Spinal hernia tissue autofluorescence spectrum

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ORIGINAL ARTICLE

Spinal hernia tissue autofluorescence spectrum

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Abstract The laser intervertebral disc decompression may provide appropriate relief in properly selected patients with contained disc herniations. The present investigation aims to characterise intervertebral disc material by autofluorescence induced by laser light. Degeneration of the intervertebral disc is associated with progressive biochemical changes in disc material. Percutaneous laser disc decompression has become rather popular for the treatment of lumbar disc herniation, but there are problems in the selection of patients. For this purpose, recognition of the disc composition is necessary. We propose a new type of spectroscopic investigation. It is advantageous to the characterization of intervertebral disc material. Intervertebral disc specimens were removed during open surgery from different disc locations. Preoperative patients' MRI was evaluated using the *Pfirrmann* disc degeneration and *Komori* scale for migrating of herniated *nucleus pulposus*. Adjacent slices of stained disc sections were evaluated by histology/histochemistry and autofluorescence spectra. Comparison of the MRI, spectral, histological and histochemical data was performed. The MRI Komori scale correlated with the histology Boos degeneration index. In the histochemistry, collagens other than collagens I and II of the disc were distinguished with best positive correlation coefficient (0.829) and best negative one (−0.904) of proteoglycans of sequester to Boos index. A correlation of the IV Gaussian component of the hernia spectra with the Boos index was established. The Gaussian component correlation with different collagen types and proteoglycan was determined for the disc and sequester. "Autofluorescence-based diagnosis" refers to the evaluation of disc degeneration by histological and histochemical evaluation; it can provide additional data on the degeneration of an intervertebral disc.

Keywords Back pain · Intervertebral disc · Autofluorescence diagnostics · Degenerative disc disease

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Introduction

Low back pain is one of the most common causes of disability for individuals of working age in developed countries [1]. There are many causes of low back pain, and it is generally believed that degenerative disc disease (*DDD*) is the most prevalent one [2]. Although the mechanisms by which DDD may cause low back pain are not

clear, the severity of DDD is associated with the onset of symptoms [3]. Degeneration of the intervertebral disc (*IVD*) is associated with progressive changes in the disc material properties, the composition and morphology of the disc extracellular matrix. Degenerative disc disease is believed to begin as early as in the second decade of life; it is viewed by most clinical practitioners as an inevitable consequence of ageing. Despite its prevalence, there is no clear distinction between disc degeneration and normal maturation, nor is it clear why disc degeneration progresses slowly in some patients, whereas in others more rapid destruction of the intervertebral discs can occur. Different methods of treatment of DDD are used; however, percutaneous laser disc decompression for lumbar disc herniation has become rather widespread [4, 5]. It was found that laser disc decompression may provide definite relief in properly selected patients with contained disc herniations, therefore methods for recognising the changes in the disc are important for the optimization of treatment.

There are numerous methods for the investigation of intervertebral disc. Visualisation methods are widely used in clinical practice [6]. Histological, immunohistochemical and biochemical ones are more common in scientific research. An accepted method for grading the severity of IVD degeneration is pathomorphological [7]. This method has been shown to be both reliable and repeatable. Biochemical methods have also been established [8]. We propose a new method of spectroscopic investigation. It can be advantageous to the characterisation of intervertebral disc material, being able to reveal biochemical change in the extracellular intervertebral disc matrix. Spectroscopic investigation is based on the autofluorescence phenomenon in the intervertebral disc tissue, which has been known since 1964 [9]. Nevertheless, only a few studies have investigated the clinical value of this phenomenon. The autofluorescence of disc tissue is triggered by ultraviolet (*UV*) light. The molecular sources of the phenomenon are specific amino acids of the collagen molecules [9]. The proteoglycan components and the liquid components of the disc do not show relevant autofluorescence. The emission wavelength from disc material was found different with different collagen composition in the intervertebral disc components, ranging from yellow–green to blue–green, and can be visualised in situ by the naked eye, and the details of the spectra were not analysed [9]. A preliminary report on the application of disc fluorescence spectroscopy was performed in [10]; however, the present work is devoted to the optimization of data processing and diagnostic methods.

