Study of the human ligamentum flavum in old age: a histological and morphometric study

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Background: Ageing is associated with many changes in the ligamentum flavum (LF): ossification, diminish of the cross-sectional area of the elastic fibres, increase of the collagen fibres and change in its mineral and matrix elements. There are limited data about the influence of the ageing process on the structure of the LF at different spinal levels. The aim of the study was to clarify the effect of the ageing process on the LF at different spinal levels. This was done through histological study and morphometric analysis using the image analyser.

Materials and methods: Vertebral column specimens were obtained from 10 human cadavers preserved in a mixture of formaldehyde and carbol. Their average age ranged 60–70 years. The vertebral blocks included 4 vertebrae at cervical level (C3, 4, 5, 6), thoracic level (T5, 6, 7, 8) and lumbar level (L2, 3, 4, 5). Sections were stained with haematoxylin and eosin, Masson’s trichrome, Orcein and Verhoeff stains. The morphometric measurements included the relative collagen area, relative elastic area, collagen area [%] and elastic area [%].

Results: The relative elastic areas reduced in all the spinal levels. The relative collagen areas increased to become 46.1 ± 2.9 in the cervical region, 51.8 ± 1.3 in the thoracic region, and 49.7 ± 2.5 in the lumbar region. The average elastic area was highest in the cervical region, and lowest in the thoracic region. The ratio of elastic area to collagen area was 1.16:1 in the cervical region, 1.01:1 in the lumbar region and 0.93:1 in the thoracic region. The elastic fibres were regular, diffuse and organised in parallel order in the cervical and thoracic regions. Their orientations were craniocaudal with a variation of some fibres in the cervical region. Most of the elastic fibres in the lumbar region were regular and organised in parallel order. Nevertheless, rupture, fragmentation, and abnormal organisation of the elastic fibres were discovered in a few specimens of the lumbar region. Increase of the vasculature and degeneration with abnormal body formations within the lumbar ligaments were observed. The LF midline gaps were present in the cervical, thoracic and lumbar regions. In 10% of lumbar specimens, the ligaments fused in the midline with absence of the midline gaps. Ossification was discovered in the thoracic and lumbar ligaments.

Conclusions: Structural differences have been observed among the LF at the different spinal levels, and all these changes were caused by the ageing process. Decrease of the relative elastic area, an increase of the relative collagen area and reduction of the elastic to collagen area ratio affected all the spinal levels of the ligaments. Many changes took place in the lumbar ligaments such as ossification, increase vasculature, degeneration with abnormal body formation, absence of the midline gaps, fragmentation and rupture of the elastic fibres. (Folia Morphol 2014; 73, 4: 492–499)

Key words: old age, human ligamentum flavum

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INTRODUCTION

The ligamentum flavum (LF) is found between spinal vertebral laminae, forming a supportive tissue in the posterior wall of the spinal canal [10]. The structural differences in the LF at different spinal levels have been analysed by light microscopy in human and monkey [10], and by electron microscopy in human and rabbit [10, 21]. In addition, the structural differences in the ligamenta flava have been investigated among some mammals [15].

Ageing is associated with many changes in the LF. Ossification was documented as part of age-related changes [2, 12]. In addition, diminish of the cross-sectional area and visco-elasticity of the elastic fibres of the ligaments was also documented [11]. Change in the mineral and matrix components (especially elastin and collagen) in the lumbar LF was reported in the Japanese population [12]. Up to our knowledge, there are limited data about the influence of the ageing process on the structure of the LF at different spinal levels.

It was hypothesised that ageing process has structural effects on the LF and these affections vary from one spinal level to another. Therefore, the aim of the study was to clarify the effect of the ageing process on the LF at different spinal levels. This was done through histological study and morphometric analysis using the image analyser.

