Microscopic And Molecular Study Of Elastin And Collagen In Human Intervertebral Disc

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Abstract : This study was carried out to analyze, biochemically and histologically, the collagen and elastin components of human intervertebral disc material obtained at operations performed in cases of lumbar intervertebral disc herniation, and to compare these data with those obtained on "ligamentum flavum" samples in the same cases. Following preparation of the residues of the specimens for analysis, histological analysis and elastin - collagen estimations were done. The results obtained were: % 1.68±0.46 elastin in disc material and % 54.60±4.48 in ligamentum flavum ($p<0.0001$); % 15±0.75 collagen in disc material and % 7.98±1.21 in ligamentum flavum ($p>0.05$). Histological evaluation revealed that elastic fibres are the primary connective tissue elements of ligamentum flavum, while the annulus fibrosus contains these fibrils only in the periosseous. The nucleus pulposus was observed microscopically not to contain elastic fibrils. These histological data confirm the molecular data obtained in this study. In conclusion, it can be stated that intervertebral disc material and yellow ligament show significant differences in elastin - elastic fibril content and elastin / collagen ratios.

Key Words : Human intervertebral disc: Elastin: Collagen

INTRODUCTION

Until now, little information has been available in the medical literature on the molecular analysis of the human intervertebral disc although studies on this tissue in experimental animals are abondant (6,8,15,17,18,21,23).

The presence of elastic fibre in the intervertebral disc has long been suspected, and investigators have observed its presence, using the electron microscope on samples obtained from humans (3,7,9,25,29). These studies have treated the subject only from the aspects of histochemistry and submicroscopic structure.

The pioneer data on the biochemical analysis of this tissue have been published by MIKADA et al. (16) and HEITH et al. (10) and these investigators concentrated on elastin. Recently, interest in the proteoglycan and water content of human intervertebral disc tissue (14,19,26) and on serine proteinase inhibitors (1) has been observed.

The objective of this investigation was to analyze human intervertebral disc material al the molecular level, including a quantitative analysis of the two connective tissue proteins, elastin and collagen. It is known that elastin imparts "elasticity" to the tissue where it is located, whereas collagen gives mechanical strength and support (5,24). Information on the elastin and collagen content from a comparative study on the same cases might shed light on the physiological relevance of the mechanical and elastic properties of disc material. In addition, confirmation on the biochemical data at microscopic level was also obtained.

This study was designed so that parallel analysis was also performed on ligamentum flavum samples obtained at operations performed in cases of lumbar intervertebral disc herniation.

MATERIALS AND METHODS

Collection of intervertebral disc and yellow ligament samples from cases of herniated disc:

Human yellow ligament and intervertebral disc samples were obtained from 25 individuals aged from 21 to 52 years (11 males and 14 females), operated for intervertebral disc herniation, at the Department of Neurosurgery, Faculty of Medicine, Fırat University, between January 1992 and January 1994 (The approval of Fırat University Institutional Review Board was obtained for this investigation which does not directly involve any extra intervention of the patient, besides the therapeutic objective). These cases were not treated with drugs which could affect the metabolism of this tissue. The investigated discs originated from the interspaces L_4-L_5 (13 cases), L_5-S_1 (10 cases), and L_3-L_4 (2 cases). Fragments of prolapsed disc and the surgically removed material were submitted to microscopic investigation in order to separate the disc material from the yellow ligament and discard foreign material not to be included in the study.

The tissue samples, after being washed in physiological saline, dried on filter paper and weighed, were stored at 1-20°C until needed for biochemical analysis.

Preparation of the specimen for analysis:

The technique used was that described by MIKAWA et al (16):

For each analysis, the mass (wet weight) of thespecimen was two grams. The samples were minced with scissors until the particle size was about 5 mm.After lyophilization, complete dryness was achived. The powder thus obtained was further pulverized at 4°C with a mortar. Using ethanol and ether, the dried samples were delipidated, and the organic extracts used for subsequent quantitative lipid analysis.

The residue, delipidated in ethanol-ether (1:1) and anhydrous ether, was dried to a constant weight in an oven at 105°C. The dry weight of the residue was measured gravimetrically. One part of the residue was used for histological analysis while the rest was kept for elastin and collagen quantitation.

