The Collagen and Crosslink Distribution in Human Thoracic Intervertebral Disc:- Preliminary Findings

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ABSTRACT

The collagen content and the extent of pyridinoline and deoxypyridinoline crosslinks in human thoracic discs were determined from a preliminary sample of human thoracic discs. Thoracic discs (n=107) were obtained from 9 human cadaveric spines (60 to 90 years old). Discs were graded macroscopically for degeneration, prior to collection of anular and nuclear samples. Pyridinoline and deoxypyridinoline crosslinks were extracted from each sample by hydrolysis and column separation, then analysed by reversed-phase HPLC.

The collagen and crosslink extent between severely degenerated anular and nuclear matrices were not significantly different. Significant increasing trends were found for the extent of deoxypyridinoline crosslinks in degenerated anulus ($p<0.001$) and the collagen content in degenerated nucleus ($p<0.001$). No significant changes in the extent of pyridinoline crosslinks were found between normal and severely degenerated discs. The degenerated disc matrix of aged thoracic disc was associated with significant changes in the collagen and crosslink content.

INTRODUCTION

Collagen is found abundantly in the anulus and nucleus of spinal intervertebral discs [1, 2]. The extracellular matrix of the intervertebral disc depends on the complex network of collagen and proteoglycan units to provide tensile strength to the disc tissue during spinal loading and movement [2, 5]. The tensile strength of the collagen network is dependent on the extent of covalent bonds or crosslinkages within the collagen fibrils in the matrix. The main collagen crosslinks formed in the disc matrix is pyridinoline and deoxypyridinoline [6, 7]. Pyridinoline and deoxypyridinoline crosslinks are mature, non-reducible trifunctional 3-hydroxypyridinium cross-links, which are the end products of lysyl oxidase-mediated reactions on lysyl and hydroxylysyl amino group residues [1]. The hydroxyllysine route of collagen crosslinking is commonly found in connective tissues that bear large mechanical loads [1].

Recent studies have reported decreased concentrations of pyridinoline crosslinks in aged and degenerated human lumbar discs, however this change was not statistically significant [7, 8]. Data for thoracic discs have not been documented in the literature. The distribution of these collagen crosslinks in the anulus and nucleus of spinal discs is also not conclusive. Duance et al [8] and Eyre [1] reported higher extents of pyridinoline crosslinks in the nucleus compared to the anulus. In contrast, Pokharna and Phillips [7] reported no significant differences between the anular and nuclear matrices of aged and degenerated lumbar discs. Reasons for these contrasting trends are not known. Further data are needed to confirm if there are regional differences in the concentration of crosslinks in the disc matrix and whether degeneration has a significant impact on this distribution.

The study sought to determine the concentration of collagen and the extent of pyridinoline and deoxypyridinoline crosslinks in a preliminary sample of human thoracic intervertebral discs. The influence of degeneration and different disc regions on these biochemical variables was also examined.

MATERIALS AND METHODS

Tissue Collection and Preparation. Human cadaveric thoracic spines, which had been removed following routine post-mortem procedures and fixed in 4% buffered formalin, were used for this investigation. Spines with disc pathology from fractures, scoliosis, previous spinal surgery or tumours were excluded from the study. In total, nine thoracic spines were selected, consisting of 4 males and 5 females, with an age range of 60 to 90 years, mean age of 78 years (SD ± 8 years, Table 1). Approval to conduct the study was granted by the institutional Human Research Ethics committee.

In the first part of the study, thoracic discs from 2 spines, were cut in the mid axial plane and removed for macroscopic grading before biochemical analysis. One mm^3 of tissue was removed from the nucleus as well as from 4 different regions of the anulus (anterior and posterior left and right quadrants) of all thoracic levels (T1 to T12). In the second part of the study, the remaining 7 spines were dissected in the mid sagittal plane and thoracic discs from both left and right hemi-sections were examined macroscopically and graded. After grading, one mm^3 disc tissues were selectively sampled from the nucleus, anterior and posterior anulus in

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the mid-sagittal plane, from each thoracic level (T1 to T12). In total, 107 thoracic discs were graded and removed for biochemical assay. One thoracic disc was excluded from analysis because it was fused.

Discs were graded using a 3-point grading scale based on the grading criteria of Thompson et al[9]. Grade I indicated normal or non-degenerate discs, grade II, moderate and grade III, severely degenerate discs. Grading of all the discs were done by the same investigator (CT). The kappa correlation coefficient for intra-rater reliability of disc grading was 0.91 for the nucleus and 0.81 for the anulus. The pilot study on the intra-rater reliability for disc grading was performed on 10 hemisected cadaveric thoracic spines (n=120 discs), over a three-month period.

