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ORIGINAL ARTICLE

Morphological changes in disc herniation in the lower cervical spine: an ultrastructural study

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Abstract
Introduction The basis of disc degeneration is still unknown, but is believed to be a cell-mediated process. Apoptosis might play a major role in degenerative disc disease (DDD). The aim of this study was to correlate the viability of disc cells with the radiological degeneration grades (rDG) in disc herniation.
Materials and methods Forty anterior IVD's (C4–C7) from 39 patients with DDD were studied histologically and ultrastructurally to quantify healthy, "balloon", chondroptotic, apoptotic and necrotic cells. Patients were classified to their rDG, as having either prolapse (P: DGII + III) and/or osteochondrosis (O: DGIV + V). Similar studies were undertaken on eight control discs.
Results Cell death by necrosis (mean 35%) was common but differed not significantly in both groups. All patients with a disc prolapse DGII + III revealed balloon cells (iAF: mean 32%). All appeared alive and sometimes were hypertrophic. However, significantly less balloon cells were found in the O-Group. Control samples revealed no evidence of "balloon" cells in DGII and only a minor rate in DGIII.
Conclusion According to the different rDG, quantitative changes were obvious in healthy and "balloon" cells, but not for cell death. At the moment it can only be hypothesized if "balloon" cells are part of a repair strategy and/or cause of disc herniation.

Keywords Intervertebral disc · Disc prolapse · Degeneration · Balloon cell · Necrosis · Chondroptosis

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Introduction

The cervical spine is the most flexible part of the whole axial skeleton due to the nature of the vertebral joints and associated discs. With ageing, a lack of diffusion was thought to cause changes in the cartilaginous endplate; subsequent dehydration with loss of elasticity and formation of fissures within the intervertebral disc (IVD) was defined as "chondrosis intervertebralis" [1]. Schmorl described the dorsal bulging of the disc as protrusions whereas herniation into the spinal canal was defined as a prolapse. Further disruption of the IVD combined with loss

of disc height and sclerotic changes of the endplates were described as "osteochondrosis intervertebralis" [1]. The reasons for these degenerative diseases still remain unclear [2]. Other abnormal features are tears either peripheral (rim lesions) which are thought to result from trauma, or circumferential and radial tears which may be related to the presence of nuclear degeneration [3]. Some authors stated that disruption of the annulus and formation of tears during degeneration are a reason for a fragmented nucleus to protrude into the annulus fibrosus [4]. However, disc prolapse was found to occur in younger patients who presumably still have a fluid nucleus and an annulus starting to become weakened by age [5]. Even now the cause of the different types of tears is discussed and mechanical considerations are still suggested [5]. Others noted that genetic factors may play an important role [2].

Histological investigations comparing healthy aged and degenerative spines reveal changes such as increased disc cell proliferation with associated cluster formation as well as increased disc cell death [6]. The mechanism of the cell death in degeneration is controversial [7–9]. Similar changes can be detected in pathological articular cartilage where a special form of apoptosis called chondroptosis has been suggested to occur, in addition to necrosis and classical apoptosis [10]. Apoptosis was also believed to play a major role in degeneration of the intervertebral disc [8, 9] and appears to be related to regression changes in thickness of the endplate [11].

Recently, a new disc cell morphology had been reported in the traumatic injured intervertebral discs of the lower cervical spine, the "balloon" cell. These were more common in injured intervertebral discs which had been subjected to extensive compressive load with subsequent fracture of the vertebra [12]. These cells have a homogeneous nucleus enveloped by an osmiophilic nuclear membrane similar to that seen elsewhere in very active cells [13].

We hypothesised that these different forms of cell morphology and cell death would be present in all disc pathologies. Therefore, in the present study, we have examined the ultrastructure of cells within the discs from patients with herniations and different degrees of degeneration. We have shown that the incidence of cells undergoing apoptosis, chondroptosis or necrosis or having "balloon" cell morphology differs between normal and degenerated discs. "Balloon" cells are present in prolapsed discs, especially higher in those with a lower grade of degeneration.

Materials and methods

Forty samples of anterior intervertebral discs were obtained from 39 patients (range 30–59 years; mean 46.7 years) undergoing routine stabilization for either soft disc herniation (prolapse) or degenerative lesions such as severe osteochondrosis (sclerosis, vertebrostenosis, uncovertebral stenosis) at levels C3–C7. Patients with trauma were excluded. Thirty-six discs were either with a disc prolapse or protrusion in the investigated segment. The remaining discs with grade V degeneration revealed disc prolapse or protrusion in the adjacent segment in the cervical spine.

All patients were classified for degeneration (DG I–V) [14] according to their appearance on anterior–posterior and lateral X-rays, CT-scans and/or MRI-scans. Discs were obtained from two main groups either with prolapse alone (P: $n = 20$; disc prolapse-grade II: $n = 10$; disc prolapse-grade III: $n = 10$), or osteochondrosis (O: $n = 20$; disc prolapse/protrusion-grade IV: $n = 10$; disc protrusion-grade V: $n = 6$; prolapse/protrusion in an adjacent segment-grade V: $n = 4$) (Table 1).

As controls, eight disc specimens (C4–Th2) from four individuals were taken 9–62 h post-mortem. Corpses were stored at 3°C. There was no known cervical spine disorder (trauma/DDD). All patients died of kidney failure or sepsis. The mean age of this group was 70 years (67–74 years) (Table 5).

