Elucidating the effect of mechanical stretch stress on the mechanism of ligamentum flavum hypertrophy: Development of a novel in vitro multi-torsional stretch loading device

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Abstract

Objective

We developed a novel multi-torsional mechanical stretch stress loading device for ligamentum flavum cells and evaluated its influence on the development of ligamentum flavum hypertrophy, a common cause of lumbar spinal canal stenosis.

Materials and methods

Stretch strength of the device was optimized by applying 5% and 15% MSS loads for 24, 48, and 72 h. A cytotoxicity assay of human ligamentum flavum cells was performed and the results were compared to control (0% stress). Inflammatory markers (interleukin [IL]-6, IL-8), vascular endothelial growth factor [VEGF], and extracellular matrix (ECM)-regulating cytokines (matrix metalloproteinase [MMP]-1, MMP-3, and MMP-9, and tissue inhibitor of metalloproteinase [TIMP]-1 and TIMP-2) were quantified via enzyme-linked immunosorbent assay.

Results

Using our multi-torsional mechanical stretch stress loading device, 5% stress for 24 hour was optimal for ligamentum flavum cells. Under this condition, the IL-6 and IL-8 levels, VEGF level, and MMP-1, MMP-3, and TIMP-2 were significantly increased, compared to the control.

Conclusion

Using the novel multi-torsional mechanical stretch stress loading device we confirmed that, mechanical stress enhances the production of inflammatory cytokines and angiogenic
Introduction

Up to 70% of the general population experiences chronic lower back pain (LBP) once or more throughout their lifetime [1]. Among the many possible causes of chronic LBP, the prevalence of lumbar spinal canal stenosis (LSCS) in the elderly population is gradually increasing [2]. LSCS is also associated with lower-extremity radiculopathy and neurogenic claudication, which greatly affects the walking distance of the elderly. These clinical symptoms are associated with daily quality of life and therefore are of great interest to spinal physicians. The pathomechanism of LSCS is unclear, but facet joint enlargement, central intervertebral disc bulging, and ligamentum flavum hypertrophy (LFH) are contributing factors [3]. Among them, LFH secondary to the aging process or mechanical stimulation induced by instability of the spinal segment are key [4]. Therefore, research on the physiologic basis of LFH has caught the attention of spinal specialists, who agree that inflammation, angiogenesis, and matrix regulation of ligamentum flavum influence the development of LFH [5–9].

Mechanical stretch stress (MSS) on the ligamentum flavum is a major contributing factor to LFH. Hayashi et al. reported that mechanical stress concentration was directly linked to LFH in a rabbit model [10, 11], and Hur et al. emphasized the link between angiogenesis and mechanical stress-induced LFH [7]. Other studies have revealed an association between inflammation triggered by mechanical stress and LFH [6–9]. Nonetheless, it is doubtful whether these studies mimic in vivo mechanical stress. In this study, we developed a novel multi-torsional cell plate stretch device that mimics in vivo mechanical stress on ligamentum flavum tissue. We evaluated the molecular biological responses related to inflammation, angiogenesis, and extracellular matrix (ECM) regulation that were reported in previous studies of ligamentum flavum cells to various stress loads to identify the stress load that best mimics LFH.

Materials and methods

Ethic declaration

This study was reviewed and approved by the local ethics committee (Research Ethics Committee of Korea University Guro Hospital: approval number 2017GR0175) and has been performed in accordance with the ethical standards as laid down in the 1964 Declaration of Helsinki and its later amendments. Informed consent was obtained from all participants.

