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ORIGINAL ARTICLE

Molecular alterations of human lumbar yellow ligament related to the process of intervertebral disk degeneration and stenosis

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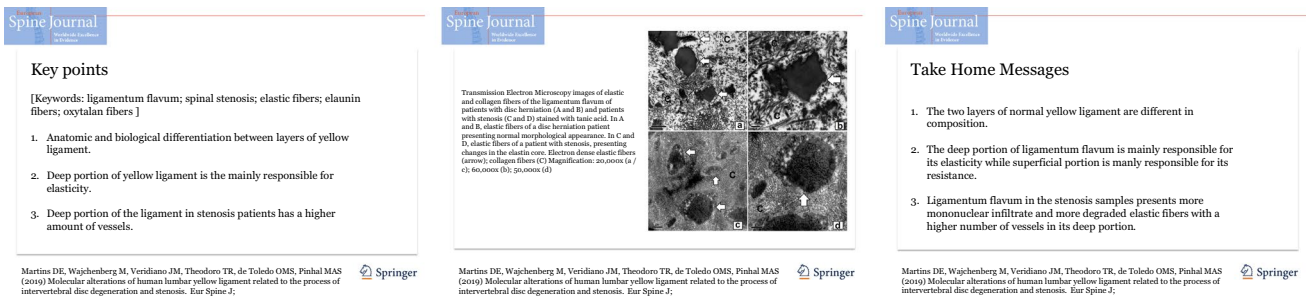
Abstract

Purpose The objective of this study was to analyze the layers of yellow ligament in lumbar canal stenosis and disk herniation.
Methods Eighteen ligaments were harvested from patients with lumbar spinal canal stenosis. Twenty-nine normal samples from lumbar spine disk herniation patients served as control. All surgical procedures were the same. Ligaments were stained in hematoxylin and eosin; picrosirius–hematoxylin for collagen; Weigert's resorcin-fuchsin for elaunin, oxytalan and elastic fibers; and transmission electron microscopy. Immunohistochemistry was performed for Il-6; Il-10; and CD-31, PGP9.5. Results are described in means and standard error (mean ± SE), and all analyses adopted the significance level of $P < 0.05$.
Results Spinal stenosis ligaments were 2.5 × thicker. Control superficial ligaments presented a large number of thick, compact collagen fibers and a significant amount of oxytalan and mature elastic fibers. The deep layer presented a large number of mature elastic fibers. In the stenosis group, collagen was thinner and compacted in both layers. There was no difference in the interleukin profile among groups. The deep portion of the stenosis group presented a higher number of vessels and nerves.
Conclusion Two layers compose the elastic system of the normal ligamentum flavum, where the deep portion is mainly responsible for its elasticity (elaunin fibers), while its resistance depends on the concentration of oxytalan fibers, which are more present in the superficial layer. Ligamentum flavum in the stenosis samples presents more mononuclear infiltrate and more degraded elastic fibers with a higher number of vessels in its deep portion.

Graphical abstract

These slides can be retrieved under Electronic Supplementary Material.



Keywords Ligamentum flavum · Spinal stenosis · Elastic fibers · Elaunin fibers · Oxytalan fibers

Introduction

The thickening of the ligamentum flavum (LF) and its biochemical and morphological changes are considered one of the most important causes of spinal stenosis [1]. The LF is important for providing spinal stability in many postures

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and maintaining a smooth surface on the posterior wall of the spinal canal and foramina [2]. LF is constituted of two portions firmly adherent to each other. The superficial layer is a light-yellow structure, adjacent to the multifidus muscles and bigger than the deep component, while the deep layer is a thin dark-yellow layer on the ventral side, adjacent to the spinal canal [3].

The elastic fiber system consists of three different kinds of fibers. The oxytalan fiber composed of parallel bundles of microfibrils that serve as a guide for the deposition of a small amount of elastin, forming the elaunin fibers, and as more elastin adheres to those fibers, it becomes thicker and forms the mature elastic fiber [4–6].

The elasticity of the tissue depends on the concentration of mature elastic fiber and elaunin fibers, while its compression resistance is related to oxytalan fibers [7]. A large amount of oxytalan fibers has been reported as a marker of strong local mechanical stress in muscles [8]. Loss of LF elasticity could be responsible for allowing it to fold into the spinal canal, thus promoting stenosis [1].

This adherence between the layers has led the LF to be treated as a single structure. However, the goal of this study was to determine whether there are differences in thickness between the layers and whether they differ in histomorphology, thus implying different function. An anatomical investigation was therefore performed on LF layers obtained from both spinal canal stenosis patients and lumbar disk herniation patients.

Methods

After Institutional Review Board approval (897.222), 29 consecutive patients with lumbar disk herniation and 18 patients with lumbar spinal canal stenosis had their LF harvested during either a microdiscectomy procedure or decompression surgery after failure of conservative treatment. All microdiscectomy procedures were performed by posterior approach using tubular retractors, and the LF was released from lamina using a curette. The two layers of the ligament were removed. The first dorsal layer was called superficial, and the second ventral layer, which lies closer to the vertebral canal, was called the deep portion. The decompression surgery was performed under direct vision.