The wet weight of all disc samples was recorded. All the samples were then dried in an oven at 80°C, for 24 hours. Drying for longer than 24 hours yielded no further loss in tissue weight. The mean dry weight of the disc samples was 3.8 mg (SD ± 1.34 mg). After drying, the disc sample was hydrolysed at 105°C with 2 ml 6 Molar hydrochloric acid for not less than 16 hours.

Pyridinoline and Deoxypyridinoline Assay. The pyridinoline and deoxypyridinoline crosslinks were analysed using a modified method described by Randall et al[10] for high performance liquid chromatography (HPLC). An aliquot of the cooled disc hydrolysate (200 µl) was further processed with glacial acetic acid and butanol to extract the collagen crosslinks. The crosslinks were separated from the other disc constituents using a cellulose column (Alltech Extract-Clean filter column with 20 µ frit). The columns were washed with a mixture of n-butanol:acetic acid:water in the ratio 4:1:1, followed by 1 µl of Tetrahydrofluran. The crosslinks were finally extracted from the cellulose column using 0.75 µl of 0.5% N-heptafluorobutyric acid, centrifuged and the resulting eluate (100 µl) was injected for reversed phase HPLC analysis.

The HPLC unit consisted of a Waters model 700 WISP (Milford, MA, USA), Model 600E pump and controller, a Shimadzu FR535 Fluorescence Detector (Tokyo, Japan) and a Waters 441 Absorbance Detector fitted with a 280 nm filter set. Data was calculated by a Waters Maxima 825 chromatography system. Detection of pyridinoline and deoxypyridinoline crosslinks was set at excitation 295 nm and emission at 395 nm wavelength. Absorbance of the internal standard, isodesmosine (bovine neck ligament, ICN Biomedicals Inc. Cat No. 191379), was set at 280 nm. The pyridinoline and deoxypyridinoline working standard was prepared as an aqueous solution from kangaroo bone and assigned values using standards provided by The Bath Institute of Rheumatic Diseases, Bath, UK[10]. The concentration of pyridinoline and deoxypyridinoline crosslinks in the discs was calculated using values from the working pyridinoline and deoxypyridinoline standards.

Collagen Assay. The collagen content in the disc samples were determined by analysing the hydroxyproline content from 40 µl of the remaining hydrolysate, using a modification of the method of Kivirikko et al[11]. Calculations for the collagen content were performed assuming 300 moles of hydroxyproline is equivalent to 1 mole of collagen[6]. The extent of pyridinoline and deoxypyridinoline crosslinks was measured as the number of crosslinks per mole of collagen.

Pilot study. A pilot study, comparing 9 matched formalin-fixed and fresh lumbar disc samples from L1 to L3 was conducted over a 2-week period. The study found no significant differences in the collagen content and extent of pyridinoline and deoxypyridinoline crosslinks (paired t-test, df = 8, p = 0.50 for collagen content, p = 0.23 for pyridinoline, p = 0.32 for deoxypyridinoline).

Statistical Analysis. Paired t-tests were used to examine for disc regional differences in the collagen content and extent of pyridinoline and deoxypyridinoline crosslinks. Analysis of variance (ANOVA) and unpaired t-tests were applied to examine for differences in the biochemical variables due to degeneration status. All statistical tests were performed using the SPSS statistical software package. A $p<0.05$ value was accepted as significant for all statistical tests.

RESULTS

Different anular regions

The 24 thoracic discs from the first 2 spines yielded 96 anular samples from each anular quadrant. All discs were graded as normal or grade I. The collagen content and extent of pyridinoline and deoxypyridinoline crosslinks were not significantly different between the left and right quadrants of the anterior and posterior anulus (Table 2). There were also no significant differences in the collagen content between the anterior and posterior anulus. However the extent of pyridinoline and deoxypyridinoline crosslinks were higher in the posterior anulus compared to the anterior anulus.

Anulus and nucleus

Data from the 2 spines in the first part of the study were regrouped as anterior and posterior anuli, and used for analysis in the second part of the study. Therefore thoracic

Table 1. The distribution of the number of anular and nuclear samples from 9 human cadaveric thoracic spines (n=107 thoracic discs), according to disc grades I (normal), II (moderate degeneration) and III (severe degeneration) status.