This study was approved by the Institutional Review Board (IRB) of our institute. Human LF tissues were collected during surgeries on the lumbar spine for herniated nucleus pulposus, following the regulations of the IRB. LF cells were isolated from the tissues of five patients of normal LF thickness. LF tissues harvested in the operating room were placed in sterile Ham’s F-12 medium (Gibco-BRL, Grand Island, NY) containing 1% penicillin/streptomycin (P/S; Gibco-BRL) and 5% fetal bovine serum (FBS; Gibco-BRL). After a phosphate-buffered saline (PBS; Welgene, Gyeongsan-si, Gyeongsangbuk-do, Korea) wash, tissues were minced and digested for 1 h at 37˚C in Dulbecco’s modified Eagle’s medium (DMEM; Welgene, Gyeongsan-si, Gyeongsangbuk-do, Korea) with 0.2% pronase (Calbiochem, La Jolla, CA). Next, LF tissues were incubated overnight at the same temperature in 0.025% collagenase I (Roche Diagnostics, Mannheim, Germany). LF cells were filtered through a sterile nylon-mesh cell
strainer (pore size, 70 μm), centrifuged, and the pellets were resuspended and cultured in DMEM containing 10% FBS and 1% P/S in a humidified atmosphere of 5% CO₂ at 37˚C. LF cultures were continued until reaching full confluence. The cells were trypsinized and replated for subculture. Subsequent experiments were conducted using these second-passage LF cells. The detailed LF isolation and culture protocols are adopted from previously reported LF experimental studies [5, 8].

Design and implementation of the novel MSS loading system

We fabricated a multiple-multidirectional mechanical stretch stress (MSS) loading chamber system capable of incubating dishes containing LF cells. The multi-torsional cell plate stretch device comprises a roofless metal frame containing fixation panels, twisting parts, culture chambers, and a controller. Multiple chambers are seated parallel on the fixation panel facing upwards (Fig 1). The sides of the chamber are fixated to two separate and parallel-oriented fixation panels, which pulls the chamber by moving in the opposite direction. In addition, the fixation panels are coupled to the twisting part to produce torsion stress on multiple chambers. The parallel chambers are aligned and stretched in the same direction and with identical power simultaneously. Each chamber is made of flexible polydimethylsiloxane (PDMS) by photolithography, that can contain cell cultures and stretch or twist. An optically transparent, ultrathin (100 μm) membrane was applied to the well bottom to render the stretch chambers compatible with optical and fluorescence microscopy (Fig 2). The MSS force developed by two step motor generators were controlled by Arduino Uno and L293D motor drivers, regulating the strength of the stretch and torsional stress. The optimal cyclic directions and loading were established after multiple virtual simulations. A 4-degree tilt away from the panel provides 2 mm stretch and 3 mm sliding of each panel beneath the chambers, resulting a in 10 degree of rotation tilt of the chamber corners and torsional stretch on the PDMS chambers (Fig 3). The PDMS chambers are designed to be assembled on the MSS device after cell attachment has been confirmed. In addition, to determine the expected stretch load force on the internal surface of the chamber, a three-dimensional (3D) simulation program (Inventor, Autodesk Inc, [14]) was utilized.
CA) was used. The torsional stress loaded on the external chamber surface was analyzed and presented as stress-strain ratios.

**Mechanical stretch stress loading on LF cells**

LF cells were plated on the PDMS chamber at a density of $5.0 \times 10^4$/mL. After 24 h of incubation, cell attachment to the cell chamber wall was verified, and the cells were subjected to MSS. Multi-torsional MSSs of 0% (no stretch–control), 5%, and 15% load were applied to multiple cell plate chambers simultaneously. The 5% and 15% MSS strength are relative strength compared to the maximal MSS that can be generated by the newly developed MSS load device. Three independent experiments were conducted.

**Lactate dehydrogenase assay**

Lactate dehydrogenase (LDH), which is normally confined within the cell is released into culture supernatant after plasma membrane breakdown as result of cytotoxicity. This is a
Fig 3. Two-step motor generators. (a) Producing a 10-degree rotational tilt of the chamber corners with resultant torsional stretch on the PDMS chambers (b) 4-degree tilt away from the panel results in 2 mm stretch and 3 mm sliding of each panel beneath the chambers.
commonly used methods allowing simultaneous analysis of specimens rapidly and cost-effectively [12]. LDH was assayed using LDH assay kit (Roche, Mannheim, Germany) after various MSS loading—0%, 5% and 15%—at various durations—24, 48, and 72 hours.

