analgesics, steroidal or non-steroidal anti-inflammatory drugs. While these agents have provided an important means of controlling the inflammation and pain in OA, their application has been overshadowed by their deleterious side effects on

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cartilage[4,5] and the gastrointestinal tract[6–8]. More recently drugs are being developed which purport to provide symptomatic relief by targeting the pathology of OA, particularly in cartilage and subchondral bone. Such agents have been classified as structural modifying steoarthritis drugs (SMOADs)[9] and would be expected to retard, stabilize or reverse the pathological changes which occur in OA joints, thereby limiting disease progression. Since detectable structural joint changes in human OA generally take several decades to develop and techniques to evaluate these changes are still under investigation, animal models of OA have been widely used to evaluate potential SMOADs and to study their mechanisms of action[10–13].

Total or partial meniscectomy is a very common orthopedic procedure which frequently leads to cartilage degeneration and the onset of OA. A contributing factor to these deleterious joint changes is the high focal stresses imposed on articular cartilage following the excision of the meniscus[14–16]. Previous studies in dogs[17–19] and sheep[20–25] have established that similar events occurred in joints of these animal species following meniscectomy. Indeed, the biochemical and histological changes which occurred in cartilage and subchondral bone following bilateral meniscectomy in sheep were shown to exhibit many similarities to early human OA[24–26]. Moreover, using the ovine model the topographical variation which exists in both the quantitative and qualitative distribution of proteoglycans (PG) in articular cartilage of different joint regions has been identified[24,26].

Diacerhein (DIA) is a slow-acting drug for OA which has been reported to provide symptomatic relief when administered orally to patients with OA of the hip[27]. The drug has also been observed to inhibit the production of interleukin-1 (IL-1) and reduced cartilage breakdown in a murine granuloma model[28], and to attenuate the development of cartilage lesions in dog[29] and rabbit models of joint arthropathy[30]. *In-vitro* studies have shown that DIA inhibited collagenase production by lapine chondrocytes and modulated IL-1 inhibition of PG synthesis[31,32]. A recent study also suggested that DIA enhanced the expression of transforming growth factor (TGF)-β1 and β2 in articular chondrocytes[33]. Collectively, these reports suggest that DIA could be classified as a SMOAD at least with respect to its effects on cartilage in animal models.

The histological grading system of articular cartilage used by Mankin *et al*.[34] and its subsequent modifications[3,5,21,24] have provided a useful means of assessing the loss of PG and the severity of AC lesions in OA joints but are limited by the inability of histochemical stains to detect small changes in PG content as may occur in early OA[35]. More precise methods for the determination of PG concentrations in articular cartilage[36] and intervertebral disc histological sections[37] have been reported using safranin-O staining and subsequent microspectrophotometric quantitation of glycosaminoglycans in these sections. However, these methods require the use of specialized spectroscopic equipment to quantitate the intensity of light transmitted through stained sections. In order to overcome these difficulties and to provide a direct, analytical means of cartilage assessment we have developed a simple method to quantitate the sulfated glycosaminoglycan content (S-GAG) in unstained histological sections of AC. This procedure was then used to determine the S-GAG content of AC in different regions and zones of joints from animals with surgically induced OA which had been treated orally with Diacerhein for 3 or 6 months. The data obtained was compared with the S-GAG content of corresponding regions of AC of non-drug-treated OA and non-operated control animals in an attempt to evaluate the ability of Diacerhein to influence the S-GAG content in different topographical regions and zones of OA joints.

Materials and methods

ANIMALS AND SURGICAL PROTOCOL

Thirty adult (2–3 years old) pure bred Merino wethers obtained from the University of Sydney farm at Camden, NSW (Department of Veterinary Clinical Sciences), were used for this study. The animals were divided into age- and weight-matched groups. Ten of these animals served as non-operated normal controls (NOC), while 20 underwent bilateral lateral meniscectomy of the stifle joints using a procedure described previously[24]. All animals were allowed to recover for 2 weeks in the animal house. The surgical procedures and the programs of the post-operative care were approved by the Animal Care and Ethics Committee of the University of Sydney (ACEC No N07/2-94/3/943).

Ten of the operated sheep were used as non-drug-treated OA controls (MEN), while the remaining 10 meniscectomized animals were given Diacerhein (DIA) using a protocol as described below. After the post-operative recovery period of 2 weeks and during drug treatment all animals were maintained in an open, grassed paddock (100 m × 22 m) with supplementary hay feeding and water *ad libitum* for the duration of the study.

DRUG ADMINISTRATION

A weighed amount of pharmaceutical grade Diacerhein powder (Negma Laboratories, Paris, France) was stirred with 2.5% of methylcellulose in water in a ceramic mortar until the suspension became homogenous. A predetermined volume of this methylcellulose suspension of the drug corresponding to the required dose was administered to the DIA groups commencing 2 weeks after the last operation. The non-drug-treated groups (MEN groups) received an equivalent volume of aqueous methylcellulose. Both the 3 month and 9 month DIA-treated groups received 25 mg/kg of the drug daily for the first 3 months; thereafter the 9 month DIA group received 50 mg/kg DIA daily for the remaining 6 month period. The treatment regimen used in this study was designed with the advice of Negma Laboratories, Paris, France and described by Hwa *et al.*[38]

PROCESSING OF SPECIMENS

At the end of the 3 and 9 month experimental period, all animals were euthanased by an intravenous overdose of sodium pentobarbitone (Lethabarb®, Virbac, Sydney, NSW, Australia). The stifle joints were removed intact and immediately transferred to the laboratory on ice. After removing the periarticular soft tissues, the joints were opened and mid-coronal bone slabs (3–4 mm thickness) were cut through the central weight-bearing region of the tibial plateaux and the femoral condyles with a band saw to obtain full-width medial and lateral osteochondral sections of 1.0 cm depth. The slabs were defined as regions of the lateral femoral condyle (LFC), and lateral tibial plateau (LTP).