Spine MRI examinations were conducted as clinical routine on a 1.5-T magnet HDX (GE Healthcare, Milwaukee, USA) according to the departmental protocol: Subjects were scanned in a supine position, and their leg extended using a multichannel spine dedicated coil. Examinations used the following sequences: sagittal T2-weighted fat suppressed (TR/TE, 2756/58; number of excitations [NEX], 2; matrix, 256 × 192; thickness, 4 mm; field of view [FOV], 40 cm), sagittal T1-weighted (357/15; 2; 320 × 256; 4 mm; 40 cm), sagittal T2-weighted (2890/58, 2; 256 × 192; 4 mm; 40 cm), coronal T2-weighted fat suppressed (2756/58, 2; 256 × 192; 4 mm; 40 cm) and axial T2-weighted (2756/58, 2; 256 × 192; 4 mm; 25 cm).

The thickness of the LF was measured at its midpoint using magnetic resonance imaging on T1-weighted axial images, which was clearly seen as a low-signal intensity mass just ventral to the vertebral lamina. All measures were done by a single investigator and performed at workstations through the Carestream Vue Motion (PACS), version 12.1.5.6529 Inc. 2009 system (Carestream Health, USA).

The average age of the disk herniation group was 40 ± 11.54 (range 19–57) with 18 males (62%); and in the stenosis group, the average age was 67 ± 9.59 (range 56–83) with 9 males (50%).

The LF was cut sagittally and fixed with 4% paraformaldehyde in 0.1 M PBS, pH 7.4, for 24 h. Samples were dehydrated in graded concentrations of ethanol and embedded in paraffin. Serial 5 µm coronal sections were made using a manual microtome LEICA RM-2145-2245 (Leica, Nussloch, Germany) and subjected to hematoxylin and eosin; picrosirius–hematoxylin for collagen analysis; Weigert's resorcin-fuchsin with previous oxidation for the detection and analysis of elastic fibers; and transmission electron microscopy (TEM). For histology and immunohistochemistry, three slides from each sample were used for analysis. All analyses were performed by the same investigator and were blinded to groups.

The histological sections were analyzed under a light microscope, the stained slides were analyzed, and images were acquired using the Nikon Eclipse E800® photomicroscope (Nikon, Tokyo, Japan) and Nis-Elements® image capture software (Nikon, Tokyo, Japan). This analysis was used for hematoxylin and eosin; picrosirius–hematoxylin and Weigert's resorcin-fuchsin.

Picrosirius–hematoxylin

Sections were deparaffinized and hydrated in distilled water, stained with Sirius Red, dissolved in a 0.1% saturated picric acid solution for 1 h at room temperature, rinsed with distilled water and counterstained with hematoxylin for two minutes. Sections were then dehydrated with increasing ethanol concentrations, diaphanized in xylene and mounted with Entellan (Merck, Darmstadt, Germany) [9].

Weigert's resorcin-fuchsin

All elastic system fibers were stained after the tissue was oxidized and prior to staining by Weigert's resorcin-fuchsin. Sections were deparaffinized and treated with a 10% potassium peroxymonosulfuric acid solution for 40 min [6] and stained by Weigert's resorcin-fuchsin method.

TEM

Small fragments of LF were fixed in 2.5% glutaraldehyde and dissolved in 0.1 M sodium cacodylate buffer (pH 7.2) containing 0.1% tannic acid for 2 h at room temperature, followed by post-fixation in 1% osmium tetroxide for 1 h at 4 °C, and overnight block staining in 0.5% aqueous uranyl acetate. The samples were embedded in EPON resin, thin-sectioned in a Leica-UCT ultratome, double-stained with uranyl acetate and lead citrate and examined with a LEO 906 electron microscope.

Immunohistochemistry

Slides with 3 µm sections were deparaffinized and rehydrated. The recovery of the antigen was performed by warming the slides at 100 °C for 30 min in 10 mmol/L citrate buffer, pH 6.0. Endogenous peroxidase was blocked with an aqueous solution of 3% hydrogen peroxide for 35 min. The samples were incubated overnight at 4 °C with the primary antibodies: anti-IL-6 (sc-130326) and anti-IL-10 (H-160/sc-7888) (Santa Cruz Biotechnology, USA) in dilution 1:100; anti-PGP9.5 (ab27053) and anti-CD31 (ab28364) (Abcam, Cambridge, UK); and anti-VEGF-A (18077) (Biorbyt, England). Finally, the slides were incubated with a complex of streptavidin marked with peroxidase following manufacturer's instructions (LSAB®) (DakoCytomation, Glostrup, Denmark). The sections were developed using 3,3′-diaminobenzidine and counterstained with hematoxylin. The presence of a brown color was considered positive for the respective molecules. The slides were analyzed with a TS100 Nikon Eclipse(r) light microscope to identify the areas that best represented the immune marking of the molecules analyzed. In each case, quantification of the immune labeling was quantified by a digital analysis. The photomicrographs with 640×480 pixels were obtained from consecutive non-coincident fields with 400× magnification using a 4300 Nikon Coolpix® digital camera adjusted for the same parameters. For analysis, ten different areas were chosen randomly and the evaluation was quantitative using ImageLab® (Softium Informatica®, Sao Paulo, Brazil), adjusted to a micrometric scale (µm) [10]. Digital quantification was expressed by the intensity of digital immune staining (ItE) for each sample [10].

Statistical analysis

Statistical analysis was performed using the Prisma5® software (GraphPad Software, La Jolla, CA, USA). All variables were considered nonparametric through the Kolmogorov–Smirnov test. Kruskal–Wallis test with the auxiliary test Dunn's was applied to associations between all the groups, and Mann–Whitney test was used to analyze the associations between two groups. Results were compared and described in means and standard error (mean ± SE). All data were analyzed using the SPSS® software (Statistical Package for Social Sciences; SPSS, Chicago, IL, USA), version 17.0. In all analyses, we adopted the significance level $P < 0.05$.