**Enzyme-linked immunosorbent assay**

Concentrations of vascular growth factor (VEGF), interleukin (IL)-6 and IL-8, matrix metalloproteinase (MMP)-1 and MMP-3, and tissue inhibitor of metalloproteinase (TIMP)-1 and TIMP-2 were analyzed by ELISA using commercially available kits (R&D Systems, Minneapolis, MN) following the manufacturer’s recommended protocols. All experiments were conducted in duplicate.

**Statistical analysis**

Data are mean ± standard error (SE) for individual experiments using independent cell cultures. P values were calculated using Student’s t-test or the Mann–Whitney U test, as appropriate according to sample size and distribution normality. P-values < 0.05 were considered to indicate statistical significance. All statistical analyses were performed using SPSS (version 20, SPSS, Chicago, IL, USA).

**Results**

**Optimizing MSS load on LF cells**

Using dual-step motor generators controlled by motor drivers, multi-torsional MSS was successfully loaded on the assembled cell chambers (S1 File). The tension-load was produced by optimizing the multi-torsional stretch strength and the cyclic load frequency, and 3D simulation was performed to visualize the expected load on the chambers (Fig 4). Morphologic

![Three-dimensional simulation of the expected load on the chambers](https://doi.org/10.1371/journal.pone.0275239.g004)
evaluation of LF cells by optical microscopy revealed no significant phenotypic change after 24 h of 5% MSS, whereas cell death was noted with more prolonged MSS loading.

**Cytotoxicity assay of LF cells**

LDH release from MSS loaded LF cells was measured to evaluate cytotoxicity at 24, 48, and 72 h after MSS loading. (Fig 5), MSS load on LF cells did not significantly affect LDH release at 5% stretch for 24 h, but at 15% stretch and exposure for 48 and 72 h LDH levels were significantly increased, indicating a cytotoxic effect. The ligamentum flavum cells did not present any phenotypic change under 5% stretch for 24h, but any further exposure to 5% MSS or any force stronger than 5% resulted in cell deaths. (Fig 6)

**Effect of MSS loading on inflammatory cytokines and vascular growth factors**

IL-6 and IL-8 release after MSS loading was 296.80±89.35 and 72.27±11.12 ng/mL, respectively, significantly higher than in the control group (174.97±58.12 and 56.43±5.59 ng/mL, respectively). Furthermore, a significant increase in VEGF level following MSS loading (141.80±19.45 ng/mL) was observed compared to the control group (23.97±8.16 ng/mL) (Fig 7, Table 1).

**Effect of MSS load on production of ECM-regulating factors**

MMP-1, MMP-3, MMP-9, TIMP-1, and TIMP-2 release levels from LF cells loaded with MSS at 5% for 24 h were measured to assess ECM remodeling. Following MSS loading, MMP-1, MMP-3, and MMP-9 release levels were 463.94±53.08, 579.92±90.43, and 25.77±1.84 ng/mL, respectively. The TIMP-2 release level was 320.00±16.34 ng/mL; TIMP-1 was undetectable. The MMP-1, MMP-3, and TIMP-2 release levels were significantly increased by MSS loading compared to the control group (330.15±35.41, 420.25±45.66, and 273.87±16.40 ng/mL, respectively) (Fig 7, Table 1).

**Discussion**

LSCS is of interest to spinal physicians due to its increasing prevalence and clinical significance. Prior reports have indicated that LFH and LSCS are significantly associated with various clinical symptoms including back pain and radiculopathy with or without neurogenic claudication [9, 13–17]. The LF is a ligamentous structure lying over the dorsal aspect of the central
spinal canal, LFH directly results in physical narrowing of the spinal canal leading to clinical LSCS. To discover a novel therapeutic candidate targeting LFH, it is important to understand the pathomechanism of LFH. Elucidating the role of mechanical stress on the LF is critical, and inflammation/angiogenesis of the LF following mechanical stress are hallmarks of LFH.