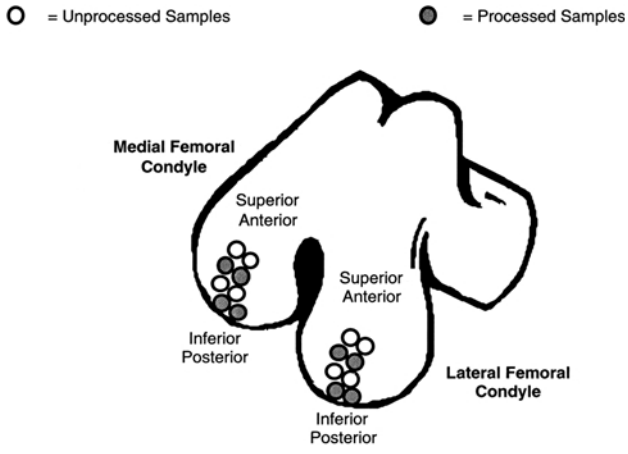

Fig. 1. Diagrammatic representation of an ovine distal femur showing the locations where 3 mm diameter full-depth plugs of condylar articular cartilage were sampled for analysis of moisture content and S-GAG content directly and after histological processing.

The specimens were placed in jars containing 10% (v/v) neutral buffered formalin and were fixed for 48 h at room temperature. The samples were then decalcified in 10% (v/v) formic acid, 5% (v/v) formalin for 7 days with continuous agitation, followed by rinsing with running tap water (30 min) and dehydration through a series of graded ethanol solutions (70%, 95% and 100% (v/v)), infiltrated in methylbenzoate/celloidin and finally embedded in paraffin. Sections were then cut and mounted on glass slides, using standard histological procedures. Four micrometer sections were also stained with toluidine blue to illustrate the histological appearance of the different articular cartilages.

A separate experiment was conducted to ascertain that the histological processing did not cause significant losses of S-GAG from the tissues. For this purpose, 3 mm diameter full-depth cartilage plugs were cut from the middle zone of femoral condyles of two non-operated joints as illustrated in Fig. 1. Two pairs of adjacent plugs obtained from the anterior and posterior part of the joint were either processed for histology as outlined above or freeze-dried and stored dry until analysis. The processed samples were then deparaffinized, taken to water and the S-GAG content determined as described below. The moisture content of the articular cartilages was determined by weighing all samples at the appropriate steps of the respective procedures. This permitted two sets of water content to be determined for the processed samples because the wet weight was assessed at the initial tissue harvest and at the point after deparaffinizing before drying.

The repeatability of the assay was assessed using 20 serial sections of tibial and femoral articular cartilage from one non-operated control animal as described below.

Serial sections of increasing thickness from 2 μm to 10 μm were also prepared and analysed in triplicate in order to determine the linearity of the assay and the optimal section thickness.

ARTICULAR CARTILAGE SAMPLE PREPARATION FOR ROUTINE S-GAG DETERMINATION

Unstained coronal sections (6 μm thick) of the lateral compartment of sheep knee joint tissues were deparaffinized, taken to water and air dried. A dissecting microscope at 15× magnification was used for the sample preparation and tissue transfer. The subchondral bone tissue was removed with a no. 11 scalpel blade after scoring along the cartilage–bone interface. The remaining articular cartilage was further divided into three equal lengths and a thin strip of tissue removed perpendicular to the articular surface. These three zones corresponded to the area nearest to the insertion of the cruciate ligaments (inner zone, I), across the middle (middle zone, M) to the outermost cartilage area (outer zone, O), respectively. Anatomically, the location of the O zone of the tibial plateau was normally fully covered (or protected) by the meniscus, the M zone was partially covered and the I zone was totally exposed. Figure 2 shows a scan of a tibial plateau section, illustrating the sequence of sample preparation for the determination of S-GAG, including the removal of the subchondral bone tissue and the arbitrary division of the AC into thirds of I, M and O zones. The glass slide containing the prepared cartilage section was then scanned on an AVR flatbed scanner (AVR Technology, Inc., San Jose, CA, U.S.A.) with an appropriate dark background for enhanced contrast. Alternatively, an image of the sections was captured with a Pixera Professional™ digital camera system and software (Pixera Corporation, Cupertino, CA, U.S.A.). A 10 mm by 10 mm microscope eyepiece graticule was also scanned (or an image captured) and used as the standard for area