Analysis of processes in the disc over degeneration can create a promising basis for fluorescence-based diagnostics. A normal intervertebral disc is an avascular fibrocartilaginous structure populated by poorly characterised cells in an extensive extracellular matrix. In an adult, the cells comprise two types resembling chondrocytes or fibrocytes [11]. These cells are thought to be predominant ones because they are better suited to withstand the avascular environment in the adult disc. Cells within the disc synthesise the matrix in which they are suspended; subsequently, they maintain and repair it. The disc matrix consists of an elaborate framework of macromolecules filled with water. The structural integrity and mechanical properties of the disc depend on the interaction between these two components. The principal macromolecules of the matrix are collagen and proteoglycans; the collagen fibrils are embedded in a gel of proteoglycans and water [12]. The proteoglycan molecule is made up of a core protein to which a variable number of glycosaminoglycan units are covalently attached. The most common glycosaminoglycan side chains in the disc are chondroitin sulphate and keratan sulphate, with the former predominating in the normal disc and the latter in the degenerated disc [11].

Collagens are important in conferring tensile strength to the disc. They are most abundant in the outer annulus, where they comprise 70 % of the dry weight, whereas they make up only 20 % of the central *nucleus pulposus* [12]. Normal intervertebral discs contain collagen types I, II, III, V, VI, IX and XI. The proportions of the different collagen types vary between the different tissue structures [13]. The intervertebral disc is composed predominantly of collagen types I and II. Under normal circumstances, collagen type I is found mainly within the outer annular region. Collagen type II, however, is present in the highest concentrations within the *nucleus* and the endplate. An inverse gradient exists across the disc, whereby the content of collagen type II is highest in the core of the disc, diminishing progressively into the periphery. The reverse occurs for collagen type I. In general, the distribution of the various types of collagen does not change with ageing [13]. The composition of collagen changes if the disc degenerates. In the early stages of degeneration, the normal density of collagen types increases within their usual areas of distribution. There is also an increase in the content of collagen type II, III, V and VI in the nucleus and an increased concentration of collagen type I in the *annulus fibrosus* [13]. Considerably less is known about other collagen types, and even less about their exact localization in normal and abnormal disc tissues. Except for a recent study on small samples of different regions of discs and experimental studies in animal models, not a single comprehensive study is available on the immunolocalization of various collagen types in completely analysed intervertebral discs. This may be due to difficulties in handling larger samples of the heterogeneous intervertebral disc tissue [13].

More advanced degeneration is also characterised by qualitative changes. Collagen type I begins to appear in the nucleus and collagen type II disappears from the endplates [13]. All these collagen molecules in all tissues emit

autofluorescent light under UV irradiation [9]. Collagen remodelling–collagen type II replacement by collagen type I in nucleus pulposus is a marker of degeneration [11]. The other well-known endogenous fluorophore presenting IVD is elastin [14].

The present analysis prompted us to analyse the possibility of using the auto-fluorescence effect of disc material as a keystone in the detection of biochemical change in disc material corresponding to DDD, and to design a minimally invasive diagnostic device based on spectroscopic analysis.

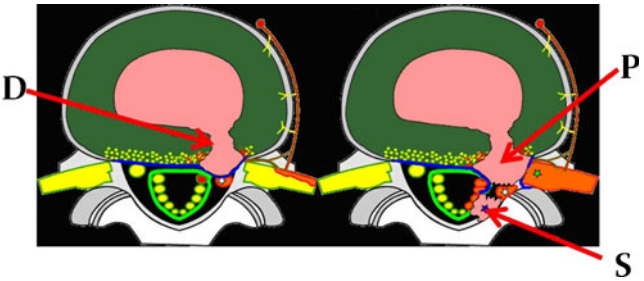

Fig. 1 Sites of disc specimen collection: *D* disc specimen from inside the disc (PLL) intact; *P* herniation from subligamentous space, PLL disrupted; *S* free sequester

Materials and methods

Patients and realisation

All patients hospitalized for open disc surgery because of intervertebral disc pathology were invited to participate in this study. They were acquainted with the chances of surgical treatment, possible complications and rehabilitation by an operating surgeon; all of them signed a separate agreement form for surgery. All patients were evaluated preoperatively by Visual Assessment Analogue Scale and Oswestry Disability Index. Objective findings appraise: motorical confusions, reflex prolapse, nerve root irritation symptoms.

