Age-Related Changes in Collagen, Pyridinoline, and Deoxypyridinoline in Normal Human Thoracic Intervertebral Discs

Celia I. Tan,[1] G. Neil Kent,[2] Andrew G. Randall,[2] Stephen J. Edmondston,[1] and Kevin P. Singer[1]

[1]Centre of Musculoskeletal Studies, Department of Surgery, University of Western Australia.
[2]Division of Clinical Pathology, The Western Australian Centre for Pathology and Medical Research (PathCentre),
Queen Elizabeth II Medical Centre, Nedlands, Western Australia.

Human thoracic discs were analyzed for collagen and collagen cross-links to determine the distribution due to segmental, age, and gender influences. Thoracic discs from 26 cadaveric spines (1 to 90 years old) were graded macroscopically, then separated into anular and nuclear samples. Only grade I (i.e., normal) disc samples were selected ($n = 209$). Pyridinoline and deoxypyridinoline cross-links were initially separated by column chromatography and analyzed by reverse-phase high-pressure liquid chromatography. The collagen content was lower and the extent of pyridinoline and deoxypyridinoline were significantly higher in the nucleus compared with the anulus ($p < .001$). The collagen content and extent of pyridinoline were significantly lower with increasing age in the anulus and nucleus ($p < .001$). Young male discs had a significantly higher extent of pyridinoline compared with females ($p < .001$). Age, gender, and disc region differences were found to have a significant influence on the biochemical composition of the normal disc extracellular matrix.

THE anulus and the nucleus consist of collagen type 1 and 2 fibrils, especially the anulus, which has a higher percentage of collagenous fibrils (1). The pyridinium cross-links formed by type 1 and 2 collagen fibers are pyridinoline and deoxypyridinoline (2). It has been hypothesized that these collagenous cross-links are essential in maintaining the structure and tensile strength of the collagen fibrillar network (2). Alteration of the collagen cross-link content in the disc matrix has been suggested to impair disc function, in particular, the disc's ability to withstand mechanical loading (3,4).

Pyridinoline and deoxypyridinoline are mature, non-reducible trifunctional 3-hydroxypyridinium cross-links, representing 2 of the end products of lysyl oxidase-mediated reactions on lysyl and hydroxylysyl amino group residues (1). Studies investigating the presence of these cross-links in spinal discs have only been reported recently. Duance and colleagues (4) and Eyre (1) reported a higher distribution of pyridinoline cross-links in the nucleus compared with the anulus of lumbar discs. Similar results were found for aged thoracic discs (5). In contrast, Pokharna and Phillips (3) found no significant difference between these disc regions. Consequently further investigation appears important to determine if there are variations in the disc collagenous matrix between the disc anulus and the nucleus. Of greater interest in disc biochemistry, recent studies on human lumbar discs have reported a significant decrease in the levels of pyridinoline cross-links with increasing age and degeneration (3,4). Similar data on thoracic discs have not been reported in the literature to our knowledge.

Studies on the influence of gender differences in the disc collagenous matrix are even fewer. There have been studies suggesting that gender-related occupation may be associated with a higher predominance of disc degeneration in lumbar discs, particularly in males (6–8). However, gender differences in the collagen and cross-link composition of spinal discs have not been discussed. In view of the association between collagen cross-links and spinal disc function, this study examined the influence of age, gender, and spinal level differences on the collagen content, and the extents of pyridinoline and deoxypyridinoline in normal human thoracic intervertebral discs.

METHODS

Tissue Collection and Preparation

Archived formalin-fixed human thoracic spinal tissues were sourced following routine post mortem procedures. An earlier study comparing the use of formalin-fixed spinal tissues with comparable unfixed samples found no significant differences after 6 months of fixation in the collagen content and extent of pyridinoline or deoxypyridinoline between the two tissue sources (9). Therefore formalin-fixed tissues were used for the purposes of this study.