Results

In the disk herniation group, the LF thickness mean was 1.68 ± 0.46 mm, while in the stenosis group, the thickness mean was 4.25 ± 0.92 mm. The distribution of levels is shown in Table 1.

Histological results from both groups show that the deep portion of the LF has a large number of fibrocytes. The collagen fibers are very thick and compacted. A large number of mature elastic fibers are observed following the collagen fibers (Fig. 1b, c). The superficial portions of the LF of the herniated group also present a large number of fibrocytes and a large number of thick, compact collagen fibers (Fig. 1d, e).

The analysis of the elastic fibers of the LF of both groups using resorcin histochemistry observed mature and oxytalan fibers in the superficial portion (Fig. 2f), while TEM allowed us to observe mature elastic fibers in the LF of stenosis patients and an irregular elastin core structure with light regions at the edges, similar to vacuoles, suggesting degradation in both regions (Fig. 3c, d).

Morphological results show that the deep portion of LF in patients with disk herniation presents a smaller proportion of cells when compared to the stenosis group. The collagen fibers are thick, compact and oriented in several directions. Mature elastic fibers are seen in abundance (Fig. 2a–c).

In the LF of all patients with stenosis, the changes occurred in the deep portion of the ligament, which presents a great number of cells, when compared to the herniated group, and a monocular infiltrate in some areas (Fig. 2d).

Yet, in the stenosis group, the histochemistry shows collagen fibers that are thinner and less compact than the

Table 1 Clinical features

	Disk herniation	Spinal stenosis
N	29	18
Age (years)	40 ± 11.5	67 ± 9.59
BMI (kg/m^2)	26.3 ± 3.47	26.8 ± 3.73
Ligamentum flavum thickness (mm)	1.68 ± 0.46	4.25 ± 0.92
Spine level		
L3–L4	4	3
L4–L5	14	15
L5–S1	11	0

BMI body mass index

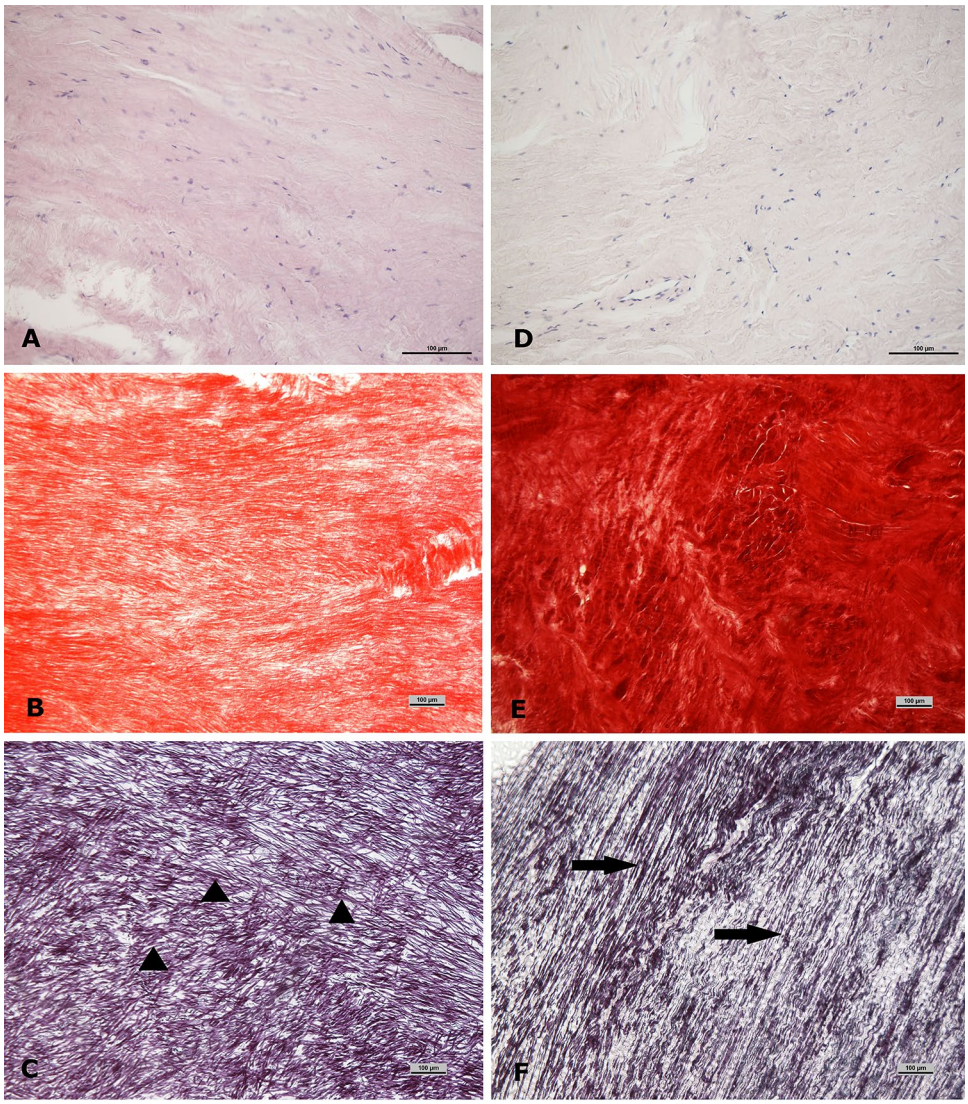

Fig. 1 Photomicrographs of the deep layer of ligamentum flavum (**a**–**c**) and the superficial layer of ligamentum flavum (**d**–**f**) of lumbar disk herniation patients (normal ligaments). In **a** and **d**, sections stained with hematoxylin and eosin. In **b** and **e**, sections stained with picrosirius–hematoxylin where the collagen fibers are red. In **c** and **f**, Weigert's resorcin-fuchsin stained with previous treatment with oxone, where the mature elastic fibers are identified in purple (arrowhead) and oxytalan fibers are identified as black fibrils (arrow). Magnification: 200× (**a** and **d**) and 100× (**b**, **c**, **e**, **f**)

herniation group (Fig. 2e). Mature elastic fibers are present in large quantities (Fig. 2f).