MATERIALS AND METHODS

Vertebral column specimens were obtained from 10 human cadavers preserved in a mixture of formaldehyde and carbol [8]. Their mean ages ranged 60–70 years. Cutaneous flaps were removed, and then the different muscular planes as well as the different anatomical parts were identified. Once the muscular planes were exposed and removed, the dorsal vertebral arches and parts were identified. Once the muscular planes were as well as the different anatomical parts were identified. Once the muscular planes were exposed and removed, the dorsal vertebral arches and the ligamentum flavum were exposed. Vertebral blocks including 4 vertebrae at cervical level (C_3, 4, 5, 6), thoracic level (T_5, 6, 7, 8) and lumbar level (L_2, 3, 4, 5) were removed. Vertebral arches were detached at the pedicles and removed en bloc. For each specimen, the right and left LF were removed. Decalcification of blocks in 5% formic acid was done before embedding in paraffin [13].

Sections were stained with haematoxylin and eosin (H & E), Masson’s trichrome, Orcein and Verhoeff stains. Masson’s trichrome stain was used for detection of the collagen fibres which were stained green. The pink colorations within the ligaments indicated normal non-fibrotic areas [13]. Orcein stain was used for detection of the elastic fibres which were identified as red fibres [18]. Verhoeff stain was used for detection of elastic and collagen fibres, the elastic fibres were identified as black fibres while the collagen fibres were identified as red fibres [18]. At least 5 sections were prepared from each specimen. The sections were separated by a given distance 20 µm to obtain roughly random choice of sections for morphometric measurements. The prepared sections were examined and photographed using a light microscope (Olympus BX53, Tokyo, Japan) with an attached camera (Olympus E-330, Olympus Co.).

Morphometric study

The sections were examined using Leica Qwin 500 image analysis software on IBM operated computer system (Leica Microsystems, Wetzlar, Germany). Five preparations from each spinal level were subjective to quantitative and semi-quantitative surveys:

- The relative collagen area was measured within a standard measuring frame of a known area in the Masson’s stained sections. The collagen fibres were selected and masked by red binary colours. Then the area of the red binary colour was assessed in relation to the area of the standard measuring frame.
- The relative elastic area was evaluated by the same way mentioned above. It was measured within a standard measuring frame of a known area in the Orcein stained sections. The elastic fibres were selected and masked by red binary colours. Then the area of the red binary colour was measured in relation to the area of the standard measuring frame.
- Collagen area [%] = relative collagen area/relative elastic area × 100%.
- Elastic area [%] = relative elastic area/relative collagen area × 100%.

The data were collected and examined using analysis of variance (one way ANOVA) followed by post hoc Bonferroni test to compare the relative collagen area and relative elastic area of the ligamenta flava among the different spinal levels using SPSS v. 20 statistical program. The data were examined by Kolmogrov-Smirnov test for normality. The level of significance was considered at p-value < 0.05.

RESULTS

Gross anatomy of the LF at different spinal levels

The ligamenta flava at different spinal levels were demonstrated with their attachments to the laminae of the vertebrae. The LF midline gaps were present in the cervical, thoracic and lumbar regions (Fig. 1A–C).
In 10% of the lumbar specimens the LF midline gaps were observed at higher levels, while at lower levels fusion with absence of the midline gaps were observed (Fig. 1D). Areas of ossification near the distal attachment of the ligaments were observed in 20% of the thoracic ligaments (Fig. 1B). In another 2 (20%) lumbar specimens, areas of ossification, mostly near the proximal attachment of the ligaments and near their middle, were demonstrated; the ossification areas were numerous so that they gave the ligaments irregularity in their appearance (Fig. 1D).

**Histological structure of the LF at different spinal levels as revealed by Masson’s trichrome and H & E stains**

The Masson’s trichrome stained sections showed the arrangement of collagen fibres within the LF at different spinal levels. The collagen fibres were regular and organised in parallel order at different spinal levels (Fig. 2A–F). The stained areas with pink colour indicated normal, non-fibrotic areas (Fig. 2B–F). Ruptured collagen fibres, areas of completely lost collagen fibres, areas with abnormally arranged collagen fibres, and areas with relatively regularly arranged collagen fibres were seen in 1 (10%) specimen in the lumbar region (Fig. 3A).