A sample of convenience of 26 thoracic spines were selected for investigation if they had no history of spinal trauma, surgery, or frank pathology to the thoracic spine (see Table 1). This resulted in a total of 303 thoracic discs, of which 9 discs were not available for investigation because 1 spine was incomplete, with only the upper 4 thoracic levels available, and another case had a completely fused T7–T8 disc. The study sample was composed of 13 males

Table 1. Demographic Details and Cause of Death for All Cases ($N = 26$)

Subject Code	Age (y)	Age Group	Gender	Cause of Death
AAT	77	Old	M	Pituitary tumor and cerebral swelling
ABT	77	Old	F	Bronchopneumonia
ACT	90	Old	M	Myocardial infarct and bronchopneumonia
ADT	75	Old	M	Myocardial infarct and emphysema
AET	79	Old	F	Bronchopneumonia and myocardial infarct
AFT	2	Child	M	Cerebral swelling and lung congestion
AT	81	Old	F	Metastatic adenoma
BT	52	Mid	M	Pneumonia
CT	18	Young	M	Spinal cord with autolysis
DT	29	Young	M	Cerebral trauma
ET	42	Mid	F	Cerebral hemorrhage from head injury
FT	77	Old	F	Myocardial infarct
GT	63	Old	F	Pulmonary congestion, death from asphyxia
HT	90	Old	M	Meningioma, death by myocardial infarct
IT	46	Mid	F	Undetermined cause of death
JT	33	Young	M	Undetermined cause of death
KT	23	Young	F	Post-traumatic epilepsy
LT	7	Child	F	Undetermined cause of death
MT	7	Child	M	Intracranial hemorrhage
NT	32	Young	F	Cervical fracture and massive hemorrhage
OT	23	Young	M	Undetermined cause of death
PT	43	Mid	F	Suicide—burn injuries
QT	40	Mid	F	Cerebral swelling and pulmonary congestion
RT	48	Mid	M	Undetermined cause of death
ST	37	Mid	M	Hypoxic encephalopathy by hanging
TT	1	Child	F	Undetermined cause of death

and 13 females with a mean age of 45.8 ± 28.2 years (age range 1–90 years) (Table 2). Approval for the study was obtained from the Institutional Ethics Committee.

Macroscopic examination of the disc was performed using the midsagittal section of each hemisected spine. A modified grading scheme from 1 to 3, based on the criteria described by Thompson and colleagues (10), was used to grade the discs. Grade I indicated normal or nondegenerate discs similar to Grade I on the Thompson scale; Grade II reflected moderate degenerative changes, which consisted of Grades II and III criteria from the Thompson scale; and grade III indicated severely degenerated disc changes, which consisted of Grades IV and V criteria from the Thompson scale. Grading of all the discs were done by the same investigator (C.I.T.). The kappa correlation coefficient for intrarater reliability of disc grading was 0.91 for the nucleus and 0.81 for the anulus (second reading repeated after a 3-month period) (5). Only discs graded as I for the anulus and the nucleus ($n = 209$), were used in this study.

In an earlier pilot study (5), significant differences in the extent of pyridinoline and deoxypyridinoline were found between the anterior and posterior anular samples ($p < .05$). Therefore, in the present study, disc tissues were selectively sampled from the nucleus, anterior anulua, and posterior anulus in the midsagittal plane, to take into consideration regional disc differences. After grading, 1 mm³ disc samples were removed from each thoracic level (T1 to T12) for pyridinoline, deoxypyridinoline, and collagen assays. The wet weight of each sample was recorded, then oven-dried 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 7.5 mg ($SD \pm 2.3$ mg). After drying, the tissue was hydrolyzed at 105°C with 2 ml of 6 M hydrochloric acid, for 16 to 24 hours.

Pyridinoline and Deoxypyridinoline Assays

The cooled sample hydrolysate (200 µl) was added to glacial acetic acid (2 ml), cellulose (0.5 ml), and butanol (8 ml), and separated via cellulose minicolumns (Alltech Extract-Clean filter columns with 20 µ frit). The column was washed sequentially with 13 ml of butanol:acetic acid:water (4:1:1), then with 1 ml of tetrahydrofuran. The cross-links were eluted with 0.75 ml of 0.5 % N-Heptafluorobutyric acid (HFBA) (Sequanal/high-pressure liquid chromatography [HPLC] grade from Pierce Biochemicals, Rockford, IL). Next, 100 µl of the resulting eluate was injected into reversed-phase HPLC, using the method described by Randall and colleagues (11). The HPLC system consisted of a Waters model 700 Satellite WISP (Milford, MA, USA), Waters 600E system controller and pump, a Shimadzu RF551 Spectrofluorometric Detector (Tokyo, Japan), and a Waters LKB 2151 variable wavelength monitor. Data acquisition and management was by a Waters Millennium 32 Chromatography Manager (Waters Corporation, Milford, MA). The column used was a Supelco-Supelcosil (Bellefonte, PA) LC-18DB (4.0 × 7.5 cm, cartridge, Prod No: PN589923). The mobile phase A was a mixture of 5% methanol and 0.12% HFBA at pH=2.0, and mobile phase B was a mixture of 80% methanol and 0.12 % HFBA.