Preoperative patients' MRI was evaluated using the *Pfirrmann* disc degeneration scale [15] and the *Komori* scale for the migration of herniated nucleus pulposus [16]. Discs of 29 patients were investigated. Interbertebral disc specimens were gathered during standard open discectomy procedures at the Department of Neurology and Neurosurgery, Vilnius University Emergency Hospital from 02 Feb 2009 to 11 Sept 2009. According to Pfirrmann method, 21 preoperative discs were given the third degeneration level, and eight discs were given the fourth degeneration level. According to the Komori scale for the migration of herniated nucleus pulposus, 20 specimens were evaluated as Komori 1, four specimens as Komori 2, and the last five specimens as Komori third-migrated sequester.

Basing on intraoperative findings, all samples were divided into three groups: *D* group—the disc specimen was gathered from the inside of the disc space, posterior longitudinal ligament (*PLL*) intact; *P* group—herniation removed from subligamentous space, PLL disrupted; *S* group—free sequester in direct contact with epidural vessels (Fig. 1). For the final assessment, 29 disc specimens were collected from different patients.

Samples, measurement and data analysis techniques

Removed during the open surgery, hernia was immediately washed in a physiological solution and placed into a clean defatted and sterile degreased plastic box for storage in a fridge at 4°C. Native disc specimens were frozen to −20°C and cut into slices 20 μm thick, placed on quartz glass and prepared for spectral analysis within 48 h after removal. Adjacent slices were taken for histological evaluation.

The fluorescence in the sample was excited by a sub nanosecond (<1 ns), high pulse repetition rate (10 kHz), single longitudinal mode, passive Q-switched Nd:YAG micro laser STA-01-TH (Standa Ltd, LT-03222 Vilnius, Lithuania), which emits ultraviolet radiation of wavelength 355 nm. The excited area was 3–4 mm^2, and the luminescence was collected by the optical fibre at the distance of 1.5 cm from the sample. The spectra in the range from 370 to 700 nm were recorded by an AvaSpec-2048TEC spectrophotometer (Avantes BV, NL-6961 RB Eerbeek, The Netherlands. Software version: AvaSoft-7.1—Basic version). The 10 spectra from the samples of each discs were measured, and more than 290 spectra were analysed in total.

The spectra of all samples (Fig. 2a) were normalised to the intensity of the luminescence signal in the long wave region (550–700 nm) where the spectrum shape in all samples was the same (Fig. 2b). Since not all biochemical components of the discs were known, the spectra were analysed by means of formal separation spectra into Gaussian components using Gaussian multi-peak analysis (*Origin 7.0* software) which fitted a Gaussian to the output of a spectral plot [17]. The example of this analysis is presented in Fig. 3.

All the spectra were analysed using four Gaussian Components. A distribution of the peak values of these components is presented in Fig. 4. The scattered data were approximated by Gaussian distribution and the components can be characterised by the peaks values at 407, 431, 467 and 507 nm. The parameters of collected spectra were compared with MRI, histological, histochemical and biochemical changes in disc specimens.