In the superficial layer of stenotic patients, mature elastic fibers and a large number of oxytalan fibers were observed distributed throughout this portion. Under light microscopy, no difference was found between the results of qualitative analysis of collagen and elastic fibers in the ligament of both groups (Fig. 2). However, samples from patients with stenosis show the presence of a mononuclear infiltrate indicative of chronic inflammation (Fig. 2d).

The elastic fibers of patients with disk herniation present mature elastic fibers with normal structure presenting an elastin core surrounded by microfibrils (Fig. 3a, b). The elastic fibers of patients with stenosis, in turn, present an elastin core with irregular borders, with clear spaces suggesting corrosion and degradation of the edges of the elastin core (Fig. 3c, d). These changes were observed in both the superficial and deep regions. Under TEM the elastic, oxytalan and collagen fibers are clearly visible (Fig. 4).

To investigate changes of inflammatory cytokines in the LF of patients with stenosis compared to patients with disk herniation, interleukin profiles were analyzed. No difference was observed between the groups (Fig. 7). Furthermore, no alterations were observed when both superficial and deep ligamentum were analyzed.

VEGF is one of the most important proangiogenic factors, which stimulates vasculogenesis and angiogenesis. However, the level of VEGF was not altered in the LF of patients with stenosis, as seen in Fig. 8.

CD31 is an integral membrane glycoprotein that might be a useful marker for total blood vessels. Figure 5 shows a significant increase in the deep LF of stenosis patients compared to the deep LF of disk herniation patients.

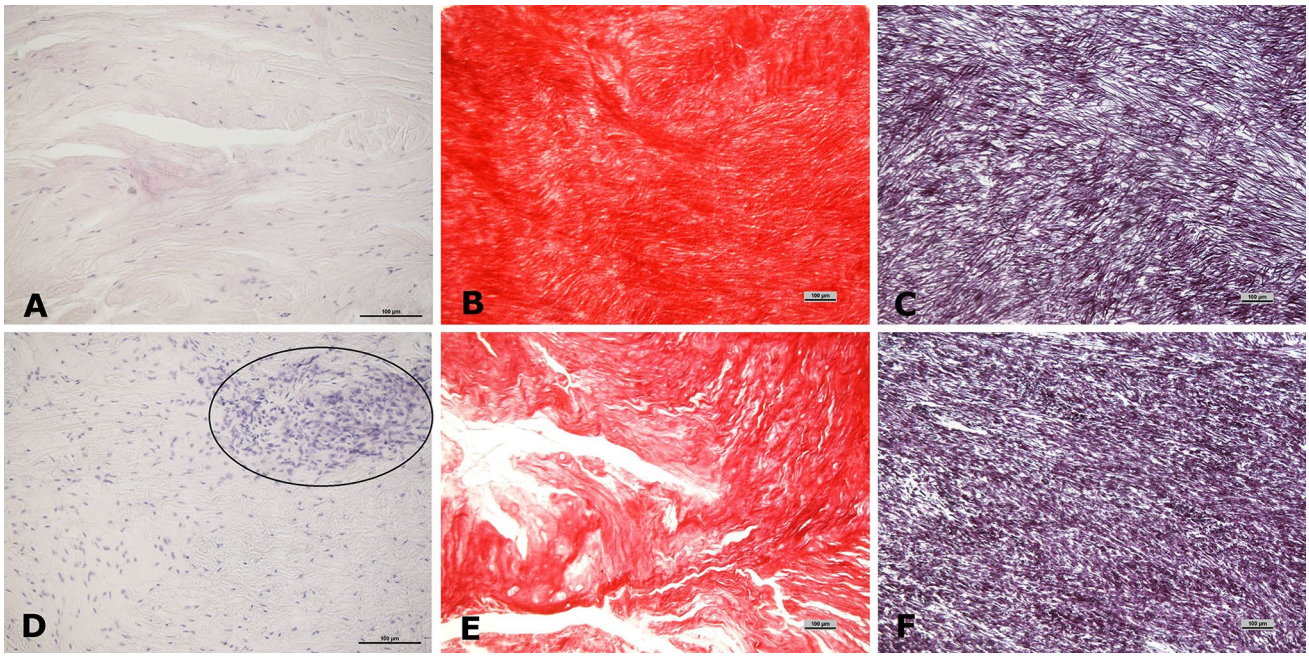

Fig. 2 Photomicrographs of ligamentum flavum of patients with disk herniation (control)—(**a**–**c**) and patients with stenosis (**d**–**f**). In **a** and **d**, sections stained with hematoxylin and eosin. In **d**, note the mononuclear infiltrate in the delimited area. In **b** and **e**, sections stained with picrosirius–hematoxylin where the collagen fibers are red. In **c** and **f**, sections stained with Weigert's resorcin-fuchsin with oxone where mature elastic fibers are identified in purple. Magnification: 200× (**a** and **d**) and 100× (**b**, **c**, **e**, **f**)

It is very important to emphasize that it was crucial to divide the LF into two groups (superficial and deep) to show the difference in the CD31 expression between stenosis and herniation tissues, since no change had been observed when total LF was analyzed (Fig. 9).