Disc grade	Anulus	Nucleus	Total number of samples	Number of anular and nuclear samples with the same grade
I	55	51	106	48
II	35	39	74	28
III	17	17	34	11
Total	107	107	214	

Table 2. The mean difference and p values for t-tests analysis on differences in the collagen, pyridinoline and deoxypyridinoline crosslinks in the different regions of the anulus from 2 thoracic spines (n=24 thoracic discs)

Annular regions	df	Collagen nmol/dry wt (p value)	Pyridinoline mol/mol collagen (p value)	Deoxypyridinoline mol/mol collagen (p value)
Anterior left vs right	23	0.020 (0.80)	0.010 (0.75)	0.0008 (0.71)
Posterior left vs right	23	0.170 (0.32)	0.190 (0.09)	0.0002 (0.96)
Anterior vs posterior	47	0.110 (0.25)	0.120 (0.04*)	0.0040 (0.04*)

* = $p < 0.05$

discs from all 9 thoracic spines were used for analysis to compare the collagen and crosslink content in the anulus and the nucleus (n=107 thoracic disc samples). Approximately 50% of the 107 disc samples (anular = 49% and nuclear = 52%) had some degree of disc degeneration (that is, grades II or III, Table 1).

Degeneration and disc regions

There were significantly higher extents of pyridinoline ($p<0.01$) and deoxypyridinoline crosslinks ($p<0.05$) in the posterior anulus compared to the anterior region in normal discs (Figure 1). These results were similar to that found in the first part of the study. For moderately degenerated anuli, the collagen content ($p<0.001$) and extent of pyridinoline crosslinks ($p<0.01$) were significantly higher in the posterior anulus compared to the anterior anulus. In contrast, the nucleus had a significantly lower collagen content ($p<0.001$) but a higher extent of pyridinoline ($p<0.01$) and deoxypyridinoline ($p<0.01$) crosslinks compared to both the anterior and posterior anuli in normal and moderately degenerated disc samples. In severely degenerated anuli and nuclei however, there were no significant differences in any of the biochemical variables, that is, between the anterior and posterior anulus, and between both anuli and the nucleus.

Degeneration influences

The changes in the biochemical matrix of the anulus and nucleus due to increasing degeneration grade were variable (Figure 2). Severely degenerated nucleus had significantly higher collagen content ($p<0.01$) and severely degenerated anterior and posterior anuli had significantly higher extents of deoxypyridinoline crosslinks ($p<0.01$) compared to normal nuclei and anuli, respectively. The collagen content in the nucleus and the extent of deoxypyridinoline in both anterior and posterior anuli showed significant increasing trends with increased degeneration grade (ANOVA, $p<0.001$). The trend for the extent of pyridinoline crosslink was different, tending to peak with moderate degeneration status. The extent of pyridinoline crosslinks was significantly higher in moderately degenerated samples compared to normal samples in all disc regions ($p<0.05$). There was no significant difference in the extent of pyridinoline crosslinks between severely degenerated and normal disc samples in all disc regions.

DISCUSSION

Significant regional differences in the biochemical matrix were noted between the anulus and nucleus of normal and moderately degenerated samples. However with severe degeneration, no regional differences in the disc matrix were found. Although the extents of pyridinoline and deoxypyridinoline crosslinks were significantly different between normal anterior and posterior anuli, however the mean differences were small (pyridinoline = 0.15 mol/mol collagen and deoxypyridinoline = 0.008 mol/mol collagen) and may require additional data to confirm these statistical findings.

The finding that the nuclei of normal and moderately degenerated discs have lower collagen content but higher extents of pyridinoline and deoxypyridinoline crosslinks compared to the anulus, is consistent with that reported by Eyre[12], and Duance et al[8]. However, this trend contradicts that of Pokharna and Phillips[7], whose anuli and nuclei data were not significantly different. Their data were however consistent with the present findings for severely degenerated anuli and nuclei. The significance of these disc regional findings suggest that the collagen and collagen crosslink content varies in different stages of disc degeneration, therefore data from a variety of normal and moderately degenerated anuli and nuclei, should not be pooled for statistical analysis, but should be analysed separately.

A possible explanation for the regional disc difference according to Eyre[1] is that the nucleus consists predominantly of type II fibers, which have twice the crosslink capabilities compared to type I fibers. Type I fibers are found in higher proportion compared to type II fibers in the anulus, therefore the crosslink extent is higher in the nucleus compared to the anulus.