The stained disc sections were evaluated histologically. We used histological grading of lumbar disc degeneration on sagittal paraffin sections stained with haematoxylin and eosin, Masson–Goldner and Alcian blue–PAS proposed by Boos et al. [18]. The protocol was additionally

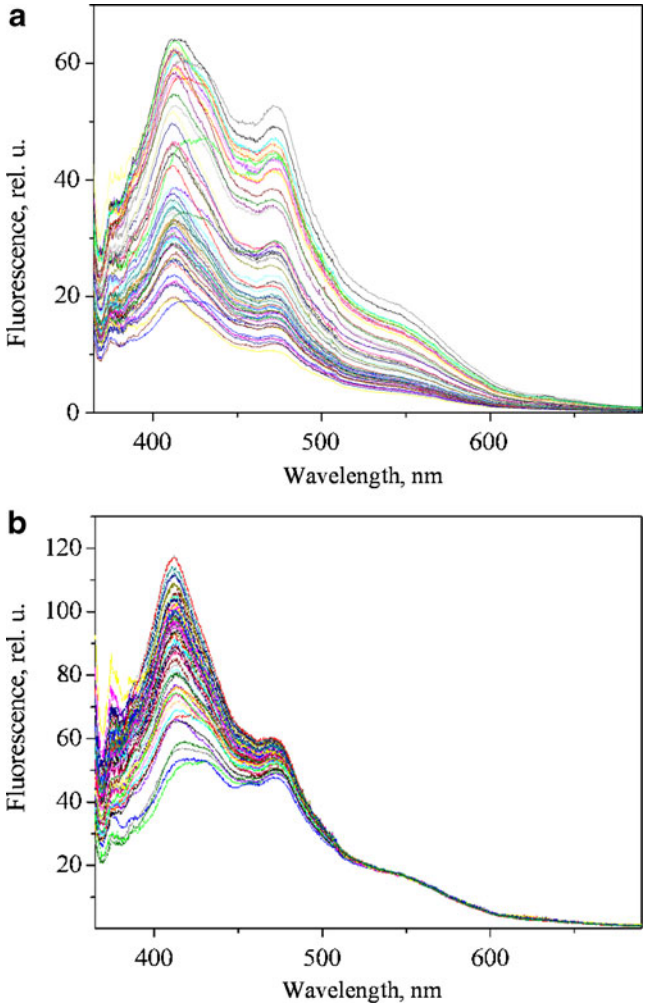

Fig. 2 **a** Spectra collected from the spectrometer, **b** spectra after normalisation (amplitude and light source error removed)

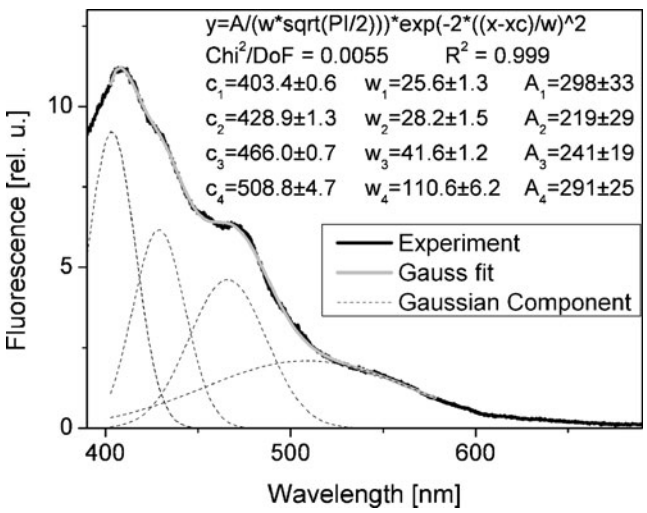

Fig. 3 A sample of an experimental spectrum (*black line*), its approximation (*grey line*) as a sum of four Gaussian components (*dash lines* and their parameters given in the *inset*)

extended by histochemical staining of slices with Picrosyrius red and evaluation in polarised light of collagen fibres (Fig. 5) and of proteoglycans by staining with Safranin O. and Toluidine blue. The ratio of different collagen fibres in disc specimens was evaluated semi-quantitatively. These data were used to compare histological data with the fluorescence spectral components of disc matrix.

As the native components of discs include collagens, we analysed the spectra of collagen types I and II to reveal the spectral regions where the main differences exist. The collagen type I spectrum is known, it has been taken from the database [19]. The spectra of collagen type II were not found, therefore the commercial collagen type II fluorescence spectrum was measured by 355 nm excitation. The fluorescence spectra of both collagen types I and II are presented in Fig. 6. These results show the main difference between these collagens in the spectral region corresponding to the first Gaussian Component of the fluorescence spactra.