Histological Analysis

The residual tissue fragments were embedded in paraffin after routine tissue processing. Bone-containing tissues were also subjected to acid decalcification before tissue processing. Then the tissue blocks were serially sectioned at five microns, with a sliding microtome. The tissue sections were affixed to glass slides with albumin, following which they were stained with haematoxylin and eosin, as well as with Verhoeff's elastin tissue stain (5,27).

—Biochemical Analysis

Preparation of soluble and residual tissue fractions:

Using the prepared residues, the subsequent preparation of soluble and residual fractions containing collagen and alastin, respectively, was done using modifications (22) of the method previously described by LANSING et al. (18).

Eight ml of 0.1 N NaOH was added to approximately 300 mg of died tissue in a tube, and the tubes were placed in a 98°C water bath for 50 minutes. The tubes were then centrifuged at 3000 rpm for 10 minutes, and the supernatants saved. The residue which constituted the insoluble "elastin" was washed twice with 3ml of distilled water; these washes, added to the supernatant, constituted the soluble fraction of each specimen, which contained the "collagen".

—Estimation of elastin:

The residue (elastin) was dehydrated using anhydrous ether and dried to a constant weight in an oven at 105°-110°c. The dry weight was the "insoluble" elastin and measured gravimetrically.

The percentages of elastin in the tissues investigated were estimated using the formula:

$$\frac{\text{mg dry tissue residue (after hydrolysis)}}{\text{mg dry tissue sample (before hydrolysis)}} \times 100\ \%$$

—Quantitation of collagen:

The soluble fraction obtained as described above, containing the collagen, was hydrolyzed in 6N HCL and neutralized with 2.5N NaOH. The collagen content was determined through the estimation of hydoxyproline, the amino acid typical of collagen,

using the standard method of WOESSNER (28). The hydroxyproline standard was purchased from SIGMA (Cat. No. 1637). According to this method, the hydroxyproline in the hydrolyzate was quantitated spectrophotometrically using measured Chloramine-T as the color reagent. The collagen content of the soluble fraction was expressed in mg, using the factor 7.46 to convert hydroxyproline to collagen. Finally, the collagen percentage of the initial wet tissue was expressed as % by the formula.

$$\frac{\text{mg collagen}}{\text{mg dry tissue}} \times 100\ \%$$

Statistical Analysis : The data obtained were analyzed using Student's "t" test.

RESULTS

The elastin content in the yellow ligament was found to be 54.60 ± 4.48 % (Means ± SE) of the total dry weight of the tissue, while that of collagen, 7.98 ± 1.21 %. In other words, elastin constituted more than half, and collagen less than one tenth of the dry weight of ligamentum flavum. By contrast, the intervertebral herniated disc material showed an elastin ratio of 1.68 ± 0.46 % (Means ± SE) and a collagen ratio of 7.98 ± 1.21 (Table 1).

The histological data have shown that the elastic fibrils are the predominant components in ligamenta flava. As to the disc material, elastic fibrils were observed only in the periosseous localization and not in the nucleus pulposus (Figs 1-5).

	n	Elastin	Collagen
Disc material	20	1.68 ± 0.46	6.15 ± 0.75
Ligamentum Flavum	25	54.60 ± 4.48	7.98 ± 1.21
	P	<0.0001	>0.05

Table 1 : Elastin and collagen content of human disc material and ligamentum flavum samples obtained at operations for prolapsed intervertebral disc. Data are given in % (mg protein/100 mg dehydrated tissue), as Means ± S.E. Statistical comparison was made between the disc material and ligamentum flavum.

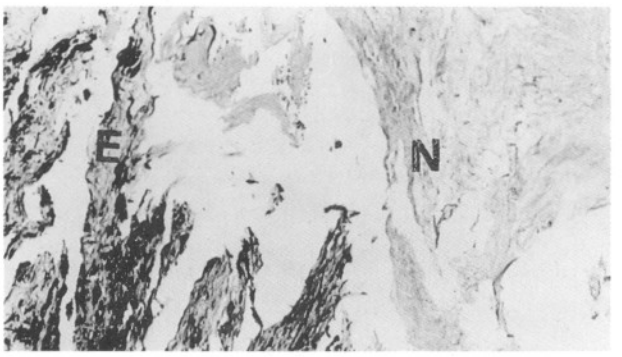

Fig. 1: Histological appearance of nucleas pulposus (N) and ligamentum flavum (E: elastic fibrils). (The staining method used was Verhoeff's elastic tissue technique - x4).