The PGP9.5 antibody reacts with human neuroendocrine marker. Expression of PGP9.5 is highly specific to neurons and to cells of the diffuse neuroendocrine system and their tumors. In order to determine whether there is an increase in the expression of PGP9.5 as a function of pain nociceptive mechanisms related to the pathophysiology of stenosis and herniation, the PGP9.5 antibody was used. Figure 6 shows no significant difference in PGP9.5 expression.

Although no significant statistical difference could be detected, it was possible to see in the samples from herniated group that there were no alterations on the number of vessels and nerves, as well as there was no degradation of elastic fibers, while in the samples collected from patients with stenosis there were a higher number of vessels and nerves and additionally the samples had significantly degradation of elastic fibers.

Discussion

We analyzed the two leaflets of LF from individuals with and without disease in the ligament separately. Samples with ligament disease were an average of 2.5 × thicker than the normal ligament. Components of the collagen and elastic system focusing on oxytalan, elaunin and mature elastic fibers were analyzed. The presence of interleukins, vessels and nerves was also investigated through immunohistochemistry. In the superficial layer of the ligament of herniated patients (normal ligament), we found a large amount of thick, compact collagen fibers, a significant amount of oxytalan fibers and mature elastic fibers, while in the deep layer, only a large amount of mature elastic fibers was observed. In the stenosis group, we observed collagen that was thinner and compacted in both layers of the ligament, with the deep portion of the ligament presenting a monocular infiltrate in some areas. However, there was no difference in the interleukin profile among the groups. In the deep portion of LF, a higher number of vessels and nerves were observed in the stenosis group.

The functions of LF have been debated over the years. It was considered the only ligament of the posterior spine that had any restrictive effect and that this provided the vertebral canal a smooth dorsal covering in all positions of the spine [11]. However, it has been demonstrated that LF accounts for a portion of the disk pressure and that its elasticity prevents it from protruding into the spinal canal [12].

Studies in rats demonstrate that elasticity depends on the concentration of the mature and elaunin elastic fibers [13]. Bearing in mind that there are differences between rodents and humans, but at the same time observing that we analyze

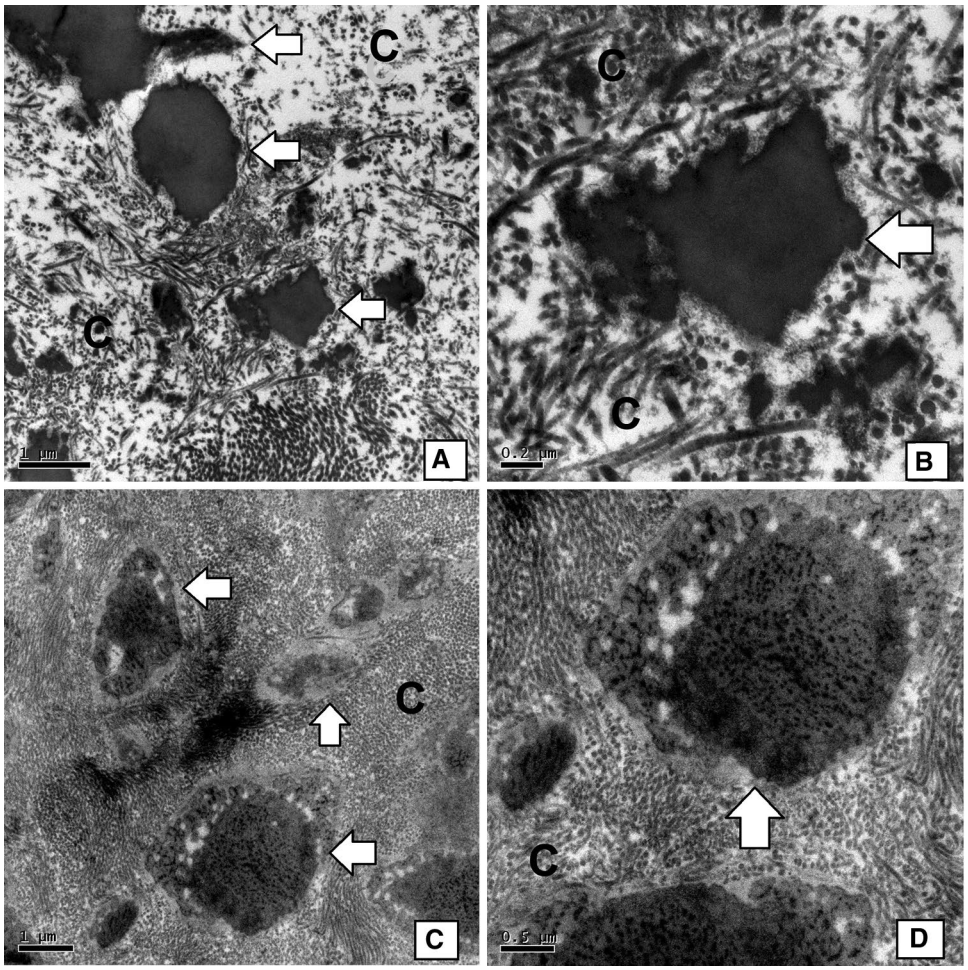

Fig. 3 Transmission electron microscopy images of elastic and collagen fibers of the ligamentum flavum of patients with disk herniation (**a**, **b**) and patients with stenosis (**c**, **d**) stained with tanic acid. In **a** and **b**, elastic fibers of a disk herniation patient presenting normal morphological appearance. In **c** and **d**, elastic fibers of a patient with stenosis, presenting changes in the elastin core. Electron dense elastic fibers (arrow); collagen fibers (C) Magnification: 20,000× (**a**, **c**); 60,000× (**b**); 50,000× (**d**)

different tissues that are subject to distinct biomechanical forces, we observed that the deep layer of the LF has a higher concentration of these fibers than the superficial layer, suggesting that this small portion of the ligament is mainly responsible for the elastic properties of the LF. While the tissue compression resistance is dependent upon the concentration of oxytalan fibers [13], we demonstrated that the superficial or dorsal layer of the LF has the highest concentration of oxytalan fibers.