The statistical data analysis was performed using SPSS statistics software for *Windows* (version 12.0). The averages (A) of the normal parameter values were calculated with standard deviations. The statistical significance of the difference in the nominal data was determined on the basis of the chi-square (χ^2) criterion, the difference in the normal quantitative data averages for two independent samples was determined by Student's *t* test, and the difference in the normal quantitative data averages for three independent samples was determined on the basis of an ANOVA single-factor analysis of variance. A significance level of $p < 0.05$, in which the results were deemed statistically reliable, was selected.

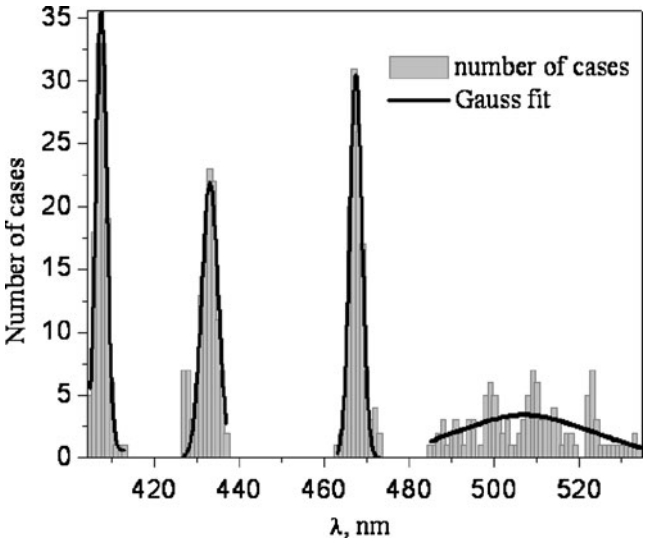

Fig. 4 The distribution of the Gaussian components' peak wavelength frequency in the samples. *Lines* approximation of the distribution by Gaussian function

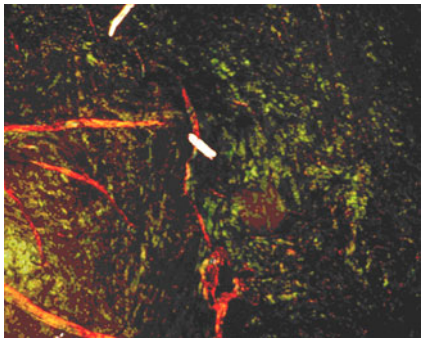

Fig. 5 Different collagen fibres composing intervertebral disc section. Stained in Picrosyrius red. *Green* collagen type II, *red* collagen type I, *yellow* other types of collagen. PS ×200

Results

Our inquiry into intervertabral discs, hernias and free sequesters produced definite morphological, histochemical and spectroscopical findings. All parameters of specimens are shown in Table 1.

Clinical specimen D, P, S group's correlation to histology and MRI

Analysis of histological data dependence by clinical location of the groups (Table 1) revealed that the Boos index in the *D* group was less than in the *P* group, and the latter was less than in the *S* specimen group ($D<P<S$). The groups are statistically different with $p=0.002$. Samples from the inside of the disc show least degeneration, free sequesters being most degenerated. Groups do not differ regarded from the point of view patients' age and relative concentration of different collagens. Groups differ according to the Komori index: $D<P<S$ ($p=0.001$), with sequesters being most distant from parent disc. Groups do not differ according to the Pfirrmann disc degeneration scale on MRI.

Clinical specimen D, P, S groups correlation with histochemistry

In histochemistry, groups differ according to proteoglycan presence D>S>P ($p<0.0001$). Samples from the inside of the disc show largest amounts of proteoglycans. Correlation analysis shows the best correlation of the Boos index with matrix proteoglycans (safranin O) in group S—a strong negative correlation coefficient −0.904 with *p* value 0.007 and coefficient of determination (R^2) equal to 0.816. Most degenerated samples show the least concentration of matrix proteoglycans.