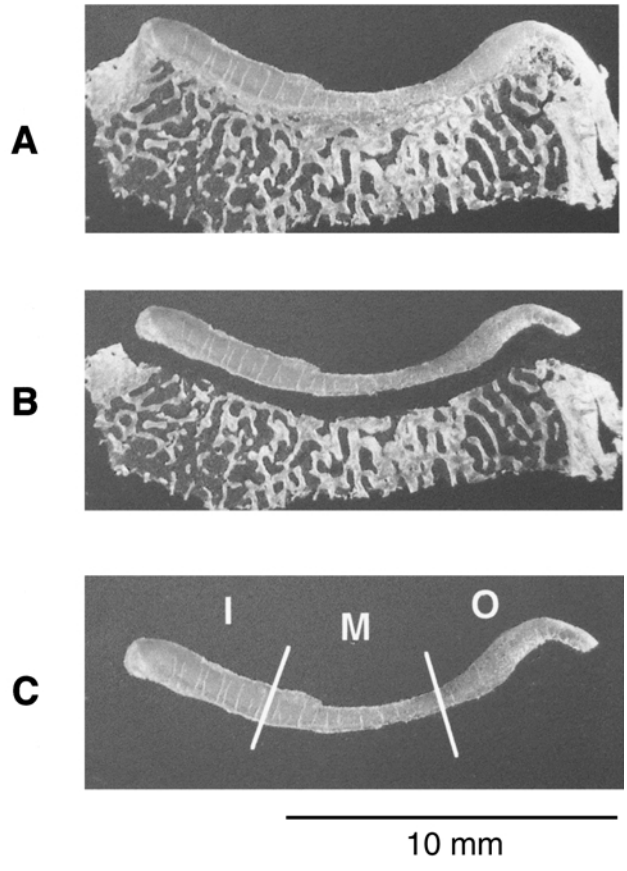

Fig. 2. Digitized scan of an unstained histological section of an ovine lateral tibial plateau (LTP) showing the sequence of sample preparation. (A) Intact unstained section of LTP. (B) Same section with subchondral bone partially removed. (C) Same section with subchondral bone completely removed and subdivided into inner (I), middle (M) and outer (O) zones, ready for area quantitation and S-GAG determination. Magnification bar represents 10 mm.

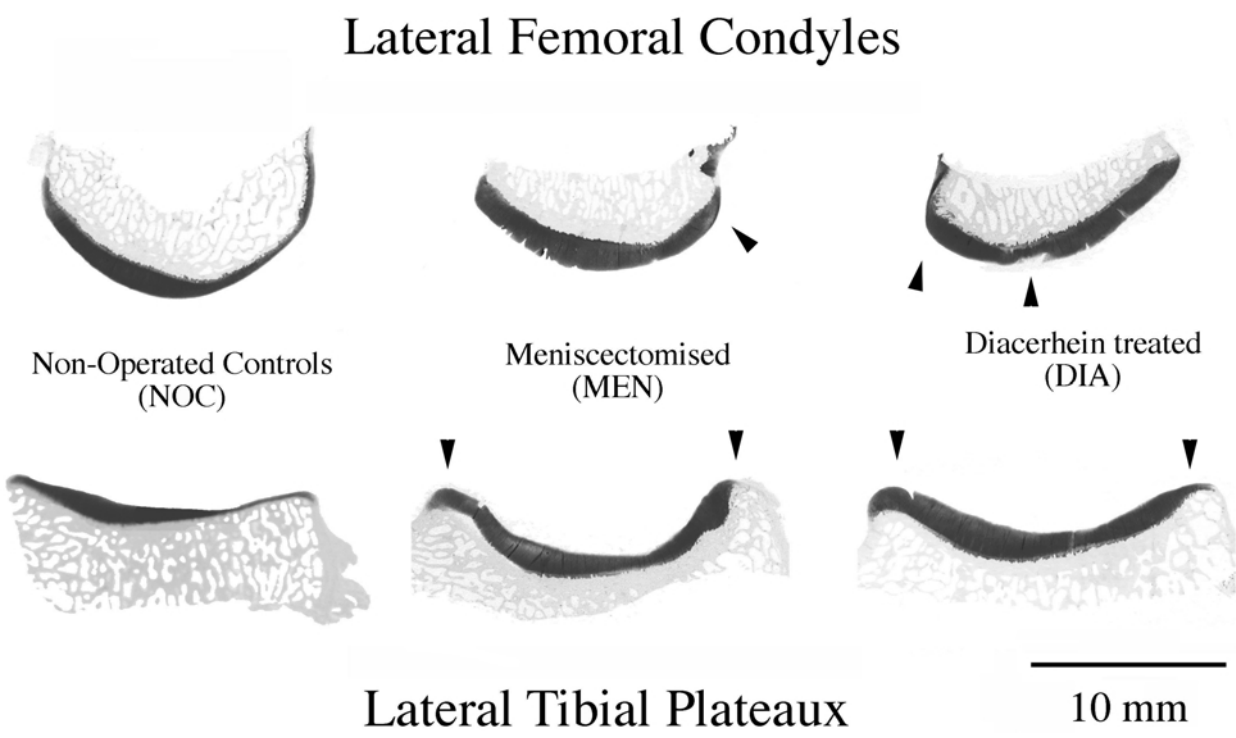

Fig. 3. Toluidine blue stained sections representative of the lateral femoral condyles (LFC) and lateral tibial plateaux (LTP) from all three treatment groups. Note the toluidin blue staining of the proteoglycans which shows little variation between zones. Lesions are indicated by 'L' and osteophyte formation by arrowheads in the operated groups. Magnification bar represents 10 mm.