We previously reported that inflammation and subsequent angiogenesis are involved in the pathomechanism of LFH in vitro, indicative of close relationships among inflammation, angiogenesis, and LFH [6]. In a follow-up study of the association between in vitro and clinical data, we discovered links among mechanical stress, angiogenesis, and LFH [7]. However, these studies were limited in that mechanical stress was not loaded directly onto the LR cells. Instead, the effects of mechanical stress were evaluated indirectly based on radiological findings. In this study, we developed a novel mechanical stress loading device with multidirectional torsion that mimics the mechanical load on LF tissue in vivo. Rather than inducing inflammation by transforming growth factor-β1 (TGF-β1) or interleukin-1β (IL-1β), we used mechanical stress on the LF and believe it reflects the effects of mechanical stress on LFH.

Fig 6. Light microscopic images of ligamentum flavum cells. Representative phase contrast light microscopic images of ligamentum flavum cells under different mechanical stretch stress (MSS) forces and different time durations. (A) Control MSS strength, (B) 5% MSS strength and (C) 15% MSS strength.

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Fig 7. Factors released in control versus 5% MSS. IL-6, IL-8, VEGF, MMP-1, MMP-3, and TIMP-2 release from ligamentum flavum cells loaded with MSS at 5% for 24 h was significantly increased compared to the control group.

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Our results demonstrated that multi-torsional MSS load for 24 h under 5% stretch force stimulation resulted in an increase in IL-6 and VEGF levels. IL-6 activates neutrophils, whose adhesion and fibrosis are promoted by increased expression of ECM-regulating molecules or cytokines [18]. A similar response leads to LFH after triggering inflammation in LF cells [6]. An increase in IL-6 can also upregulate mRNA expression and DNA synthesis of LF cells, resulting in ossification or fibrosis [19]. Our finding of a significant increase in IL-6 expression confirms that MSS loading induced inflammation in LF cells, mimicking the initial inflammatory phase of LFH. Likewise, VEGF initiates and stimulates the angiogenic cascade of LFH, and its concentration in degenerated or hypertrophied LF is significantly higher than that in healthy ligaments [6, 7, 10, 20]. MSS stimulation for 24 h resulted in marked elevation of VEGF expression in LF cells, indicating that MSS loading mimics the angiogenic cascade that occurs after an inflammatory reaction.

As well as inflammation and angiogenesis, the resultant changes in ECM-modulating factors (such as the elastin to collagen ratio) are important. Our data on ECM-regulating enzymes provide insight into the response of LF cells to mechanical stress. MMPs are endopeptidases involved in ECM homeostasis and in cell–cell interactions and angiogenesis. Significant changes or dysregulation of MMPs occur in cells during inflammation [21, 22], as well as in LF fibroblasts [8, 23]. MMP-1 is a collagenase for all collagen subtypes, and MMP-3 a broad-spectrum proteinase that regulates activation of other MMPs [24]. Elevated MMP-1 and MMP-3 levels after MSS stimulation by our novel multi-torsional stress loading device are compatible with prior reports confirming an association with LFH [25, 26]. This suggests the key role of mechanical stress in LFH as a result of altered ECM regulation in LF cells, indicating the dysregulation of regenerative potential and vulnerability to mechanical stress. However, Kim et al. reported increased expression of MMP-9 after inflammatory stimulation of LF cells in vitro [8], and Lakemeier et al. indicated that MMP-9 expression is higher in LFH tissue [27]. TIMPs also regulate ECM homeostasis, and TIMP-1 and TIMP-2 play key roles in fibrosis in various cell types by increasing proliferation. Park et al. hypothesized that TIMP-1 and TIMP-2 influence LFH by increasing ECM density and promoting hypertrophy by suppressing MMP activities [4]. This hypothesis was confirmed by the significant association between elevated TIMP-1 and TIMP-2 expression in LF fibroblasts and spinal stenosis, a reproducible finding of several different experiments of various methods [28]. This is compatible with our TIMP-1 and TIMP-2 expression data.