Finally, ossification of the lumbar LF was established in the H & E stained sections (Fig. 3B, C).

**Histological structure of the LF at different spinal levels as revealed by Orcein and Verhoeff stains**

The Orcin-stained sections demonstrated the order of arrangement of elastic fibres within the LF. The elastic fibres were regular, diffuse and organised in parallel order in the cervical and thoracic regions (Fig. 4A, B). Their orientation was craniocaudal with a variation of some fibres in the cervical region (Fig. 4A, B). The elastic fibres were regular and organised in parallel order in the most of the lumbar specimens (Fig. 4C). However, rupture of the elastic fibres was observed in 3 (30%) specimens of the lumbar region (Fig. 4D–F). In addition, the elastic fibres were fragmented and disorganised in the lumbar region (Fig. 4D–G). Increase of the vasculature and degeneration with abnormal body formation within the lumbar ligaments were found in one specimen for each (Fig. 4F, G).

The Verhoeff stained sections demonstrated the order of arrangement of elastic fibres (black fibres) and collagen fibres (pink fibres). Most of the collagen fibres were masked by the black appearance of the elastic fibres. The elastic fibres in the cervical and thoracic regions were regular and organised in parallel order (Fig. 5A, B). Most of the specimens in the lumbar level displayed regular and dense arrangement of elastic fibres (Fig. 5C, D). However, the fibres were fragmented with no parallel order in many specimens (Fig. 5E). Rupture of the elastic fibres, increase the vasculature and ossification of the lumbar ligaments were observed in the lumbar ligaments (Fig. 5D, E).

**Morphometric evaluation of the elastic and collagen fibres**

Structural differences between the LF at different spinal level were observed in the old age. The relative elastic area in the cervical region was 20% more than that of the thoracic region (p = 0.000) and 4% more
Figure 2. Representative histological sections of the ligamentum flavum (LF) stained with Masson's trichrome stain. The collagen fibres (arrows) were regular and organised in parallel order at different spinal levels. The areas stained pink colour (arrowheads) indicated normal, non-fibrotic areas; A. Cervical LF (×40); B. Cervical LF (×100); C. Thoracic LF (×40); D. Lumbar LF (×40); E, F. Lumbar LF (×100).

Figure 3. Representative histological sections of the lumbar ligamentum flavum (LF); A. Ruptured collagen fibres (encircle) within normal non fibrotic areas (arrows). Areas of completely lost collagen fibres (*), areas with abnormally arranged collagen fibres (rectangle), and areas with relatively regularly arranged collagen fibres (arrowheads) were seen (Masson's trichrome ×40); B. Ossified area (OLF) within the LF (H & E ×100); C. Ossified area (OA) within the LF (H & E ×400).
Figure 4. Representative histological sections of the ligamentum flavum (LF) stained with Orcein stain showing the order of arrangement of elastic fibres: A. Elastic fibres were regular, diffuse and organised in parallel order in the cervical region. The orientation of most elastic fibres (arrows) was craniocaudal with variation of some fibres (arrowheads) (×40); B. Elastic fibres were regular, diffuse and organised in parallel order in the thoracic region. The orientation of the fibres was craniocaudal (arrows) (×40); C. Elastic fibres were regular and organised in parallel order (arrows) in the lumbar region (×100); D. Rupture of the elastic fibres (encircle) in the lumbar region; the fibres were fragmented and disorganised (×40); E. Some elastic fibres in the lumbar region were ruptured (encircles), some fibres were regular and organised in craniocaudal organisation (arrows); other fibres were disorderly arranged (×40); F. The elastic fibres were ruptured (encircle), and disorderly arranged (arrows) in the lumbar LF. Note the presence of multiple blood vessels (V) within the ligament (×40); G. Some elastic fibres in the lumbar region were regular and organised in craniocaudal organisation (arrows); other fibres were fragmented and disorderly arranged (arrowheads). Degeneration of the elastic fibres with abnormal body formations (encircles) had also been observed.