Histochemical section staining by picrosyrius red allowed us to evaluate the qualitative presence of different types of collagens and make semi-quantitative calculation of their distribution (collagen type II/collagen type I/other types of collagens) throughout the section. Less degenerated disc samples have predominately collagen type II as the major fibrous matrix component. More degenerated disc samples show a decrease of collagen type II and increase of collagen type I. Other collagens in specimen group D were distinguished by the best correlation coefficient (0.829) to the Boos index ($p=0.011$; $R^2=0.687$).

Spectra and histochemistry correlation in D, P, S groups

With collagen type I increase in the disc, the Gaussian component 1 area decreases and 2, 3, 4 increase, while in sequester component 1 area increases and 2 decreases. With increase of other collagen types, the area of component 1 in disc and component 2 in sequester increases, while the areas of components 4 in disc and 1 and 3 in sequester decrease. These data are given in Table 2.

With the proteoglycan (safranin O) increase in group D (samples from the inside of the disc), the Gaussian components 1 and 3 increased. Proteoglycan (TW) in group P (samples with posterior longitudinal ligament disrupted) decreases with an increase in Gaussian component 1 and a decrease in Gaussian component 2 (Table 3).

In group D samples, the first Gaussian component has a strong negative correlation with collagen type I ($r=-0.78$, $p=0.022$, $R^2=0.608$), and strong positive correlation with matrix proteoglycans ($r=0.801$, $p=0.022$, $R^2=0.642$). The least degenerated samples from the inside of the vertebral disc have the least concentration of collagen type I and highest concentration of proteoglycans. In the samples of group S, the second Gaussian component has a strong positive correlation with other collagens ($r=0.869$, $p=0.011$, $R^2=0.755$).

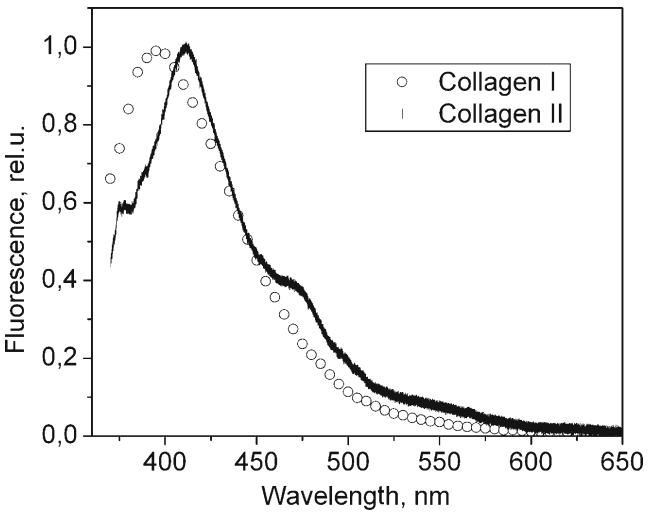

Fig. 6 Collagen type I and collagen type II fluorescence spectra at 355 nm excitation

Table 1 Morphological, histochemical and spectral parameters in the intervertebral discs (D), hernias (P) and free sequesters (S)