| Table 1. Inflammatory mediators, angiogenic factor, and ECM-regulating Molecule Production from LF cells. |
|-----------------|-----------------|-----------------|
|                | Control         | MSS load (5%, 24hrs) | p-value |
| IL-6           | 174.97±58.12    | 296.80±89.35     | 0.013*   |
| IL-8           | 56.43±5.59      | 72.27±11.12      | 0.015*   |
| VEGF           | 23.97±8.16      | 141.80±19.45     | 0.028*   |
| MMP-1          | 330.15±35.41    | 463.94±53.08     | 0.049*   |
| MMP-3          | 420.25±45.66    | 579.92±90.43     | 0.024*   |
| MMP-9          | 35.46±4.82      | 25.77±1.84       | 0.009**  |
| TIMP-1         | NC              | NC               | -        |
| TIMP-2         | 273.87±16.40    | 320.00±16.34     | 0.049*   |

ECM, extracellular matrix; LF, ligamentum flavum; IL, interleukin; VEGF, vascular endothelial growth factor; MMP, matrix metalloproteinases; TIMP, tissue inhibitors of metalloproteinase; NC, Not Competent

Values unit: ng/mL ± SE

* P<0.05, significant increase in concentration.
** P<0.05, significant decrease in concentration.

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As well as inflammation and angiogenesis, the resultant changes in ECM–modulating factors (such as the elastin to collagen ratio) are important. Our data on ECM-regulating enzymes provide insight into the response of LF cells to mechanical stress. MMPs are endopeptidases involved in ECM homeostasis and in cell–cell interactions and angiogenesis. Significant changes or dysregulation of MMPs occur in cells during inflammation [21, 22], as well as in LF fibroblasts [8, 23]. MMP-1 is a collagenase for all collagen subtypes, and MMP-3 a broad-spectrum proteinase that regulates activation of other MMPs [24]. Elevated MMP-1 and MMP-3 levels after MSS stimulation by our novel multi-torsional stress loading device are compatible with prior reports confirming an association with LFH [25, 26]. This suggests the key role of mechanical stress in LFH as a result of altered ECM regulation in LF cells, indicating the dysregulation of regenerative potential and vulnerability to mechanical stress. However, Kim et al. reported increased expression of MMP-9 after inflammatory stimulation of LF cells in vitro [8], and Lakemeier et al. indicated that MMP-9 expression is higher in LFH tissue [27]. TIMPs also regulate ECM homeostasis, and TIMP-1 and TIMP-2 play key roles in fibrosis in various cell types by increasing proliferation. Park et al. hypothesized that TIMP-1 and TIMP-2 influence LFH by increasing ECM density and promoting hypertrophy by suppressing MMP activities [4]. This hypothesis was confirmed by the significant association between elevated TIMP-1 and TIMP-2 expression in LF fibroblasts and spinal stenosis, a reproducible finding of several different experiments of various methods [28]. This is compatible with our TIMP-1 and TIMP-2 expression data.
Mechanical stress is a key factor in LFH, as confirmed by *in vitro* [29–33], *in vivo* [10, 11], and clinical studies [7]. Chao *et al.* developed an *in vitro* method of loading stress on LF cells by centrifuging them in a horizontal microplate rotor [33]. Nakamura *et al.* loaded a cyclic uniaxial load to LF cells by attaching the cell culture chamber to a stretching apparatus [29], and Nakatani *et al.* loaded mechanical stress using a vacuum unit to pull a flexible cell culture plate from the center [31]. It is meaningful that centrifugal and cyclic one-dimensional mechanical forces on LF fibroblasts affected the mechanostress pathway. However, because one- and two-dimensional forces are unlike that on LF tissue *in vivo*, the accuracy of the model is unknown. Therefore, it is significant that we developed a reproducible repetitive mechanical stress loading device that recapitulates the mechanical stress on LF cells. The device will be used to provide insight into the role of direct mechanical stress on LFH *in vitro* and the cells’ fate after mechanical stress loading.