calibration necessary for the subsequent conversion of pixels into area units, i.e. mm^2. The scans were stored as compressed JPEG files and the areas quantitated (in triplicate) using an image analysis program (Image Pro® Plus, Version 3.0, Media Cybernetics, Silver Spring, MD, U.S.A.). The glass slide with the area/volume quantitated sections was briefly dipped in water to wet and soften the articular cartilage for removal and transfer. The individual zones were then carefully peeled off the wet surface with the tip of a no. 11 scalpel blade and placed into a well of a 96-well microtiter plate containing 50 μl papain digestion buffer. Duplicate sections were thus prepared, digested and analysed.

PAPAIN DIGESTION

The papain digestion buffer consisted of 2 μl papain suspension (Sigma P-3125) per milliliter of calcium and magnesium free phosphate buffered saline containing 5 mM cysteine (Sigma Chemical Co., St Louis, MO, U.S.A.) and 10 mM EDTA, pH 7.4. Each plate contained a series (0–5 μg/well) of chondroitin sulfate (CS) (Sigma C-8529) standards prepared from working solutions (100 μg CS/ml) in identical papain digestion buffer. The plates were then covered with a self-adhesive plate sealer and incubated within a humidified container in a 60°C oven for 16 h. After reaching room temperature the plates were centrifuged for 5 min at 1500 ***g*** with the plate sealer in place to collect condensation droplets. The cartilage plugs prepared for the assessment of potential S-GAG losses during processing were similarly digested in 1.0 ml papain buffer together with a separate CS standard (100 μg/ml) prepared in papain buffer. Appropriate dilutions were made for the subsequent S-GAG quantitation using the dimethylmethylene blue binding assay in microtiter plates.

1,9-DIMETHYLMETHYLENE BLUE BINDING ASSAY

1,9-Dimethylmethylene blue (DMMB) (Aldrich 34,108-8, Sigma-Aldrich, Castle Hill, NSW) solutions (50 mg/l) was prepared by dissolving 50 mg of DMMB in 5 ml of ethanol and subsequent dilution to a volume of 1000 ml with 0.2% (w/v) sodium formate buffer, pH 3.5.

Using an electronic multi-channel pipette 150 μl of DMMB reagent was rapidly added to each well containing the digested cartilage sample. The plate was briefly mixed on a plate shaker (5–10 s) and the absorbance at 650 nm measured immediately in a plate reader. The GAG content was calculated from the standard curve constructed by the plate reader software (Softmax®, Molecular Devices Corporation, Menlo Park, CA, U.S.A.).

DATA ANALYSIS AND STATISTICS

All data were entered into a spreadsheet and the subsequent calculations and statistical analyses [ANOVA with a Fisher's PLSD (protected least significant difference) post-hoc treatment] were performed using a statistical software package (StatView®, Version 5.0, Abacus Concepts, Inc, Berkeley, CA, U.S.A.). The threshold value for significance was set to 0.05.

Results

Representative sections of the lateral femoral condyles and tibial plateaux from all three groups with lesions and

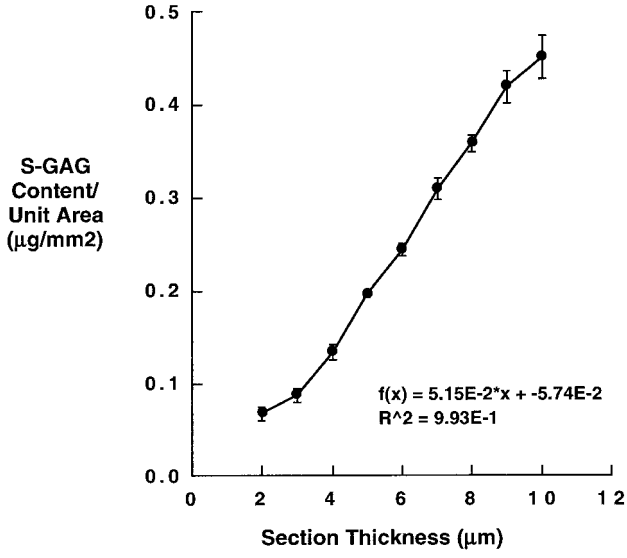

Fig. 4. Relationship between thickness of the histological sections of articular cartilage and the sulfated glycosaminoglycan (S-GAG) content per unit area determined as described in Methods.