End chromatogram took approximately 33 minutes and pyridinoline and deoxypyridinoline were detected at 7 and 9 minutes. Fluorescence detection of pyridinoline and deoxypyridinoline was set at excitation 295 nm and emission 395 nm wavelength and 280 nm absorbance for the internal

Table 2. Information on 26 Cadaveric Thoracic Spines Showing Number of Discs (Anulus and Nucleus) Graded I (Nondegenerate) According to Age Groupings

Age Groups	Age Range (y)	Number of Thoracic Spines	Mean Age (y ± SD)	Female	Male	Number of Grade I Discs	Total Number of Discs Graded
Child	1–15	4	4 ± 3.2	2	2	40	40
Young	16–35	5	25 ± 5.5	2	4	52	72
Mid	36–60	7	44 ± 5.1	4	3	69	84
Old	>60	10	78 ± 8.0	5	4	48	107
Total	1–90	26	45.8 ± 28.2	13	13	209	303

standard, isodesmosine (bovine neck ligament, ICN Biomedicals, Inc. Australasia, Sydney, Australia). The extent of pyridinoline and deoxypyridinoline cross-links in the discs was calculated against the working standards and expressed as number of cross-links per mole of collagen.

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, England (11). Collagen was determined by analyzing the hydroxyproline content using a modification of the method of Kivirikko and colleagues (12). Calculations for the collagen content were performed, assuming 300 moles of hydroxyproline was equivalent to 1 mole of collagen (13). The extents of pyridinoline and deoxypyridinoline were measured as the number of cross-links formed per mole of collagen (mol/mol collagen).

Preparation of Pyridinoline and Deoxypyridinoline Stock Standard From Kangaroo Bone

The working calibrator is an aqueous preparation of pyridinoline and deoxypyridinoline prepared from kangaroo bones. Semipurified pyridinoline (mW = 429) and deoxypyridinoline (mW = 413) were combined with 0.5% HFBA in aqueous solution at a suitable ratio. The standard is stored at −70°C in 1-ml aliquots. Concentration assigned to the working standard is as follows: Pyd = 714 nmol/L and Dpd = 360 nmol/L. Set points were assigned by running this standard in parallel with the old standard. Values were assigned by comparison with the original standard supplied from the Institute for Rheumatic Diseases (Bath, England).

Statistical Analysis

Paired *t* tests were applied to examine for intradisc or disc regional differences, and analysis of variance (ANOVA) was used to examine the age, gender, and thoracic intersegmental influences on the collagen content and extents of pyridinoline and deoxypyridinoline. For statistical comparison, each disc was considered an independent sample. However, due to the small number of spines, spanning a wide age range (1–90 years), samples were grouped according to age ranges for statistical analysis in order to determine age trends. The age groups: Child (0 to 15 years), Young (16 to 35 years), Mid (36 to 60 years), and Old (>61 years) (Table 1) were employed. Similarly, thoracic segmental levels were grouped to form upper (T1 to T4), mid (T5 to T8), and
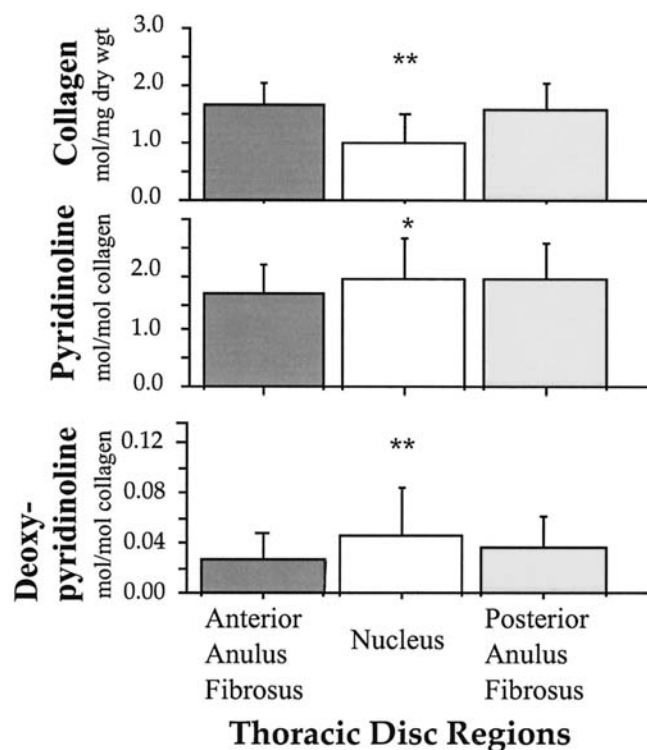