In conclusion, we developed a novel multi-torsional mechanical stress loading device which can mimic the physiologic multi-dimensional mechanical stress that ligamentum flavum gets in vivo. Using this device, we confirmed that mechanical stress does enhance the inflammatory reaction in ligamentum flavum cells and enhances the production of angiogenic factors subsequently leading to possible LFH. ECM regulating enzymes’ expression were also notably compatible with that of previous studies. We believe this development will help us understand the effect of MSS on LFH by providing a reproducible stress loading platform for ligamentum flavum *in vitro* experiments. Nevertheless, our study still has certain limitations which must be overcome by further future studies. We focused on the ligamentum flavum cellular protein production and expression and is currently lack of intracellular signaling RNA studies, and therefore cannot provide a comprehensive conclusion including subsequent cellular changes such as cytoskeletal remodeling or mechano-transduction signaling pathways. Further studies concerning intracellular signaling RNA with additional assay experiment, such as MTT assay, and various cell staining technique to validate cytoskeletal changes and cellular viability might lead us to a more information regarding the bio-mechanism. Another limitation is that we do not have in vivo results yet, and cannot have a concrete conclusion that our device perfectly mimics the actual multi-dimensional stretch stress on ligamentum flavum tissue. In order to do so, in vivo and further follow up experimental studies with more various loading stress are needed.

**Supporting information**

S1 File. **Novel multi-torsional mechanical stretch stress loading device.** An open metal frame with multiple chambers seated parallel to the fixation panels. These chambers incorporate two-step motor generators producing rotational tilt and stretch forces. (MP4)

**Author Contributions**

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**References**

1. Andersson GB. Epidemiological features of chronic low-back pain. Lancet. 1999; 354(9178):581–5. https://doi.org/10.1016/S0140-6736(99)01312-4 PMID: 10470716

2. Kalichman L, Cole R, Kim DH, Li L, Suri P, Guermazi A, et al. Spinal stenosis prevalence and association with symptoms: the Framingham Study. Spine J. 2009; 9(7):545–50. https://doi.org/10.1016/j.spinee.2009.03.005 PMID: 19398386

3. Szpalski M, Gunzburg R. Lumbar spinal stenosis in the elderly: an overview. Eur Spine J. 2003;12 Suppl 2:S170–5. https://doi.org/10.1007/s00586-003-0612-1 PMID: 13680315

4. Park JB, Lee JK, Park SJ, Riew KD. Hypertrophy of ligamentum flavum in lumbar spinal stenosis associated with increased protease inhibitor concentration. J Bone Joint Surg Am. 2005; 87(12):2750–7. https://doi.org/10.2106/JBJS.E.00251 PMID: 16322626

5. Hur JW, Bae T, Ye S, Kim JH, Lee S, Kim K, et al. Myofibroblast in the ligamentum flavum hypertrophic activity. Eur Spine J. 2017; 26(8):2021–30. https://doi.org/10.1007/s00586-017-4981-2 PMID: 28180980

6. Moon HJ, Park YK, Ryu Y, Kim JH, Kwon TH, Chung HS, et al. The angiogenic capacity from ligamentum flavum subsequent to inflammation: a critical component of the pathomechanism of hypertrophy. Spine (Phila Pa 1976). 2012; 37(3):E147–55. https://doi.org/10.1097/BRS.0b013e3182269b19 PMID: 21673619

7. Hur JW, Kim BJ, Park JH, Kim JH, Park YK, Kwon TH, et al. The Mechanism of Ligamentum Flavum Hypertrophy: Introducing Angiogenesis as a Critical Link That Couples Mechanical Stress and Hypertrophy. Neurosurgery. 2015; 77(2):274–81; discussion 81–2. https://doi.org/10.1227/NEU.0000000000000755 PMID: 25850600

8. Kim BJ, Hur JW, Park JS, Kim JH, Kwon TH, Park YK, et al. Expression of matrix metalloproteinase-2 and -9 in human ligamentum flavum cells treated with tumor necrosis factor-alpha and interleukin-1beta. J Neurosurg Spine. 2016; 24(3):428–35.

9. Sairyo K, Biyani A, Goel V, Leaman D, Booth R Jr., Thomas J, et al. Pathomechanism of ligamentum flavum hypertrophy: a multidisciplinary investigation based on clinical, biomechanical, histologic, and biologic assessments. Spine (Phila Pa 1976). 2005; 30(23):2649–56.