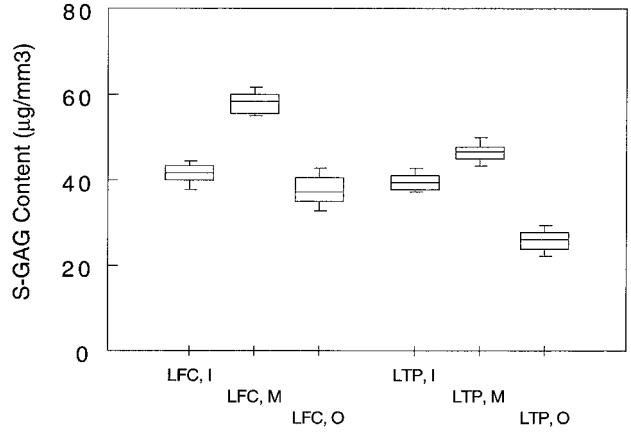

Fig. 5. Box plot of S-GAG content of 20 serial sections of the lateral femoral condyle (LFC) and tibial plateau (LTP), divided into the three different zones [inner (I), middle (M), and outer (O)] from a nonoperated control sample illustrating the repeatability of the assay.

osteophytes identified are shown in Fig. 3. From this figure it is evident that the toluidine blue histochemical staining of sections did not reflect the true variation in S-GAG content, as determined using the present method (see results below). This difference was most marked in the outer zone of the meniscectomized group where the S-GAG content was increased but staining appeared normal. The areas of coronal AC sections from sheep stifle joints ranged between 2–10 mm^2 (corresponding to values of 0.012–0.06 mm^3) and could be very accurately determined by image analysis using the calibrated graticule as a standard area. A clear linear relationship (R^2=0.993) between section thickness and sulfated GAG content per unit area was demonstrated as illustrated in Fig. 4. A tissue section thickness of 6 µm (verified by digital micrometer) was found to be optimal and was used for all subsequent analyses of sections. The results could were therefore expressed as micrograms S-GAG per cubic millimeter of tissue (µg/mm^3).

The modified 1,9-dimethylmethylene blue (DMMB) dye binding assay used here was adapted from the method originally described by Farndale *et al.*[39,40] and Templeton[41]. However, it was modified and improved to provide for the greatly extended working range required of this assay. The absorbance measurement of the band of uncomplexed DMMB at 650 nm, rather than the α-band of the S-GAG-DMMB complex at 535 nm, resulted in a two-fold increase in sensitivity. In addition, by varying the DMMB concentration (10 mg/l to 500 mg/l), we were able to determine S-GAG levels over a 500-fold range from 0.1 µg to 50 µg per well (results not shown).

Figure 5 and Table I show the results of a validation study to demonstrate the repeatability of the assay. Twenty serial sections (assayed in triplicate) of the lateral femoral condyle and lateral tibial plateau cartilages from a non-operated control joint were used. It is evident that the coefficients of variation ranged from 4.9% to 10.2%, which we considered were acceptable for the purpose of this assay and the present comparative study.

Table II provides data which showed that the tissue processing protocol used did not generally affect the S-GAG levels measured in articular cartilage. Except for one region (anterior LFC), insignificant differences were found between adjacent cartilage plugs which were analysed for S-GAG either with or without histological processing. Furthermore both methods detected the normal topographical variations in S-GAG content between the anterior and posterior joint regions. It was also evident that the moisture content of all samples, irrespective of their origin (MFC, LFC, left or right), were within a very narrow range averaging 69.1±1.8% (mean±S.D.) for the unprocessed control samples. The wet weight of the processed samples was determined twice for each sample; first at tissue harvest and then at the penultimate step of the deparaffinizing procedure, before drying. The two resultant assessments of moisture content of these processed samples correlated very closely at 71.8±1.7% and 73.1±2.1%. Although the difference in moisture content between processed and unprocessed cartilage was small, it reached statistical significance (P<0.05).

The results of the S-GAG analysis of articular cartilage from the three treatment groups, using the new technique, are summarized in Figs 6 and 7. In Fig. 6 the levels of S-GAG for the femoral condyles are represented with regard to cartilage zones (I,M,O) for each treatment group thus facilitating comparisons of S-GAG between zones [Fig. 6(A) and (B)], as well as for each zone to enable comparisons for all experimental groups to be readily made [Fig. 6(C) and (D)]. Figure 7 shows the data for the corresponding lateral tibial cartilages. In both Figs 6 and 7, (A) and (C) represent the 3 months and (B) and (D) the 9 month experimental period, respectively.

In the non-operated control groups (NOC) there were highly significant differences between the S-GAG content of the I, M and O zones of both the LFC and LTP. The outer zone of the NOC, which is covered by the lateral meniscus, contained approximately half the S-GAG content of the middle and inner zones [Fig. 6(A),(B) and 7(A),(B)].

Three or 9 months after lateral meniscectomy (MEN) the S-GAG levels were progressively increased in the O zones, particularly in the LTP after 9 months [Fig. 7(D)]. At the same time there was a corresponding decrease in S-GAG levels in all the M zones (which included the main lesion) compared to those of the NOC groups, especially in the LFC after 9 months [Fig. 6(D)]. As a result, there was no

Table I
Repeatability of the assay

Joint region and zone	Number (*N*)	Mean (μg S-GAG/mm^3)	S.D.	S.E.	CV
LFC—inner	60	41.5	2.7	0.35	6.5%
LFC—middle	60	58.1	2.8	0.37	4.9%
LFC—outer	60	37.6	3.6	0.46	9.5%
LTP—inner	60	39.7	2.2	0.28	5.5%
LTP—middle	60	46.5	2.4	0.31	5.2%
LTP—outer	60	25.8	2.6	0.34	10.2%

Sulfated glycosaminoglycan (S-GAG) content of 20 serial sections of zones (inner, middle and outer) from the lateral femoral condyle (LFC) and tibial plateau (LTP) of a nonoperated control sample analysed in triplicate using the method described in the text, showing the number of data points (*N*), mean, standard deviation, standard error, and coefficients of variation.