Figure 1. The collagen content and extent of pyridinoline and deoxypyridinoline in the nucleus, anterior anulus fibrosis (AF), and posterior AF of grade I (nondegenerate) thoracic discs (*n* = 209). The collagen content was significantly higher in the anterior and posterior AFs compared with the nucleus (*p* < .001). However, the extent of pyridinoline and deoxypyridinoline were highest in the nucleus. This difference was statistically significant between the nucleus and the anterior anulus for the extent of pyridinoline (*p* < .001) and between the nucleus and both anuli for the extent of deoxypyridinoline (*p* < .001). Error bars represent 1 standard deviation. **t* test between anterior AF and nucleus, where *p* < .001. ***t* test between both anuli and nucleus, where *p* < .001.

lower (T9 to T12) thoracic regions to determine segmental level trends. To determine the correlation coefficient between age and biochemical variables, partial correlation coefficient analysis were performed. All statistical tests were performed using the SPSS (Chicago, IL) and Statview (Abacus Concepts Inc., CA) statistical software packages. A probability level of *p* < .05 was accepted as representing a meaningful difference in all tests of statistical significance.

RESULTS

Influence of Disc Region

Significant differences in collagen content and extents of pyridinoline and deoxypyridinoline were found between the nucleus and the anulus of normal or nondegenerate thoracic discs (Figure 1). The anterior anulus had significantly lower extents of pyridinoline and deoxypyridinoline compared with the nucleus and posterior anulus (*p* < .001). The nucleus, however, had significantly lower collagen content (*p* < .001) compared with both anuli, but significantly higher extents of pyridinoline compared with the anterior anuli (*p* < .001), and significantly higher extent of deoxypyridinoline compared with both anuli (*p* < .001).
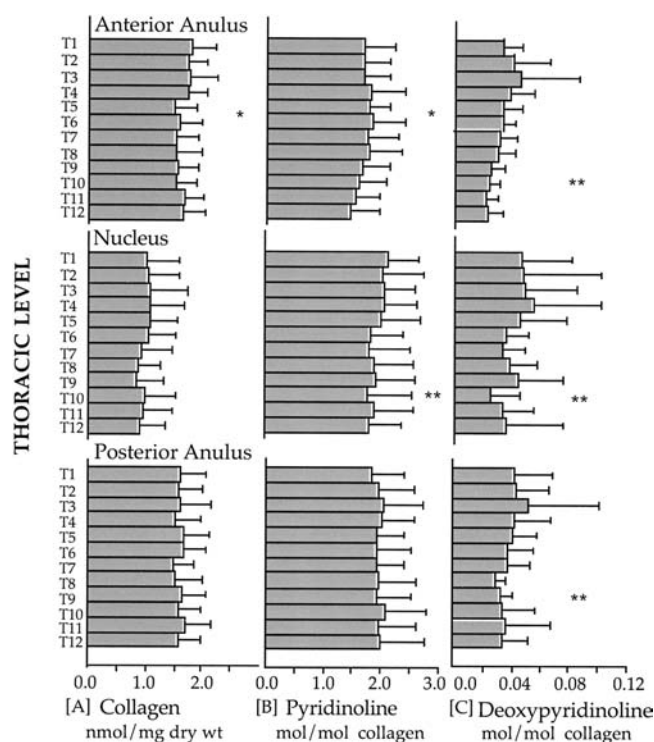
Figure 2. The collagen content [A] and extent of pyridinoline [B] and deoxypyridinoline [C] in the nucleus, anterior anulus, and posterior anulus of grade I (nondegenerate) thoracic discs in the different thoracic levels (T1 to T12, $n = 209$). Significant decreasing craniocaudal trends were noted in all disc regions for the extent of deoxypyridinoline ($p < .05$) [C]. The dip in the collagen content [A] and the peak in the extent of pyridinoline cross-link in the midthoracic region (T5 to T8) of the anterior anulus [B] also were statistically significant ($p < .05$). In the nucleus, the extent of pyridinoline in the lower thoracic region had significantly lower values compared with the upper thoracic region ($p < .05$). Error bars represent 1 standard deviation. *Analysis of variance test for comparison of means of each thoracic level from T1 to T12, where $p < .05$. **t-test comparison between lower and upper thoracic segments, where $p < .05$.
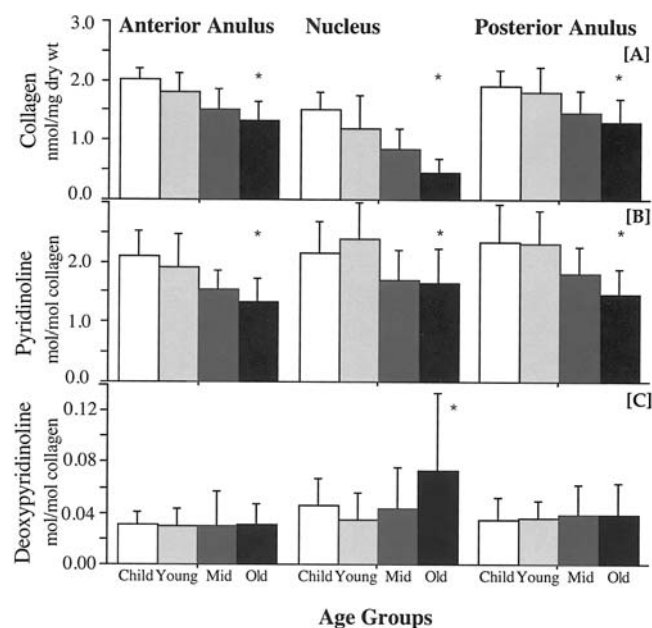