10. Hayashi K, Suzuki A, Abdullah Ahmadi S, Terai H, Yamada K, Hoshino M, et al. Mechanical stress induces elastic fibre disruption and cartilage matrix increase in ligamentum flavum. Sci Rep. 2017; 7 (1):13092. https://doi.org/10.1038/s41598-017-13360-w PMID: 29026131

11. Hayashi K, Suzuki A, Terai H, Ahmadi SA, Rahman MS, Maruf MH, et al. Fibroblast Growth Factor 9 Is Upregulated Upon Intervertebral Mechanical Stress-Induced Ligamentum Flavum Hypertrophy in a Rabbit Model. Spine (Phila Pa 1976). 2019; 44(20):E1172–E80. https://doi.org/10.1097/BRS.0000000000003089 PMID: 31022154
12. Galluzzi L, Aaronson SA, Abrams J, Alnemri ES, Andrews DW, Baehrecke EH, et al. Guidelines for the use and interpretation of assays for monitoring cell death in higher eukaryotes. Cell Death & Differentiation. 2009; 16(8):1093–107. https://doi.org/10.1038/cdd.2009.44 PMID: 19373242

13. Beamer YB, Garner JT, Shelden CH. Hypertrophied ligamentum flavum. Clinical and surgical significance. Arch Surg. 1973; 106(3):289–92. https://doi.org/10.1001/archsurg.1973.01350150029008 PMID: 4689802

14. Park JB, Chang H, Lee JK. Quantitative analysis of transforming growth factor-beta 1 in ligamentum flavum of lumbar spinal stenosis and disc herniation. Spine (Phila Pa 1976). 2001; 26(21):E492–5. https://doi.org/10.1097/00007632-200111010-00007 PMID: 11679833

15. Towne EB, Reichert FL. Compression of the Lumbosacral Roots of the Spinal Cord by Thickened Ligamenta Flava. Ann Surg. 1931; 94(3):327–36. https://doi.org/10.1097/00000658-193109000-00002 PMID: 1786626

16. Okuda T, Baba I, Fujimoto Y, Tanaka N, Sumida T, Manabe H, et al. The pathology of ligamentum flavum in degenerative lumbar disease. Spine (Phila Pa 1976). 2004; 29(15):1689–97. https://doi.org/10.1097/01.brs.0000132510.25378.8c PMID: 15284518

17. Yoshida M, Shimada Y, Tamaki Y, Tanaka T, Tanaka T. Hypertrophied ligamentum flavum in lumbar spinal canal stenosis. Pathogenesis and morphologic and immunohistochemical observations. Spine (Phila Pa 1976). 1992; 17(11):1353–60. https://doi.org/10.1097/00007632-199211000-00015 PMID: 14622118

18. Romano M, Sironi M, Tonianti C, Polentarutti N, Fruscella P, Ghezzi P, et al. Role of IL-6 and its soluble receptor in induction of chemokines and leukocyte recruitment. Immunity. 1997; 6(3):315–25. https://doi.org/10.1016/s1074-7613(00)80334-9 PMID: 9075932

19. Park JO, Lee BH, Kang YM, Kim TH, Yoon JY, Kim H, et al. Inflammatory cytokines induce fibrosis and ossification of human ligamentum flavum cells. J Spinal Disord Tech. 2013; 26(1):E6–12. https://doi.org/10.1097/01.BRS.0000132510.25378.8c PMID: 15284518

20. Yayama T, Kobayashi S, Sato R, Uchida K, Kokubo Y, Nakajima H, et al. Calcium pyrophosphate crystal deposition in the ligamentum flavum of degenerated lumbar spine: histopathological and immunohistochemical findings. J Rheumatol. 2008; 27(5):597–604. https://doi.org/10.1007/s10067-007-0754-3 PMID: 17934688

21. Visse R, Nagase H. Matrix metalloproteinases and tissue inhibitors of metalloproteinases: structure, function, and biochemistry. Circ Res. 2003; 92(8):827–39. https://doi.org/10.1161/01.RES.0000070112.80711.3D PMID: 12730128