Table II
Comparison of sulfated glycosaminoglycan (S-GAG) content of unprocessed articular cartilage and samples processed for histological assessment

Joint region	Left/ right	Anterior/ posterior	Unprocessed* Mean±S.D. (μg S-GAG/mg dry tissue)	Processed† Mean±S.D. (μg S-GAG/mg dry tissue)	*P*-value (processed vs unprocessed)
LFC‡	Left	Anterior	168±4.3	148±5.5	0.01
LFC	Left	Posterior	172±4.0	170±4.0	0.61
LFC	Right	Anterior	159±8.5	154±3.0	0.37
LFC	Right	Posterior	190±3.2	192±6.2	0.60
MFC§	Left	Anterior	143±2.3	142±7.6	0.78
MFC	Left	Posterior	207±2.5	202±2.5	0.10
MFC	Right	Anterior	118±2.5	123±6.0	0.23
MFC	Right	Posterior	244±1.7	246±8.6	0.67

*Determined by direct analysis using the modified DMMB binding assay.
†After processing for histology and deparaffinized.
‡Lateral femoral condyle cartilage.
§Medial femoral condyle cartilage.
The value for significance was set to 0.05.

significant difference in S-GAG content in the meniscectomized group between the O and M zones of the LFC and LTP after 9 months [Fig. 6(B) and 7(B)].

DIA treatment further increased the S-GAG content of the O zone of the LFC after 3 and 9 months compared to NOC and MEN groups but had no significant effect on the O zone of the LTP compared with the MEN group [Fig. 7(C) and (D)]. In the M zone of both LFC and LTP of the DIA treated animals the S-GAG content was lower than in the MEN group after 3 months but only in the LTP after 9 months [Fig. 6(C),(D) and 7(C),(D)].

There were minimal changes in the S-GAG content of the inner (I) zones of the treated groups except that the DIA treatment decreased the S-GAG levels in the LTP after three months [Fig. 7(C)] and increased it in the LFC after nine months relative to NOC [Fig. 6(D)]

Discussion

The methodology described in this study was dependent on the accurate determination of the physical dimensions of unstained histological sections. This was achieved by digital scanning techniques and subsequent computer assisted image analysis. In addition, the thickness (6 μm) of the cartilage sections was verified by repeated random measurements with a digital micrometer. The study also confirmed that the modification of the DMMB assay employed to improve its sensitivity and the use of the same microtiter wells for both the cartilage digestion and the quantitation of S-GAG content in tissue micro-sections provided a convenient and rapid method of analysis. Methodical errors due to sample dilutions or transfers were thus eliminated or kept to a minimum.

The question of whether proteoglycans (and therefore S-GAGs) were lost from cartilage sections during histological processing was also addressed in this study. Analysis of adjacent cartilage plugs with and without processing confirmed that losses were small. One region (anterior LFC) did show a difference between methods but the reasons for this are unclear and appear to be the exception rather than the rule. Moreover, since all sections were treated identically, the losses which did occur would be of a systematic nature allowing meaningful comparisons between treatments to be determined.

The S-GAG levels in the outer zones of the LFC or LTP regions of the non-operated control joint cartilages were significantly lower than in adjacent middle and inner zones. This difference arises from the reduced mechanical loading