Figure 3. Changes in the collagen content [A] and extent of pyridinoline [B] and deoxypyridinoline [C] in the different age groups, Child (1–15 years), Young (16–35 years), Mid (36–60 years), and Old (>60 years) for all grade I (nondegenerate) thoracic discs ($n = 209$), in the different disc regions. In all disc regions, the collagen content and the extent of pyridinoline were significantly lower with increased age ($p < .001$) [A and B]. In contrast, the extent of deoxypyridinoline was higher with age but was only significant in the nucleus ($p < .001$) [C]. Error bars represent 1 standard deviation. *Analysis of variance tests for comparison of means of each age group, where $p < .001$.

There were no significant differences in the collagen content between the anterior and posterior anulus. These differences in the collagen content and extent of collagen cross-links in the anulus and nucleus were also observed when data were examined for each age group.

Influence of Spinal Segmental Level

The collagen content and extent of collagen cross-links in the disc in different thoracic segmental levels showed significant trends in the anterior anulus (Figure 2). In the midthoracic region of the anterior anulus, the collagen content was lower, while the extent of pyridinoline was higher compared with the upper and lower thoracic regions ($p < .05$).

In the nucleus, the extent of pyridinoline had a decreasing craniocaudal trend (Figure 2), where the lower thoracic region had significantly lower extent compared with the upper thoracic region ($p < .05$).

There was also significant decreasing craniocaudal trend for the extent of deoxypyridinoline in all disc regions (Figure 2). The extent of deoxypyridinoline in the lower thoracic region was significantly lower than the upper thoracic region ($p < .05$).

Age Influence

With increasing age, the collagen content and extent of pyridinoline decreased significantly in all disc regions ($p < .001$, Figure 3). In contrast, the extent of deoxypyridinoline increased with age, but this trend was only significant in the nucleus ($p < .001$). The correlation coefficients ranged from -0.5 to -0.7 between age and collagen content for all disc regions ($p < .001$); and -0.4 to -0.5 for extent of pyridinoline ($p < .001$). The only significant positive partial correlation coefficient for the extent of deoxypyridinoline was 0.3 ($p < .001$) for nuclear samples.