The results for collagen crosslink changes in the disc matrix due to different degeneration grades, have not been previously reported and showed interesting trends. The finding that the collagen content increased with degeneration is not new, and is consistent with most studies for lumbar discs[13, 14]. However the non-significant change in the anulus has not been reported. Although the extent of pyridinoline crosslinks was significantly different with different grades of degeneration, however between normal and severely degenerated thoracic anuli and nuclei there was no significant difference. This latter finding in the study is consistent with other studies on human lumbar discs[7, 8], and is surprising, considering the proposed function of pyridinoline crosslinks, which is to provide tensile support to the disc matrix[2, 7, 8]. It would be expected that as the collagen content increased with degeneration, that the extent of pyridinoline and deoxypyridinoline crosslinks would also increase correspondingly. A significant increase in the extent

of pyridinoline crosslink was noted for moderately degenerated discs, however the increase was not sustained in severely degenerated disc samples. Reasons for this change in pyridinoline crosslink extent are not known, and may be influenced by a change in the other disc biochemical components, such as water, proteoglycans and non-collagenous proteins with degeneration[3, 13].

Although the extent of deoxypyridinoline crosslinks is higher in degenerated discs, the ratio of deoxypyridinoline to pyridinoline crosslinks is very low, approximately 1: 50, in disc tissue[12]. The ability of deoxypyridinoline crosslinks to provide support to the collagen network is questionable due to the small increases (0.04 mol/mol of collagen). There is currently little literature on deoxypyridinoline crosslinks in spinal discs as it is more commonly used as a urinary marker for bone collagen turnover[10, 12]. Reasons for the higher extent of deoxypyridinoline crosslinks with degeneration, in particular in the anulus, are still not known and is worthy of further investigation.

In conclusion, the present preliminary study with a predominantly aged disc sample, revealed that there were significant differences in the disc matrices between normal and moderately degenerated anuli and nuclei. The significant difference in the disc regions however, is not seen in severely degenerated discs. Although the extent of pyridinoline and deoxypyridinoline crosslinks was significantly different between the anterior and posterior anuli, however the mean differences were small and would require further data to confirm statistical findings. Disc degeneration was found to be associated with significantly increasing trends in the collagen content in the nucleus, and for the extent of deoxypyridinoline crosslinks in the anulus. The extent of pyridinoline crosslinks increased significantly with moderate degeneration, however it was not significantly different between severely degenerated and normal discs.

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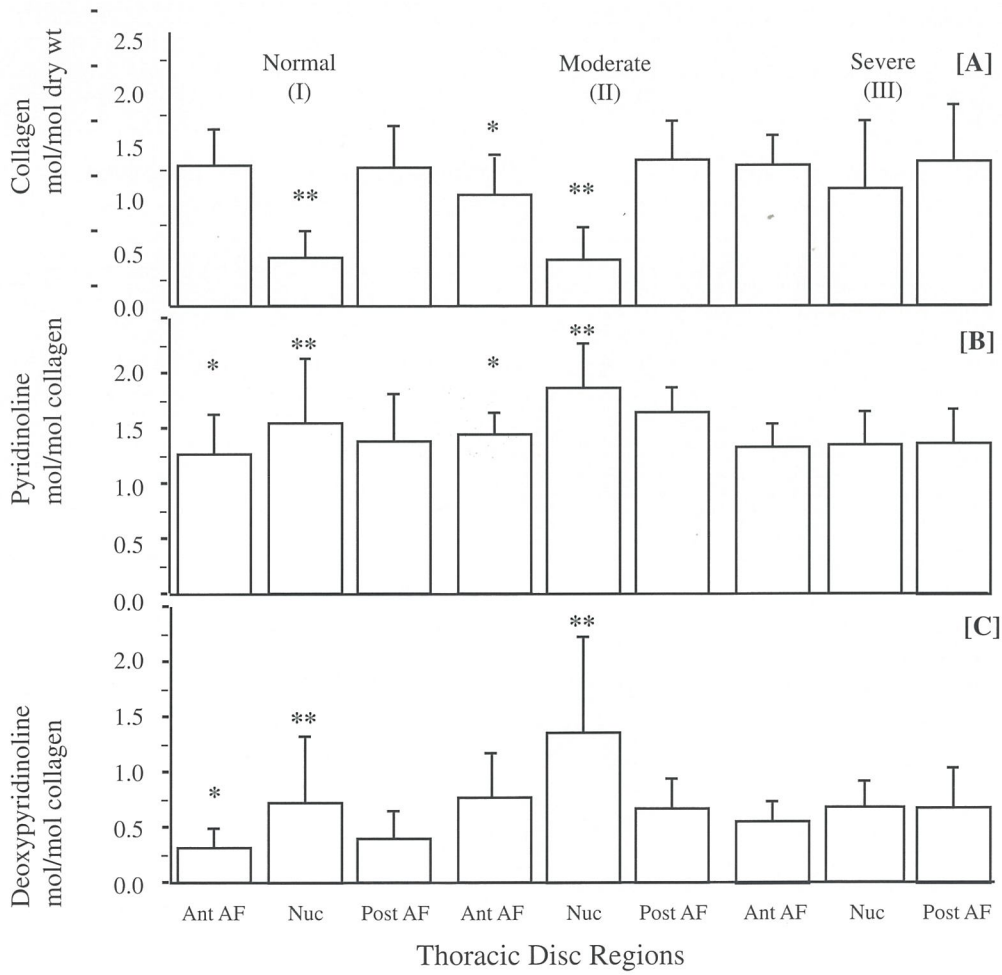