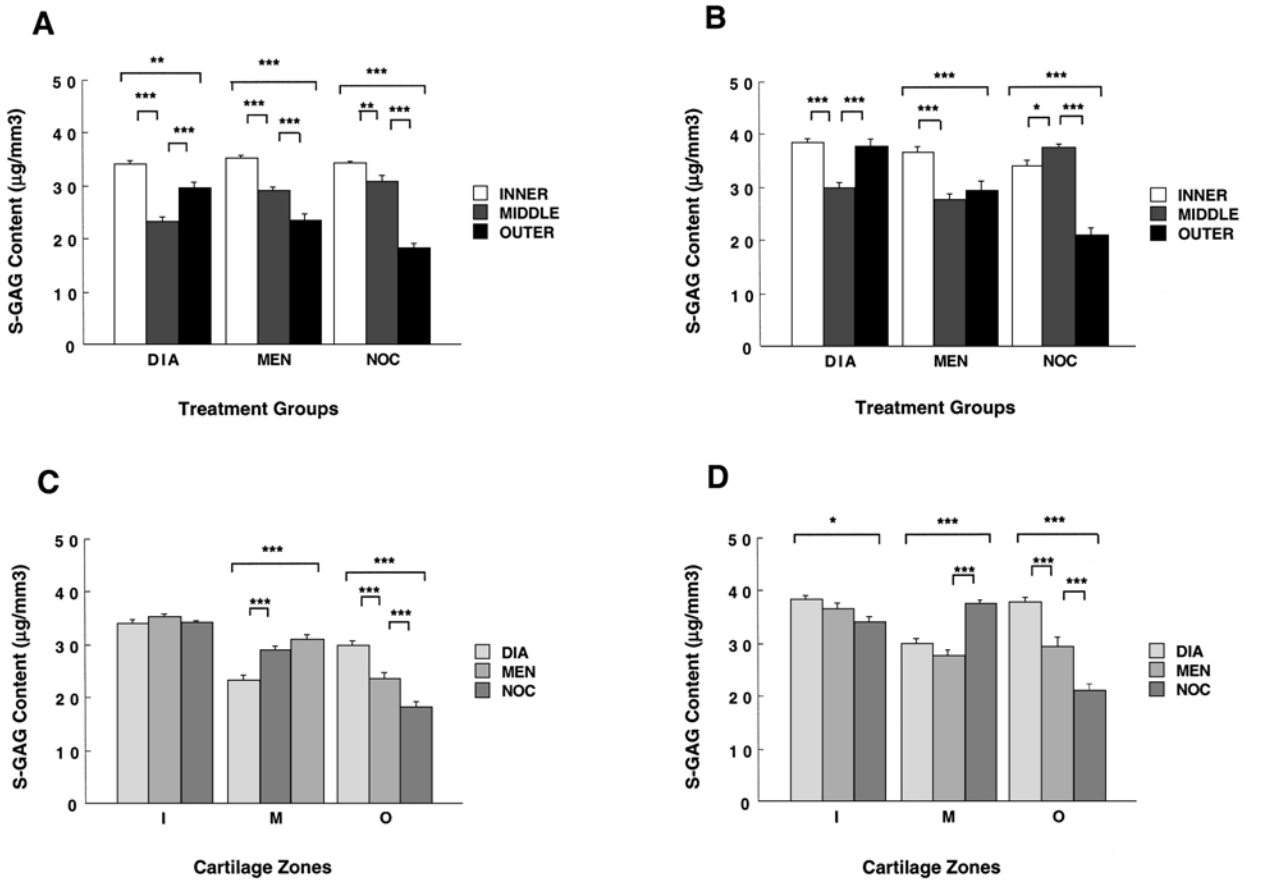

Fig. 6. Sulfated glycosaminoglycan (GAG) content of inner (I), middle (M) and outer (O) zones of articular cartilage from the lateral femoral condyles (LFC) of ovine stifle joints from the 3 and 9 month experimental periods. (A) and (C) represent the 3 month and (B) and (D) the 9 month experimental period, respectively. GAG levels are expressed as µg/mm^3 using chondroitin sulfate as a standard. (A) and (B) Arranged by treatment groups (DIA, MEN, NOC); each group is split by zone (I,M,O) to illustrate changes occurring in different zones within a particular treatment group. (C) and (D) Arranged by zone, (I,M,O); each zone is split by treatment (DIA, MEN, NOC) to illustrate changes occurring due to treatment within a particular zone. *Indicates $P<0.05$; **indicates $P<0.005$; ***indicates $P<0.0005$.

due to the overlying meniscus which normally distributes up to 50% of the load across the joint[2–6]. When these same three cartilage zones were examined using traditional histochemical staining with toluidine blue, uniform staining intensity across the whole section was observed, demonstrating the limited sensitivity of such dyes to quantitate S-GAG concentration in histological sections. Similar observations have been reported by Ostergaard *et al.*[35] using safranin-O staining of human cartilage sections.

As has been noted previously[26,38,47], lateral meniscectomy, while producing high focal stress and cartilage degeneration in the middle zone of the tibial plateau also initiated an anabolic (hypertrophic) response by chondrocytes in the outer zones as identified by the increased PG content in these areas. This hypertrophic response of chondrocytes in the outer zone of the LFC of meniscectomized ovine joints was further enhanced in the Diacerhein treatment group. The explanation for the increased anabolic response of the chondrocytes in the outer zones of joints of the Diacerhein-treated meniscectomized animals is not entirely clear, but may be a result of up-regulation of TGF-β expression by chondrocytes exposed to this drug[32,33]. While TGF-β is known to increase the synthesis of PG by articular cartilage *in vitro*[48] its injection into murine knee joints stimulates osteophytosis[49,50] which also occurred in the outer zones of meniscectomized joints in this study.

As reported previously[24,38,47], it is the M zone of the femoral and tibial cartilages where the classical pathological lesions of early OA, such as softening and cartilage erosion, are first evident. The corresponding loss of S-GAG and thus PG content from these zones therefore reflects the increased catabolic activity in these areas. This loss of S-GAGs from the M zone was more pronounced in the DIA treated groups than non-drug-treated control. While the reasons for this enhancement are still to be determined, and are contrary to previous reports using other models[29–30], it could be speculated that increased weight bearing due to decreased pain could be a contributory factor. Diacerhein does exhibit antiinflammatory activity and diminishes the pain associated with osteoarthritis[27–28]. O'Connor *et al.*[51] have shown that reducing the physiological protective effects on anterior cruciate ligament deficient canine joints by dorsal route ganglionectomy can accelerate progression of osteoarthritic lesions. The possibility of increased weight bearing activity in meniscectomized animals receiving DIA might also account for the elevated hypertrophic response of cartilages and increase in subchondral bone thickness in the outer zones of their joints[47].