Consequently, the highest concentration of oxytalan fibers may be related to the increase in compression strength of the superficial LF, since oxytalan fibers support more compression than elastic and elaulinic fibers.

Reports vary since around 60% of the patients with lumbar disk herniation have a thick LF and around 59% or them exhibit marked scarring with fibrous connective tissue [14], while other reports show slight fibrotic changes with aging but without alterations with disk herniation [15]. Although in this study we have used LF from patients with lumbar disk herniation as controls, the LF of all these samples has normal thickness and was considered normal ligaments. Sample size was a limitation of this study, and additional studies must be done comparing LF from cervical, thoracic and even upper levels of the lumbar spine. Another limitation is the use of LF from lumbar disk herniation patients instead of cadavers or trauma patients without degenerative disease.

An important point that must be emphasized is that the LF from patients with lumbar spinal canal stenosis has irregular elastin core edges with vacuoles, suggesting degradation, which may impact elasticity and could explain why the LF bulge into the spinal canal in the standing position even if normal in thickness [16].

Despite stenosis, it is well known that intervertebral disk herniation pain is a result of spinal nerve inflammation and swelling caused by the pressure of the herniated disk. The stenosis process narrows the spinal canal (central stenosis) and presses on the spinal cord and can also cause inflammation and weakness. The increased thickness of the LF is a common feature of patients with spinal stenosis, while it is not a characteristic of patients with herniation.

The role of inflammatory mediators in the pathophysiology of herniation and stenosis is still controversial. Several findings suggest that the inflammatory response occurs in the early stage of disk herniation and stenosis, but the

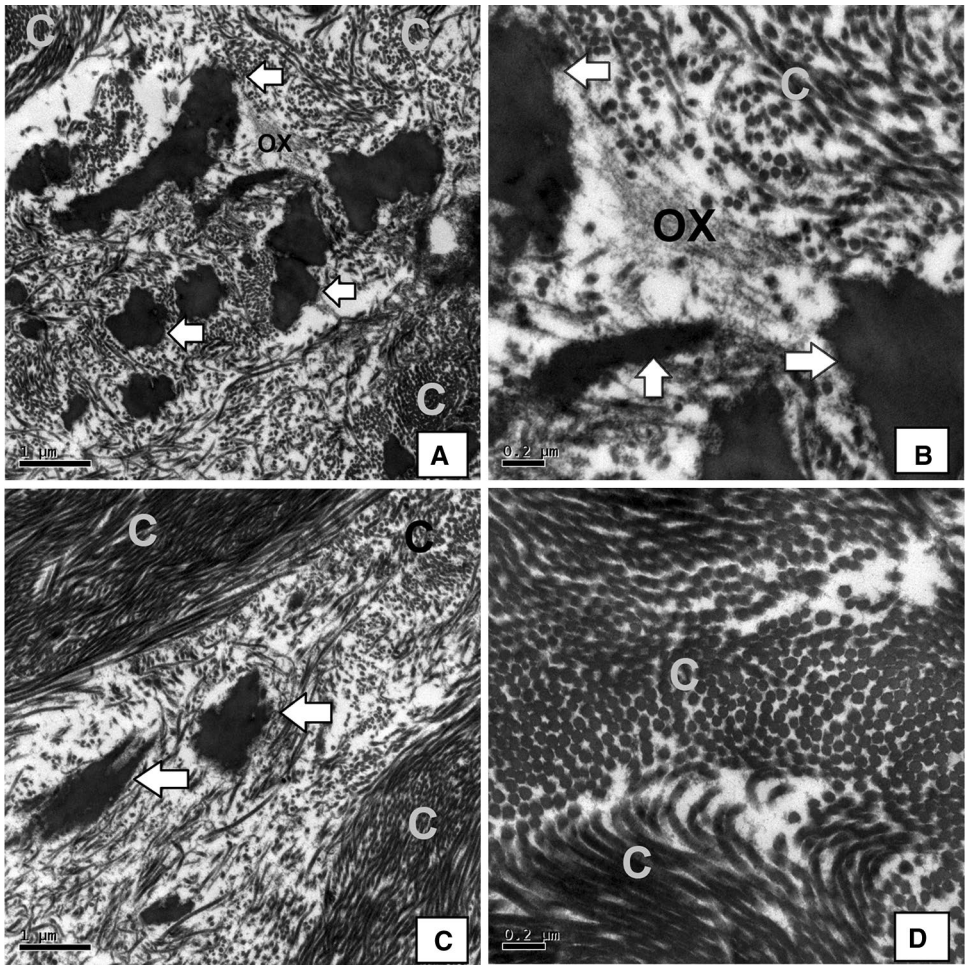

Fig. 4 Transmission electron microscopy images of elastic and collagen fibers of the superficial portion of the ligamentum flavum of patients with disk herniation stained with tanic acid. Electron dense elastic fibers (arrow); collagen fibers (C), microfibrils (OX). Magnification: 20,000× (**a**, **c**); 60,000× (**b**, **d**)