Gender Influence

The overall extent of pyridinoline was significantly higher in male subjects compared with female subjects in all disc regions ($p < .001$, Figure 4). When data were ordered according to age groups, the extent of pyridinoline in male discs dropped below that of females in all the disc regions; however, this gender difference in the Old age group was not statistically significant.

The collagen content was also significantly higher in males compared with females but only for the anterior and posterior anulus ($p < .05$). In contrast, there were no significant gender differences in the extent of deoxypyridinoline in all disc regions and in all age groups. Similar differences
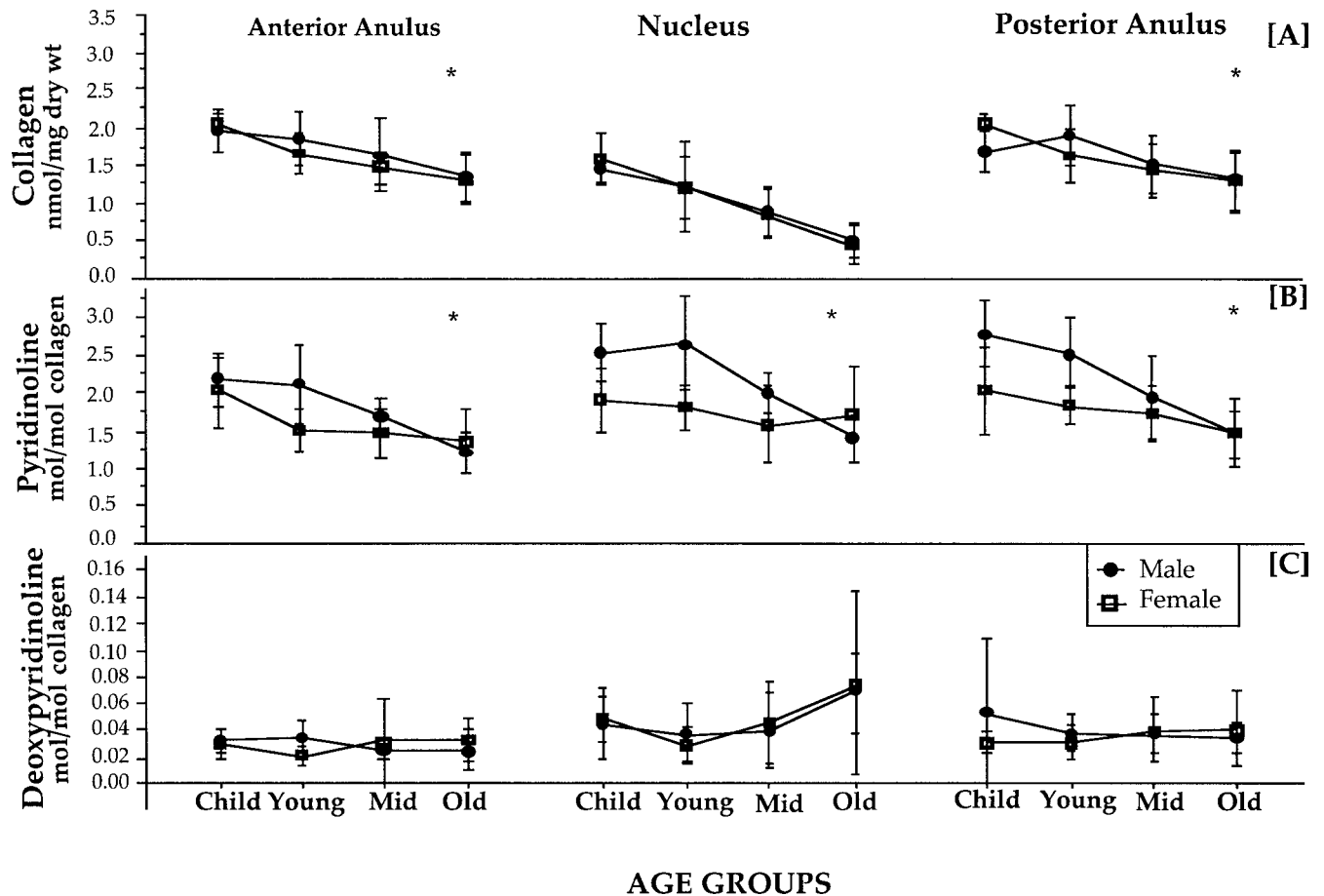
Figure 4. The collagen content [A] and extent of pyridinoline [B] and deoxypyridinoline [C] in the nucleus, anterior anulus, and posterior anulus of grade I (nondegenerate) male and female thoracic discs ($n = 209$). The collagen content was significantly higher in males compared with females for both the anterior and posterior anulus ($p < .05$) [A]. The extent of pyridinoline also was significantly higher in male disc samples compared with female samples in all discs regions and decreased significantly with age ($p < .001$) [B]. There was no significant gender difference for the extent of deoxypyridinoline [C]. Error bars represent 1 standard deviation. *t-test comparison of means of male and female samples, where $p < .05$.