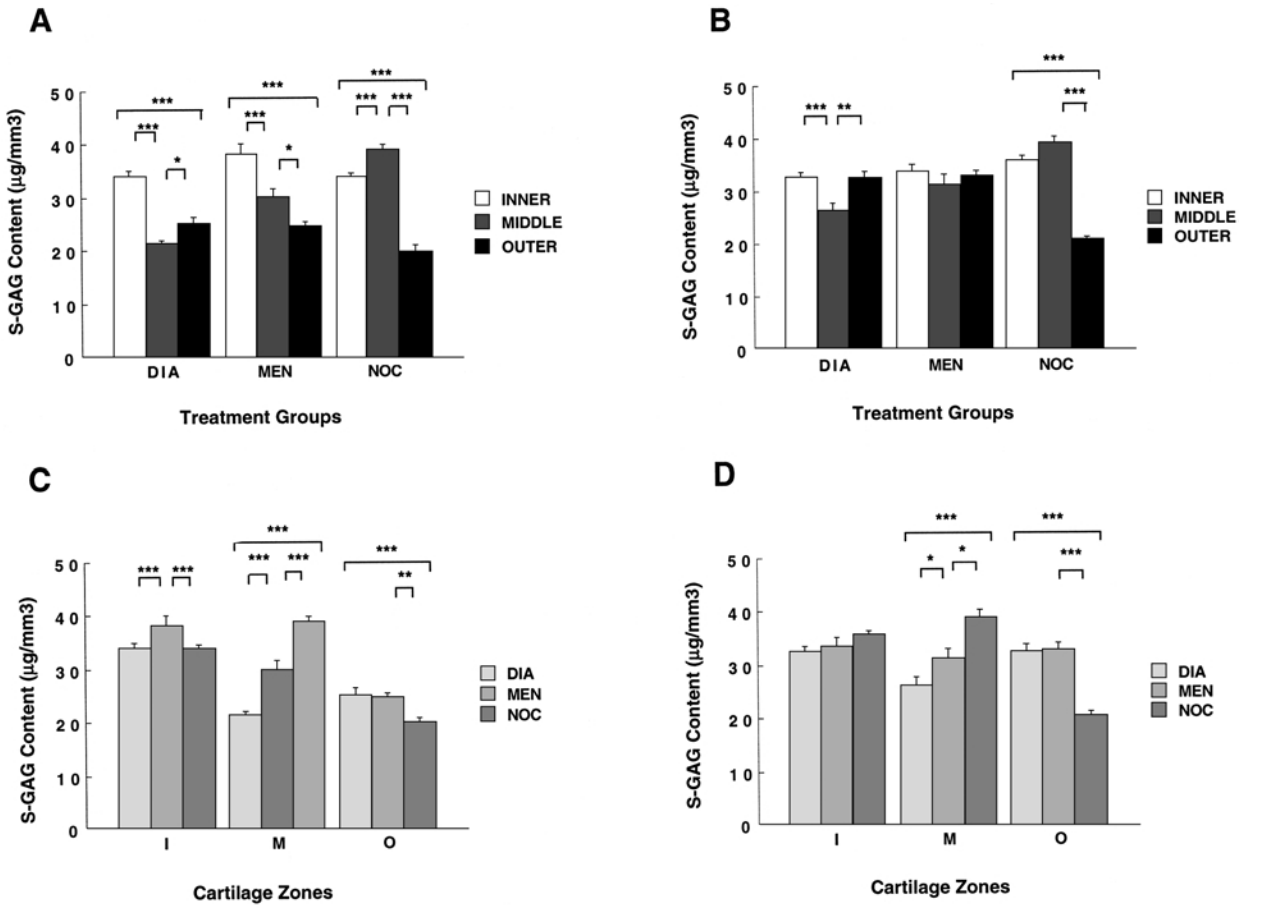

Fig. 7. Sulfated glycosaminoglycan content of inner (I), middle (M) and outer (O) zones of articular cartilage from the lateral tibial plateaux (LTP) of ovine stifle joints from the 3 and 9 month experimental periods. (A) and (C) represent the 3 month and (B) and (D) the 9 month experimental period, respectively. GAG levels are expressed as µg/mm^3 using chondroitin sulfate as a standard. (A) and (B) Arranged by treatment groups (DIA, MEN, NOC); each group is split by zone (I,M,O) to illustrate changes occurring in different zones within a particular treatment group. (C) and (D) Arranged by zone, (I,M,O); each zone is split by treatment (DIA, MEN, NOC) to illustrate changes occurring due to treatment within a particular zone. *Indicates $P<0.05$; **indicates $P<0.005$; ***indicates $P<0.0005$.

Conclusions

The determination of the sulfated glycosaminoglycan content in unstained histological sections as described here offers a sensitive and reproducible technique for monitoring subtle changes in articular cartilage PG metabolism in arthritic joints. This quantitative technique offers several advantages over the non-parametric histochemical staining methods which have been traditionally used to assess the levels and distribution of proteoglycans in articular cartilage sections.

Using this technique it was shown that Diacerhein, when given orally to meniscectomized sheep for 3 and 9 months, induced significant changes in the S-GAG composition of the outer and middle zones of articular cartilage from the OA joints. Diacerhein treatment increased the S-GAG levels in the outer zones of joint AC whereas levels in the lesion (middle) zone were reduced relative to non-drug-treated controls. Whether these cartilage changes occurred from a direct effect of high dose Diacerhein on chondrocyte metabolism or arose because of increased weight bearing of the meniscectomized joint remains to be resolved.

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