Specimen number	Histologic data			Spectroscopic parameters–Gaussian component relative area			
	Structural changes (Boos points)	Matrix proteoglycans (Safranin O.)	II/I/other collagens	1st	2nd	3rd	4th
18-D_1	18	Mx+2	80/0/20	22.4	9.0	24.1	21.6
19-D_2	22	Mx+2	40/40/20	26.5	16.0	18.1	37.1
23-D_3	14	Mx+2	10/40/50	27.9	11.8	20.8	25.7
28-D_4	15	Mx+1	60/10/30	26.5	13.1	16.3	34.3
29-D_5	17	Mx+1	60/40/0	10.4	9.3	9.8	38.4
30-D_6	17	Mx+0	30/70/0	11.2	10.0	13.7	26.9
38-D_7	14	Mx+3	50/20/30	28.9	13.5	21.9	26.3
40-D_9	15	Mx+1	30/60/10	6.4	11.8	17.5	23.2
42-D_{10}	16	Mx+2	70/0/30	30.6	10.9	22.6	23.5
14-P_1	9	Mx+2	30/20/50	23.5	4.4	26.4	17.3
15-P_2	16	Mx+1	40/30/30	11.3	11.0	9.4	46.6
17-P_3	14	Mx+2	20/40/40	27.9	17.4	19.0	31.1
21-P_4	11	Mx+1	10/80/10	51.8	9.2	27.6	25.4
22-P_5	13	Mx+2	60/30/10	34.7	1.9	45.5	9.3
24-$P_{5'}$	13	Mx+1	60/30/10	15.6	8.4	15.9	24.4
25-P_6	15	Mx+0	20/60/20	11.2	11.4	12.7	30.1
32-P_8	16	Mx+2	70/10/20	29.4	14.0	21.1	28.6
34-P_9	14	Mx+2	50/10/40	40.4	14.9	19.3	35.1
35-P_{10}	13	Mx+2	85/5/10	15.7	24.6	13.9	20.6
36-P_{11}	15	Mx+2	20/40/40	25.7	32.2	19.2	36.4
43-P_{12}	20	Mx+0	70/0/30	19.5	13.0	13.0	38.6
16-S_3	17	Mx+2	20/30/50	17.2	9.0	21.4	23.4
26-S_5	22	Mx+1	50/0/50	23.8	16.0	16.5	35.1
27-S_6	14	Mx+3	50/10/40	25.5	11.8	21.3	22.5
31-S_7	18	Mx+3	60/10/30	14.3	13.1	16.5	23.3
38-S_8	19	Mx+3	60/10/30	32.2	9.3	19.4	29.9
41-S_9	21	Mx+2	40/10/50	16.2	10.0	17.4	29.1
44-S_{10}	22	Mx+2	20/50/30	18.1	13.5	24.2	21.7
45-S_{11}	21	Mx+2	40/40/20	33.1	11.8	21.8	26.1

Comparison of the Boos index and spectra

The area of Gaussian Component 4 (505.2±5.0 nm) in specimen group P has shown significant correlation with the Boos index. The positive correlation coefficient is 0.604; p value 0.049; R^2=0.364. Moreover, specimens in group P were heterogeneous. By means of Boos index, specimens in group P (Table 1) were divided into two groups in terms of the level of structural changes (from Boos index 9 to 13 and from 14 to 20). The areas of the fourth Gaussian Component of the two groups differ, using Student's t criteria, with p=0.001 (the average value of the fourth Gaussian component in the group with Boos index 9–13 was 19.5±2.9 relative units, and in the group with Boos index 14–20 it was 35.3±2.3 relative units).

Discussion

Clinical D, P, S groups chosen by us clearly reflect the dynamics of the degeneration process and serve as a model of high grade degeneration. The clinical location of groups D (disc), P (hernia) and S (sequester) shows increasing histological degeneration according to the Boos index, MRI-based Komori index, but not on the disc degeneration Pfirrmann scale. Clinical groups D, P and S represent progressive dynamical changes in the disc material degeneration process. Groups D, P and S do not differ according to age—one can find markedly degenerate disc specimens in relatively young individuals. The groups differ depending on proteoglycan D>S>P (p<0.0001). Samples from the

Table 2 The correlation of collagen type I and other types of collagens in the disc and sequester samples with the fluorescence spectrum Gaussian Components

Collagens	Specimen group	Gaussian number or ratio	Correlation coefficient	*p* Values	Coefficients of determination (R^2)
I	D	1	−0.780	0.022	0.608
I	D	1/2	−0.812	0.014	0.659
I	S	1/2	0.925	0.003	0.856
I	D	1/3	−0.741	0.035	0.550
I	D	1/4	−0.746	0.034	0.556
other col	D	1	0.829	0.011	0.687
other col	S	2	0.869	0.011	0.755
other col	D	3	0.783	0.022	0.613
other col	D	1/2	0.802	0.017	0.644
other col	S	1/2	−0.793	0.033	0.629
other col	D	1/4	0.840	0.009	0.706
other col	S	2/3	0.951	0.001	0.905