22. Parks WC, Wilson CL, Lopez-Boado YS. Matrix metalloproteinases as modulators of inflammation and innate immunity. Nat Rev Immunol. 2004; 4(8):617–29. https://doi.org/10.1038/nri1418 PMID: 15286728

23. Warner RL, Bhagavathula N, Nerusu KC, Lateef H, Younkin E, Johnson KJ, et al. Matrix metalloproteinases in acute inflammation: induction of MMP-3 and MMP-9 in fibroblasts and epithelial cells following exposure to pro-inflammatory mediators in vitro. Exp Mol Pathol. 2004; 76(3):189–95. https://doi.org/10.1016/j.yexmp.2004.01.003 PMID: 15126100

24. Pasternak B, Aspenberg P. Metalloproteinases and their inhibitors-diagnostic and therapeutic opportunities in orthopedics. Acta Orthop. 2009; 80(6):693–703. https://doi.org/10.3109/1745367090344825 PMID: 19986900

25. Park JB, Kong CG, Suhl KH, Chang ED, Riew KD. The increased expression of matrix metalloproteinases associated with elastin degradation and fibrosis of the ligamentum flavum in patients with lumbar spinal stenosis. Clin Orthop Surg. 2009; 1(2):81–9. https://doi.org/10.4055/cios.2009.1.2.81 PMID: 19885059

26. Oshisaka T, Ha KY. Matrix metalloproteinase-3 on ligamentum flavum in degenerative lumbar spondylolisthesis. Spine (Phila Pa 1976). 2009; 34(16):E552–7. https://doi.org/10.1097/01.BRS.0b013e318191aa0232 PMID: 19770597

27. Lakemeier S, Schofer MD, Foltz L, Schmid R, Efe T, Rohlf J, et al. Expression of hypoxia-inducible factor-1alpha, vascular endothelial growth factor, and matrix metalloproteinases 1, 3, and 9 in hypertrophied ligamentum flavum. J Spinal Disord Tech. 2013; 26(7):400–6.

28. Xu YQ, Zhang ZH, Zheng YF, Feng SQ. MicroRNA-221 Regulates Hypertrophy of Ligamentum Flavum in Lumbar Spinal Stenosis by Targeting TIMP-2. Spine (Phila Pa 1976). 2016; 41(4):275–82. https://doi.org/10.1097/BRS.0000000000001225 PMID: 26571175

29. Nakamura T, Okada T, Endo M, Kadomatsu T, Tanikawa T, Sei A, et al. Angiopoietin-like protein 2 induced by mechanical stress accelerates degeneration and hypertrophy of the ligamentum flavum in lumbar spinal canal stenosis. PLoS One. 2014; 9(1):e85542. https://doi.org/10.1371/journal.pone.0085542 PMID: 2446599
30. Nakamura T, Okada T, Endo M, Nakamura T, Oike Y, Mizuta H. Angiopoietin-like protein 2 promotes inflammatory conditions in the ligamentum flavum in the pathogenesis of lumbar spinal canal stenosis by activating interleukin-6 expression. Eur Spine J. 2015; 24(9):2001–9. https://doi.org/10.1007/s00586-015-3835-z PMID: 25735609

31. Nakatani T, Marui T, Hitora T, Doita M, Nishida K, Kurosaka M. Mechanical stretching force promotes collagen synthesis by cultured cells from human ligamentum flavum via transforming growth factor-beta1. J Orthop Res. 2002; 20(6):1380–6. https://doi.org/10.1016/S0736-0266(02)00046-3 PMID: 12472256

32. Cai HX, Yayama T, Uchida K, Nakajima H, Sugita D, Guerrero AR, et al. Cyclic tensile strain facilitates the ossification of ligamentum flavum through beta-catenin signaling pathway: in vitro analysis. Spine (Phila Pa 1976). 2012; 37(11):E639–46.

33. Chao YH, Tsuang YH, Sun JS, Sun MG, Chen MH. Centrifugal force induces human ligamentum flavum fibroblasts inflammation through activation of JNK and p38 pathways. Connect Tissue Res. 2012; 53(5):422–9. https://doi.org/10.3109/03008207.2012.685132 PMID: 22506718