Figure 5. Representative histological sections of the ligamentum flavum (LF) stained with Verhoeff stain showing the order of arrangement of elastic fibres (black fibres) and collagen fibres (pink fibres): A. Elastic fibres in the cervical region were regular and organised in parallel order (arrows) (×100); B. Elastic fibres in the thoracic region were regular and organised in parallel order (arrows) (×40); C. Elastic fibres were regular and displayed a dense arrangement (arrows) (×100); D. Elastic fibres were regular and displayed a dense arrangement (arrows) in the lumbar region. Rupture of the elastic fibres (*) and presence of areas of ossification (arrowheads) had also been observed (×100); E. The elastic fibres were fragmented with no parallel order (arrows) in the lumbar LF. Note rupture of the elastic fibres (arrowheads), and presence of multiple blood vessels (V) within the ligament (×40).
than that of the lumbar region \((p = 0.8)\); moreover, the relative elastic area in the lumbar region was 15\% more than that of the thoracic region \((p = 0.000\); Tables 1, 2).

The relative collagen area in the cervical region was 3\% less than that of the thoracic region \((p = 0.8)\) and 9\% less than that of the lumbar region \((p = 0.01)\); moreover, the relative collagen area was 6\% less in the thoracic region than that of the lumbar region \((p = 0.1)\) (Tables 1, 2).

In the cervical region, the elastic area \[%\] was higher (53.9\%) than the collagen area \[%\] (46.1\%) and the ratio of elastic to collagen fibres was 1.16:1. In the thoracic region, the elastic area \[%\] (48.2\%) was lower than the collagen area \[%\] (51.8\%) and the ratio of elastic to collagen fibres was 0.93:1. In the lumbar region, the elastic area \[%\] and the collagen area \[%\] were nearly the same (50.3\% and 49.7\%, respectively) and the ratio of elastic to collagen fibres was 1.01:1 (Table 1).

**DISCUSSION**

The LF is the most elastic tissue in the human body. It is rich in elastic fibres compared with other spinal ligaments [9]. The elastic fibres account for 60\% to 70\% of the normal ligament dry weight [12] and they may reach up to 80\% of the ligament dry weight at young age [21]. They provide elasticity and give the ligament a high compliance and elastic recoil [13]. In the current study, the elastic area \[%\] of the old age ligaments reduced in all the spinal levels, it was 53.9\% in the cervical region, 48.2\% in the thoracic region, and 50.3\% in the lumbar region. The diminution of the elastic area \[%\] in old age is a part of the age-related changes [22] and reflects the pathologic process of degeneration that affects the LF [18, 23].

The difference of the average elastic area of the ligaments among the different spinal levels was further noticed. The average elastic area was highest in the cervical region, and lowest in the thoracic region.
Contrary to our findings, Nihei et al. [10] reported more elastic fibres in the upper thoracic region compared with that of the cervical region, with greater dominance of elastic fibres in the lower spinal levels, but their samples were obtained from rabbits. The ligamenta flava of the thoracolumbar spine of mammals with great spinal mobility, like rabbits, have a larger content of elastin than in animals with little spinal motion [15].

The elastic fibres were regular, diffuse and organised in parallel order in the cervical and thoracic regions. Their orientations were craniocaudal with a variation of some fibres in the cervical region. This diversity in fibre orientation could be due to the need for a larger degree of forward and backward curvature and rotation in the cervical region [10]. In the lumbar specimens, the elastic fibres were fragmented and disorderly arranged which might be imputed to the pathologic process of degeneration of the ligament caused by the ageing process [16, 18]. Further degeneration was presented in the lumbar specimens by rupture of the elastic fibres. The elastic fibre bundles showed gradual fragmentation and extinction, probably because of the action of proteases such as elastase and chymotrypsin [1]. As these age-related degenerative changes progress, the LF tissues become more disorganised, which may contribute to the development of spinal stenosis [13].