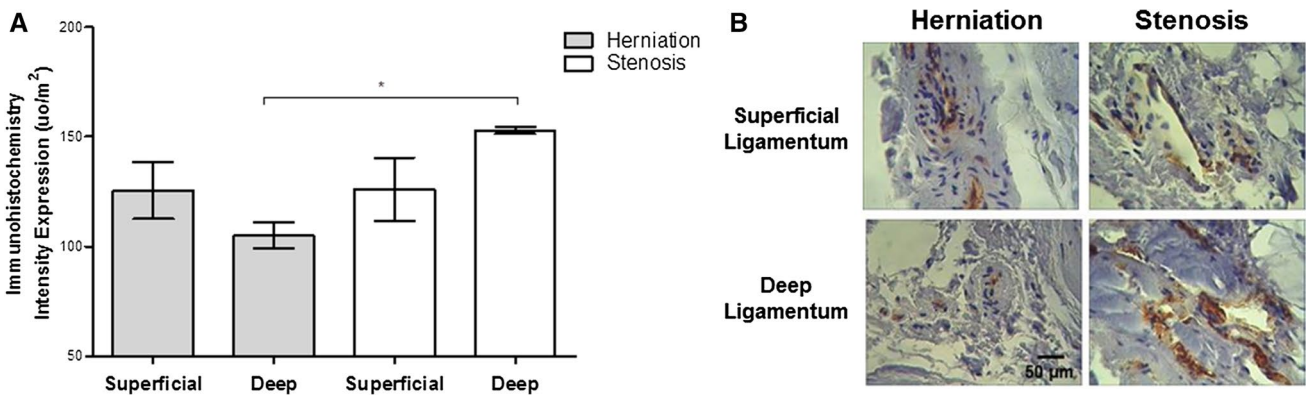

Fig. 5 Immunohistochemistry quantification of CD31 in flavum ligamentum tissues. The results represent the intensity of expression of CD31. **a** Digital quantification from tissues of the ligamentum flavum obtained after surgery in patients with herniated disk (gray bars) or with stenosis (white bars) and **b** images from immunohistochemistry reaction in patients with herniated disk (left) or with stenosis (right). In both groups, tissues were removed from the superficial and deep region of the ligamentum flavum. The values represent the mean and standard error of the intensity of expression (ItE) obtained as described in methods. The lines indicate mean and standard error value of CD31 expression in each group ($P^* = 0.0167$; nonparametric Mann–Whitney test)

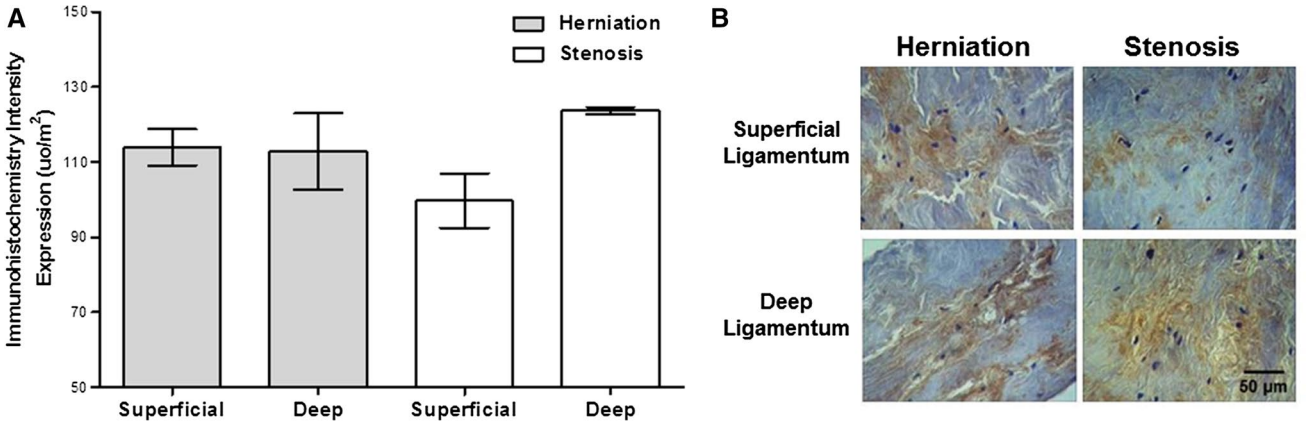

Fig. 6 Immunohistochemistry quantification of PGP9.5 in flavum ligamentum tissues. The results represent the intensity of expression of PGP9.5. **a** Digital quantification from tissues of the flavum ligamentum obtained after surgery in patients with herniated disk (gray bars) or with stenosis (white bars) and **b** images from immunohistochemistry reaction in patients with herniated disk (left) or with stenosis (right). In both groups, tissues were removed from the superficial and deep region of the flavum ligament. The values represent the mean and standard error of the intensity of expression (ItE) obtained as described in methods. The lines indicate mean and standard error value of PGP9.5 expression in each group ($P=0.5962$; nonparametric Kruskal–Wallis test)

increase in inflammatory cytokines is transient. Indeed, most studies failed to demonstrate inflammatory cytokines increased during chronic disk herniation samples or stenosis. Our results corroborate such data, since Interleukin-6 (IL-6), a pro-inflammatory cytokine, as well as Interleukin-10 (IL-10), an anti-inflammatory cytokine, was not changed in the yellow ligament when patients with intervertebral disk herniation and stenosis were compared.