in gender results were noted for collagen content, and extent of pyridinoline and deoxypyridinoline, when data were regrouped according to thoracic segmental levels.

DISCUSSION

Literature on the biochemical cross-links in human thoracic discs is limited when compared with studies on lumbar discs (3,4,14). The extent of pyridinoline reported in the literature ranges from 0.7 to 1.66 mol/mol collagen in the anulus and 1.0 to 1.59 mol/mol collagen in the nucleus of lumbar discs (4,15). Interestingly, Pokharna and Phillips (3) reported values of 3.4 to 4.2 mol/mol collagen in lumbar discs, despite Eyre's (1) contention that the nucleus was only able to form 3 moles of cross-link per mole of collagen. Reasons for the high values reported by Pokharna and Phillips are not known (16). The present study on thoracic discs found a mean of 1.83 mol/mol collagen in the anulus and 1.95 mol/mol collagen in the nucleus of thoracic discs. This value is considerably lower than that of Pokharna and Phillips (3), but slightly higher than that of Eyre (15) and Duance and colleagues (4). The variation in the extent of pyridinoline the different lumbar studies could be due to

the age and degenerative status of the disc (4). The results of the present study of the collagen content, and extents of pyridinoline and deoxypyridinoline in normal thoracic discs only, provide a basis for future comparison with degenerated thoracic discs.

Significant regional differences in the collagenous matrix were found between the anulus and the nucleus of nondegenerate thoracic discs. The present study found higher collagen content in the anulus compared with the nucleus, but a higher extent of pyridinoline and deoxypyridinoline in the nucleus, which was consistent in all the age groups. This regional difference in collagen content and extent of collagen cross-links is similar to most lumbar disc studies (1,4,17,18). The higher extent of collagen cross-links in the nucleus observed in this study is not surprising because of the abundance of type II collagen fibers in the nucleus compared with the anulus (19). According to Eyre (1), type 2 collagen has the potential to form up to 3 moles of cross-links per mole of collagen, while type 1 collagen is able to form half the number of cross-links as type 2 collagen.

Pokharna and Phillips (3), however, found no significant difference in the extent of pyridinoline between the anulus

and the nucleus in lumbar discs. Their nonsignificant result may be due to the inclusion of degenerated lumbar discs in their study (3). With degeneration, the proportion of type 2 collagen fibers in the nucleus has been reported to decrease and type 1 fibers to increase due to the formation of granulation tissue (20).

This study found that the collagen content and extent of pyridinoline was significantly lower with increased age for all disc regions. The lower collagen content is consistent with the observations of Crean and colleagues (14); however; Olczyk (17) and Scott and colleagues (18) found an increase in collagen content with increasing age in lumbar discs instead. A plausible explanation for these conflicting reports could be the inclusion of degenerated discs in the samples of the latter two studies. Degeneration may stimulate and encourage new collagen synthesis in the disc matrix, which is part of the tissue repair and regeneration process (14). This may explain the high collagen content, as opposed to the low collagen content noted in the present group of normal disc samples. The lower collagen content seen in these normal aged discs also may be due to an increase in other matrix constituents, such as keratin sulphate and other noncollagenous components (4,21,22), which were not measured in the present study.

The lower extent of pyridinoline with increasing age was consistent with that reported by Pokharna and Phillips and Duance and colleagues (3,4). However, these two studies did not separate the effects of degeneration and age on the discs. The results from the present study, although using a sample of convenience, included only nondegenerate discs from a wide age range, suggesting that the natural aging process may have a negative influence on the collagen content and the extent of pyridinoline in the disc matrix (23). The possible consequence of a low collagen content and extent of pyridinoline in a normal-aged disc is a disc matrix that is susceptible to injury under load-bearing conditions (3,24). Whether degenerative processes will expedite or alter the normal age changes on the disc collagenous matrix is worthy of further investigation.