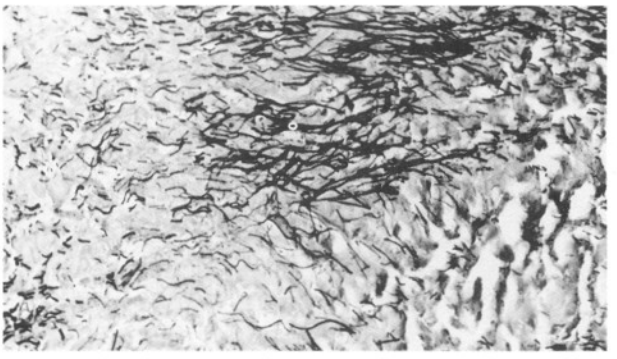

Fig. 2: Histological appearance of ligamentum flavum, with the elastic fibrils stained black using the Verhoeff's technique - (x10).

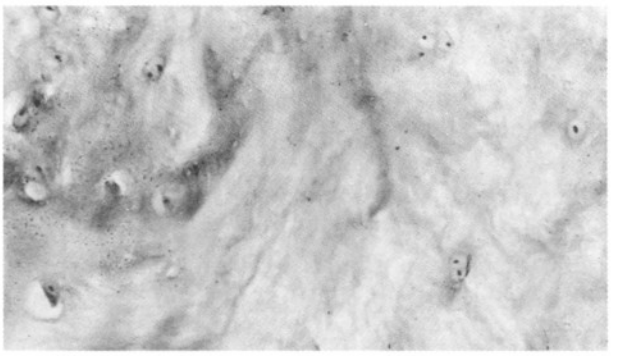

Fig. 3: Histological appearance of nucleus pulposus, using the hematoxylene-eosine staining technique - (x20). No elastic fibrils are observed.

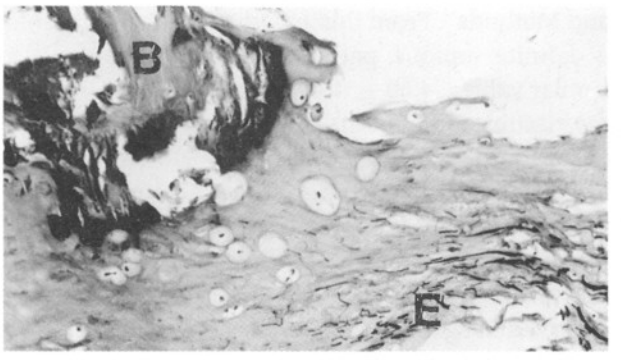

Fig. 4: Histological appearance of the annulus fibrosus in periosseous localization, using Verhoeffs technique (x10). (B: bone tissue; E: elastic fibrils, stained black).

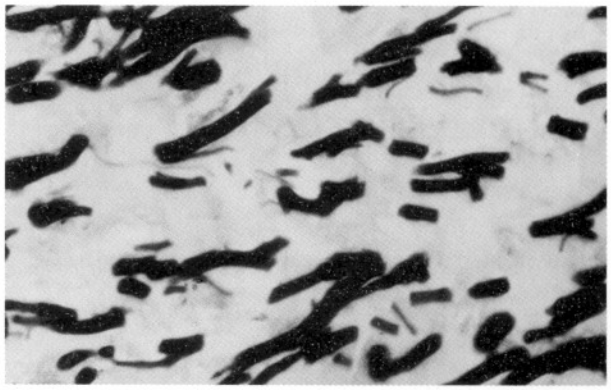

Fig. 5: Histological appearance of elastic fibrils, stained black, using the Verhoeff's technique - (x20)

DISCUSSION

A study of the intervertebral disc to determine the structural components that are responsible for its flexibility and shock absorbing capacity, as well as its mechanical strength, is essential to understanding the normal functions and diseases of the vertebral column (7).