Fig. 1 - The distribution of the collagen content [A] and extent of pyridinoline [B] and deoxypyridinoline crosslinks [C] in the nucleus (Nuc), anterior (Ant) and posterior (Post) anulus (AF), with normal (I, n= 48 discs) findings, moderate (II, n= 28 discs) and severe (III, n=11 discs) degeneration changes.
Significant differences in collagen content [A] and extent of pyridinoline crosslinks [B] and deoxypyridinoline crosslinks [C], were found between the anulus and nucleus graded as I and II ($p<0.01$), except for deoxypyridinoline crosslinks in grade II discs. However no significant differences were found for all three biochemical variables between the anulus and the nucleus in severely degenerated discs (III). Error bars represent one standard deviation from the mean.
(= $p < 0.05$ between Ant AF and Post AF, ** = $p < 0.01$ between Ant AF and Nuc, and Post AF and Nuc)*

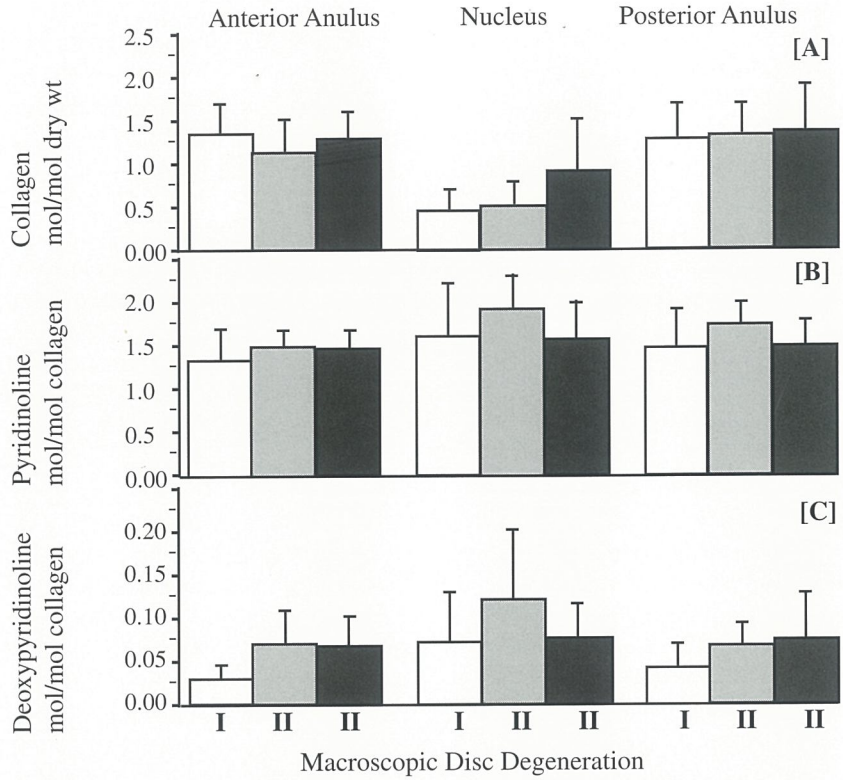

Fig. 2 - The collagen content and extent of pyridinoline and deoxypyridinoline crosslinks in the nucleus, anterior and posterior anulus of thoracic discs, with normal (I) findings, moderate (II) and severe (III) degeneration changes. Grade I anulus = 55 samples, grade II = 35 and grade III = 17 samples. Grade I nucleus = 51 samples, grade II = 39 samples, and grade III = 17 samples.
The collagen content was significantly higher in grade III samples compared to grade I, but only in the nucleus ($p<0.01$) [A]. The extent of pyridinoline crosslinks was significantly higher in grade II samples ($p<0.05$) but was lower in grade III samples in all disc regions. However there were no significant differences in the extent of pyridinoline crosslinks between grade I and III samples for all disc regions [B]. The extent of deoxypyridinoline crosslinks was higher in grades II and III samples, but only in the anterior and posterior anulus ($p<0.01$) [C]. Error bars represent one standard deviation from the mean.
(t-test = $p<0.01$ between grade III and I samples)*

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