The thoracic spinal level trends for pyridinoline and deoxypyridinoline have not been reported previously. Scott and colleagues (18) found a decreasing craniocaudal trend in the collagen content for thoracic discs. The present study provides preliminary findings on the distribution of collagen, pyridinoline, and deoxypyridinoline in the different levels of the thoracic spine. However, the only significant findings were a lower collagen content and higher extent of pyridinoline in the anterior anulus, lower extent of pyridinoline in the nucleus, and significant decreasing craniocaudal trends for the extent of deoxypyridinoline in all disc regions.

The significantly lower collagen and higher extent of pyridinoline noted in the midthoracic region of the anterior anulus is an interesting finding. The anterior thoracic region, especially with increasing age, is subjected to sustained compressive loading due to the natural kyphotic posture (25,26), which is associated with lower anterior disc heights in the midthoracic region (27,28). The greater loading experienced by the anterior anulus may be the reason for the different disc matrix composition compared with the posterior anulus. Similar stress reactions are seen in middle thoracic vertebral

bodies as a function of the spinal configuration and habitual kyphosis (29). However, in order to test this hypothesis, an investigation correlating biomechanical stress with the disc collagenous matrix would be necessary.

Few studies have described gender differences in the collagen content or collagen cross-links in spinal discs. In the present study, significantly higher collagen content was noted in the anulus of male samples, and significantly higher extent of pyridinoline was observed in male thoracic discs, compared with females, in all disc regions. In the Old age group, this gender difference was reversed. Aged female disc samples tended to have a higher extent of pyridinoline, possibly due to a faster rate of collagen and cross-link degradation in males with increasing age.

The low extent of deoxypyridinoline compared with pyridinoline in the thoracic disc matrix seen in the present study is consistent with Eyre's report (15). The present study found an average ratio of pyridinoline to deoxypyridinoline cross-links of $>40:1$, comparable with the $>50:1$ ratio by Eyre (15). Similar to pyridinoline, the extent of deoxypyridinoline was highest in the nucleus and lowest in the anterior anulus of nondegenerate thoracic discs (Figure 2). With increasing age, however, in contrast to collagen and pyridinoline, the extent of deoxypyridinoline was significantly higher, but only in the nucleus (Figure 2). This finding differs from that reported by Takahashi and colleagues (30), who found no significant change in the extent of deoxypyridinoline in human bone, cartilage, ligament, tendon, meniscus, and muscle with increasing age. Although this difference is small, the mean difference being 0.04 mol/mol collagen (Figure 3); however, the physiological significance is not known, as this cross-link is present in very small amounts in the disc matrix (0.02 to 0.08 mol/mol collagen). A possible reason for this higher extent of deoxypyridinoline with age is that in aged nondegenerate nuclei; the enzyme lysyl hydroxylase, which has been suggested as favoring the formation of pyridinoline cross-links, is not as active as in the younger nuclear matrix. In clinical syndromes resulting from a deficiency of lysyl hydroxylase, a higher extent of deoxypyridinoline is found in the tissues (31). This differing age influence on pyridinoline and deoxypyridinoline in thoracic discs is an unexpected finding and worthy of further investigation.

Conclusion

In conclusion, this study provides baseline data on the distribution of the collagenous matrix in nondegenerate thoracic discs in relation to disc region, thoracic spinal level, age, and gender and serves for future comparisons with degenerative discs. The collagen content was lower and the extent of pyridinoline and deoxypyridinoline were significantly higher in the nucleus compared with the anulus. The collagen content and extent of pyridinoline were significantly lower with increasing age in the anulus and nucleus. Young male discs had a significantly higher extent of pyridinoline compared with females ($p < .001$). Age, gender, and disc region differences were found to have a significant influence on the biochemical composition of the normal disc extracellular matrix.

Address correspondence to Kevin P. Singer, PhD, Centre for Musculoskeletal Studies, Department of Surgery, The University of Western Australia, Level 2, Medical Research Foundation Building, Royal Perth Hospital, Rear 50, Murray Street, Perth, WA 6000. E-mail: kps@cyllene.uwa.edu.au

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