This study was carried out at the molecular as well as microscopic level, with the objective of comparing the elastin and collagen content in the disc material and the yellow ligament.

Mikawa et al (16) performed a histological and biochemical analysis of the elastic fibre and elastin in human yellow ligament and intervertebral disc material obtained from a veriety of cases, including cervical spondylosis, ossification of the posterior longitudinal ligament, spinal cord tumour, scoliosis, intervertebral disc herniation, spinal cord stenosis, and primary spinal tumour. In our study, the smaples were collected only from cases of intervertebral disc herniation, as specified under the section "Materials and Methods". From this aspect, our data represents a definite, unique pathology group. We found a similar value (54.60 $\pm$ 4.48, versus 46.7 $\pm$ 0.9 %) for the elastin content in the yellow ligament, while that of collagen (7.89 $\pm$ 1.21 versus 31.0 %) was observed to be notably lower in our study. Our microscopic findings seem to support this result, and the data of Johnson et al. (7) on the fact that "elastic" fibres are the "predominant" type of connective tissue fibres observed in ligamenta flava give similar support. The discrepancy of our collagen value and that of Mikawa et al. (16) may result from the heterogeneity of their clinical cases, as described above. We would also like to point out that although our cases are definitely less heterogenous that those of Mikawa et al. (16), we can not consider our samples as "totally homogeneous", due to the fact that our material is "peroperative" and is expected to show different degrees of degeneration in the different cases.

In addition, our low results on the collagen content of human disc intervertebral tissue may also signify the possible lack of mechanical strength in cases of herniated disc, a finding which correlates a biochemical result with the thysiological function very well. On the other hand, the elastic fibres are claimed to assist prevertebral muscles in maintaining erect posture and the return to this position following extension of the vertebral column, that is, provide "elasticity".

The elastin content was found to be much lower in the intervertebral disc tissue: 1.68 $\pm$ 0.46 (Means $\pm$ SE) of the total dry weight, comparable with the finding of Mikawa et al. (16), which was. 1.7 $\pm$0.2 % (Means $\pm$ 0.2 % (Means $\pm$ SE). Elastin and collagen have been found to exist in varying rations in different tissues (4,11,12). The percentage reported in this study for elastin is small; however equally small amounts of elastin are found in loose connective tissues; in the skin, for example, where the functional role of elastin as the "elastic" component of the skin can not be underestimated. Elastin in tih intervertebral disc may serve the function of helping the disc in retaking its shape after mechanical deformity (2), that is, rendering the disc "elastic".

From the point of view of microscopic study, we made an analysis of "annulus fibrosus" and "nucleus pulposus", the two predominant structures of the disc material, besides the hyaline cartilage plates. The nucleus pulposus is a deep, gelatinous, hydrophylic body of fibrocartilage that is confined superiorly and inferiorly by th hyaline cartilage plates and superficially by the annulus fibrosus. The annulus fibrosis is a superficial, highly collagenous sleeve of lamellated connective tissue that connects the hyalin cartilage plates and the adjacent compact bone of the vertebral epiphysis (7). In our study, the annulus fibrosus was shown to contain elastic fibrils in periosseous localization. On the other hand, elastic fibrils were observed not to be constituents of the matrix of the nucleus pulposus. This last finding is not in agreement with that of some other

investigators (7,16) who did report, though sparse and irregular, the distribution of elastic fibres throughout the nucleus pulposus. The information that "elastic fibres are not observed throughout the intervertebral disc, but are restricted to, or better developed in areas of intervertebral disc which are connected or attached to adjacent vertebrae" may bring out the possibility that part of the disc nearest the adjacent vertebrae. Another explanation would be the fact that the disc tissue samples included in this study were representative of material from herniated disc cases, while the other studies were performed either on autopsy material or on non-homogeneous disc material.

Evaluating together our data on the microscopic and biochemical analysis of human intervertebral disc material, we state that in herniated disc cases, the intervertebral disc material contains a small, but microscopically and functionally significant elastin/elastin fibre component, whereas the yellow ligament is rich in both, data which are in keeping with the latter's physiological role. In our future studies, the now well-known polymorphism of disc collagen and the possible polymorphism (20) of elastin will be investigated in disc herniation.

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