Increase of the vasculature within the ligament of the lumbar region was observed in many specimens. The normal ligament is highly elastic, based on the large proportion of elastic fibres with less blood flow [13]. So increase vasculature of the ligament accompanied with the reduced elastic fibres [%] contributes to the loss of elasticity within the ligament.

The collagen fibres contents in the LF are about 20% at young age [21]; they provide stiffness and stability to the ligaments [13]. In this study the collagen area [%] increased to become 46.1% in the cervical region, 51.8% in the thoracic region, and 49.7% in the lumbar region. Increase of the collagen fibres is one of the age-related degenerative changes [16, 18]. The collagen has very high tensile strength and is less likely to stretch [10] and its dominance may affect the range of motion of vertebral column and reduce the elasticity of the LF.

Ordinarily, the elastin and collagen fibre ratio is 4:1 at young age [21] and may reach up to 2:1 [13]. In the current study these ratio deceased to become 1.16:1 in the cervical region and 1.01:1 in the lumbar region. In the thoracic region, the ratio was inverted (0.93:1). The decrease of the ratio mostly attributed to the aging process and resulted in decreased elasticity and increased stiffness or fibrosis [13].

In this study, presence of the LF midline gaps in the cervical, thoracic and lumbar regions was observed. In addition, it was noticed in 10% of the lumbar ligaments in this study that some levels featured midline gaps, whereas at lower levels the ligaments were fused in the midline with absence of gaps. These gaps are intervals for the passage of veins connecting the posterior external vertebral venous plexus with the posterior internal vertebral venous plexus [6]. The exact incidence of mid-line gaps has been controversially discussed. Panjabi et al. [14] dissected 6 cervical columns and found the ligaments to be continuous in the mid-line in all instances. Hogan [4] conducted his study on 28 cadavers and found the incidence of midline gaps in the cervical region is about 50%. Zarzur [24] and Olszewski et al. [11] dissected 10 and 6 lumbar vertebral columns, respectively, and found no evidence of LF midline gaps. Others found that LF midline fusion could be absent “to a variable degree”, no exact incidences were given [3]. Recent investigations at cervical, high thoracic and lumbar levels have confirmed failure of fusion of the ligamenta flava in the midline may reach up to 74% [6]. The loss-of-resistance technique is most commonly used to identify the epidural space in the lower thoracic and lumbar region. This technique depends upon the resistance to injection of saline and needle advancement offered by spinal ligaments, which abruptly decreases once the tip of the needle has come through the LF, and reached the epidural space [8]. One of the most important problems encountered when administering epidural anaesthesia is failure to identify the epidural space. The LF midline gaps could be responsible for the failure to recognise the loss of resistance in some patients [7].

One of the most significant findings in the lumbar region was the presence of ossification of the LF. Ossification was discovered in the thoracic and lumbar ligaments, but no ossification was discovered in the cervical region. The difference in incidence at different spinal levels suggests the existence of local factors acting with systemic factors; however, the cause of this condition has not been fully elucidated [10]. Ossification of the LF compresses the spinal cord inducing various symptoms [10]; it is often presented as thoracic spinal stenosis [17] and the main cause of thoracic myelopathy in Asia [20]. The incidence...
of ossified LF is relatively high and can be detected radiographically in 25% of adults [10]; its prevalence is highest at the age of 50–59 years [5].

**CONCLUSIONS**

In conclusion, structural differences among the LF were found at the different spinal levels, and all these changes were caused by the ageing process. Decrease of the relative elastic area, an increase of the relative collagen area ratio affected all the spinal levels of the ligaments. Many changes took place in the lumbar ligaments such as ossification, increase vasculature, degeneration with abnormal body formation, absence of the midline gaps, fragmentation and rupture of the elastic fibres.

**REFERENCES**