In this study, a few potential sample errors inherent to the nature of the work must be remembered such as the age of patients that may interfere with elastic fibers and collagen features, as well as the fact that the ligaments have been removed from different levels of the lumbar spine. Although most came from the L4–L5 level (Table 1), it was not assessed whether there was a difference between lumbar levels.

In this study, we also did not evaluate which type of innervation is present in the LF, but it was possible to demonstrate an increase in vessels and nerves in the deep region of LF from patients with stenosis, suggesting that LF could have some function in the proprioception of the spine. Therefore, LF may present some function in the physiopathology of the stenosis beyond the compression caused by the thickening that occurs in the development of the disease. In summary, according to the results of the present study, the two layers of normal LF are different in composition, the deep portion being mainly responsible for its elasticity. LF in the stenosis samples presents more mononuclear infiltrate and more degraded elastic fibers with a higher number of vessels in its deep portion.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interest.

Appendix

See Figs. 7, 8 and 9.

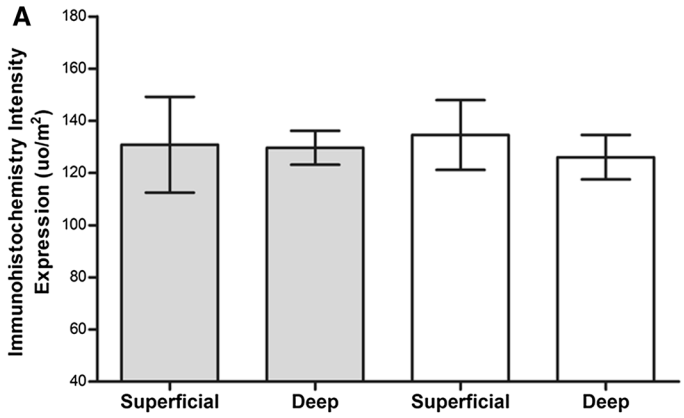

Fig. 7 Digital quantification of IL-6 and IL-10 in flavum ligamentum tissues. The results represent the intensity of expression of interleukins −6 (**a**) and −10 (**b**) in tissues of the ligamentum flavum obtained after surgery in patients with herniated disk (gray bars) or with stenosis (white bars). In both groups, tissues were removed from the superficial and deep region of the flavum ligament. The values represent the mean and standard error of the intensity of expression (ItE) obtained as described in methods. The statistical analysis was performed using Kruskal–Wallis test with the auxiliary test Dunn's to associations between all the groups and Mann–Whitney test to analyze associations between two groups. Results described in means and standard error (IL-6, $P=0.9225$ and IL-10, $P=0.6407$)

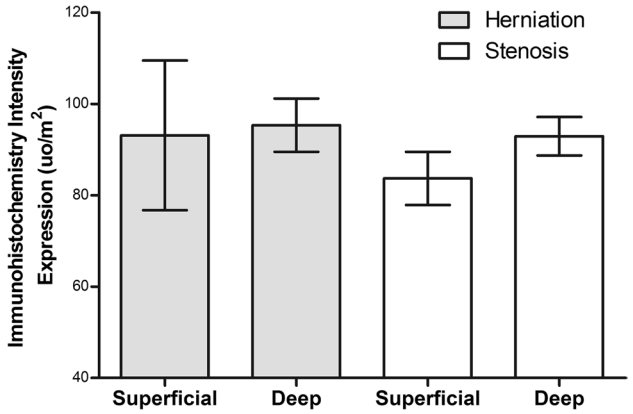

Fig. 8 Digital quantification of VEGF-A in flavum ligamentum tissues. The results represent the intensity of expression of VEGF-A in tissues of the ligamentum flavum obtained after surgery in patients with herniated disk (gray bars) or with stenosis (white bars). In both groups, tissues were removed from the superficial and deep region of the flavum ligament. The values represent the mean and standard error of the intensity of expression (ItE) obtained as described in methods. The statistical analysis was performed using Kruskal–Wallis test with the auxiliary test Dunn's to associations between all the groups and Mann–Whitney test to analyze associations between two groups. Results described in means and standard error ($P=0.7277$)

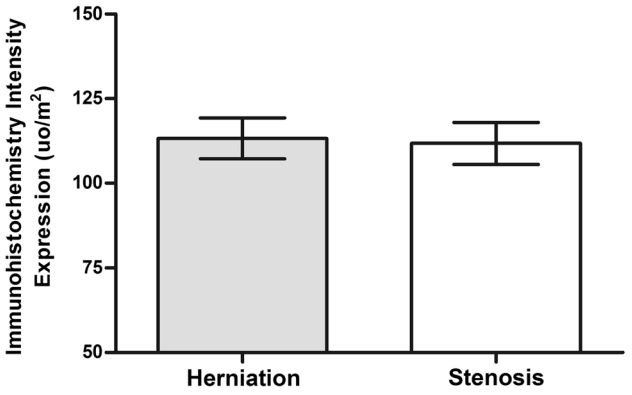

Fig. 9 Immunohistochemistry quantification of CD31 in flavum ligamentum tissues. The results represent the intensity of expression of CD31 in patients with herniated disk (gray bars) or with stenosis (white bars) without differentiating them into superficial and deep portions. The values represent the mean and standard error of the intensity of expression (ItE) obtained as described in methods. The lines indicate mean and standard error value of CD31 ($P=0.7431$; nonparametric Mann–Whitney test)

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