inside of the disc show the largest amounts of proteoglycans. Other collagen types in group D (disc) reveal the best positive correlation coefficient to the Boos degeneration index (0.829; p=0.011; R^2=0.687). The best negative correlation of Boos index with matrix proteoglycans (Safranin O.) specimen is in group S (sequester), with correlation coefficient −0.904 (p=0.007; R^2=0.816). The area of Gaussian component 4 in the P specimen group showed significant correlation with the Boos index, with positive correlation coefficient 0.604; p values 0.049; R^2=0.364. Two groups of less and more severe structural changes in the P group Gaussian component 4 were distinguished. In the D-group, disc with PLL intact, there was no correlation of Gaussian components to the Boos degeneration index. Probably less degenerate disc, which is the object of minimal invasive surgery requires the fitting of optimal excitation parameters during clinical study.

Biochemistry approved vertebral disc degeneration markers reveal correspondence with spectral data in our investigation. If the collagen type I increases in the disc, the area of Gaussian component 1 decreases and components 2, 3 and 4 increase. If the component area 1 increases in sequester, Gaussian component 2 decreases. With the increase of other collagen types the areas of component 1 in the disc and 2 in the sequester increase. The proteoglycan (Safranin O) increase in the disc follows the increase of Gaussian components 1 and 3. The proteoglycan (TW) in hernia decreases with the increase of Gaussian component 1 and the decrease of component 2. The spectral Gaussian components respectively correlate strongly with chosen histochemistry markers in degenerate disc D, P and S groups. Spectral component source fluorophores are different in the clinical groups, and their fluorescence overlaps. The known important fluorophores in disc degeneration—collagen type II and elastin—are represented in the Gaussian components 1 and 2, respectively, [14] of the degenerate disc spectra. Several degeneration markers were found recently in the degenerating disc substance. In our study, collagen X, specific for more advanced degeneration, could probably be related to other collagen types which correlate strongly with the Boos index in disc and Gaussian component 2 in sequester. Osteoprotegerin, alkaline phosphatase could probably be related to Gaussian component 4 in the disc periphery, correlating significantly with the Boos index [20]. In future an autofluorescence spectral marker set and diagnostic algorithm will be proposed for the evaluation of the intervertebral disc degeneration.

Conclusion

Low back pain caused by intervertebral disc degeneration is an insufficiently researched problem of modern medicine. Subtle biochemical change of the intervertebral disc extracellular matrix is the core of the disc degeneration problem.

Table 3 The correlation of proteoglycans safranin O and TW with the fluorescence spectrum Gaussian Components in P and D sample groups

Histochemical changes	Specimen group	Gaussian number or ratio	Correlation coefficient	*p* Values	Coefficients of determination (R^2)
TW	P	1/2	−0.765	0.009	0.585
Safr. O	D	1	0.801	0.022	0.642
Safr. O	D	3	0.851	0.011	0.724

The comparison of fluorescence spectra and histology data has demonstrated a promising character of spectroscopy in the biochemical evaluation of degenerate disc, indicating its possible use for the *in vivo* disc biochemical investigation, especially during the minimally invasive procedures where a histological specimen is not available. Intervertebral disc, during minimally invasive intradiscal procedures, is accessed by a needle puncture and affected by laser light or a radiofrequency beam.

Photodiagnostic investigation of the disc material performed directly prior to treatment could give additional information on the status of intervertebral disc degeneration, and influence decision parameters relating to minimal invasive treatment. Fluorescence spectra and histology data have demonstrated that spectroscopy could be used in vivo for the qualification of the degeneration level in disc periphery and nucleus pulposus before laser treatment, and could support decisions on minimal invasive treatment parameters. The photodiagnosis information obtained by us supports further investigation into the optical properties of disc, encouraging adaptations for the effective improvement of minimal invasive treatment of disc degeneration. The laser diagnosis and laser treatment could be realised by a single device operated through a triple-furcated fibre cable using the first fibre for the treatment, the second one for the fluorescence excitation, and the third one for the spectrum collection.

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