Anterior segments (7–9 mm wide, i.e. opposite to the side of herniation), containing the outer annulus fibrosus (oAF), inner annulus fibrosus (iAF) and occasionally the nucleus pulposus (NP) were removed during surgery (autopsy) and immediately dissected into four pieces. Due to sequestration in the posterior portion of the discs in approximately 70% of the investigated discs, only five cases could be studied, where the posterior portion with mostly iAF dorsal and oAF dorsal remained. These specimens were processed for standard histology, vital staining with trypan blue, cell death with "TUNEL" and ultrastructural examination.

Imaging

Standard radiographs were carried out in the anterior/posterior and lateral projection of the cervical spine. CT-scans were performed on a GE Lightspeed 64. MRI scans were performed on a Siemens Avanto 1.5T (sagittal and axial T1 + T2 weighted). Four observers (two radiologists and two traumatologists) examined all images separately; discrepancies in scores were resolved by consensus opinion. The score is able to distinguish different stages (a five-grade scale) of degeneration with specific parameters for standard radiographs and/or MRI-scans. The score is based on the Thompson's classification for degeneration and biochemical parameters. Multiple parameters for the radiological score (e.g. disc height, osteophytes, intradiscal calcification, sclerosis, endplate shape) and for the MRI score (e.g. T2 intensity loss, Modic changes, Debit score, annular tears, osteophytes, NP shape, endplate integrity) lead combined or for their own to comparable results [14].

For the control group, photographs from the anterior aspect were taken during autopsy.

Morphology at the light microscopical level

Samples were fixed in Schaffer solution [15] and in paraformaldehyde followed by dehydration in an ascending series of ethanols and embedded in methyl-methacrylate (MMC) or paraffin wax, respectively [15]. The MMC sections were stained with Goldner's trichrome and the paraplast sections with haematoxylin and eosin. Morphological features of the disc were noted such as ruptures, cluster formations, blood vessel in-growth and the general tissue integrity. In addition, total cell counts/mm^2 were undertaken by photographing one field of view ($\times$10 objective) of a haematoxylin- and eosin-stained section.

Trypan blue exclusion test and "TUNEL" investigations were set to demonstrate cell viability and apoptosis, respectively, as previously described [12].

Ultrastructural studies

Samples were dissected into three or four cubes, from the outer, mid- and inner annulus and, when present, the nucleus pulposus. Samples were fixed in 2.5% glutaraldehyde for 12 h and post-fixed with 2% osmium tetroxide. After embedding in Araldite, ultra-thin sections were cut and stained with uranyl acetate and lead citrate [16]. Sections were examined in a Zeiss EM 10 transmission electron microscope. Both matrix and cell morphologies were assessed. At least 27 cells were examined in each sample of the oAF, 23 in the iAF and a minimum of 13 cells in the NP.

Table 1 Prolapse group (P) and osteochondrosis group (O): morphological investigations

Pat. Group Pain onset	Age / Sex	DG O P Pt	Level	Cluster >10	Cluster 4 –10	Vessel ingrowths	Balloon Cells in %	adjacent segment herniation
1 P >4yrs	33 f	II P	C5/6	NP	iAF-NP		oAF 4 iAF 18	
2 P >2yrs	35 f	II P	C5/6			oAF	oAF 12 iAF 36	Pt:C6/7
3 P >2yrs	36 f	II P	C6/7	iAF	oAF-iAF	oAF	iAF 21 NP 34	P:C4/5 P:C5/6
4 P >1yrs	40 f	II P	C5/6	iAF	iAF	oAF	oAF 20 iAF 17 NP 23	
5 P 6 m	43 f	II P	C6/7		NP	oAF	oAF 17 iAF 34 NP 15	Pt:C5/6
6 P >1yrs	45 f	II P	C6/7	NP	NP	oAF	oAF 24 iAF 40 NP 11	
7 P >1yrs	45 f	II P	C5/6			oAF	iAF 19	
8 P >1yrs	48 m	II P	C5/6	NP	NP		oAF 4 iAF 44	P:C6/7
9 P >2yrs	55 m	II P	C5/6	iAF-NP	iAF	oAF	oAF 36 iAF 17 NP 8	Pt:C6/7
10 P 4 m	57 m	II P	C6/7			oAF	iAF 38	
11 P 3 w	30 m	III P-O	C5/6	NP	iAF-NP	oAF	iAF 14 NP 23	
12 P <1yrs	39 f	III P-O	C5/6	NP	NP	oAF	iAF 48	
13 P 1yrs	39 m	III P-O	C4/5		NP	oAF	iAF 54 NP 17	P: C5/6 Pt:C6/7
14 P 1yrs	42 m	III P-O	C6/7		NP	oAF	iAF 48 NP 14	
15 P 2yrs	43 m	III P-O	C5/6		NP	oAF	iAF 23 NP 8	P: C6/7
16 P >2yrs	43 m	III P-O	C4/5	NP-EP	NP	oAF	oAF 13 iAF 22 NP 23	P: C6/7
17 P 3 w	44 m	III P-O	C6/7	NP	iAF-NP	oAF	iAF 63	
18 P >2yrs	49 m	III P-O	C5/6		NP	oAF	iAF 32	Pt: C6/7
19 P >2yrs	49 f	III P-O	C5/6		oAF-iAF	oAF	oAF 13 iAF 24	
20 P >2yrs	51 f	III P-O	C6/7	iAF	iAF	oAF	iAF 33	

Table 1 continued

Pat. Group Pain onset	Age / Sex	DG O P Pt	Level	Cluster >10	Cluster 4 –10	Vessel ingrowths	Balloon Cells in %	adjacent segment herniation
1 O > 3yrs	37 f	IV O-P	C5/6	NP	NP	oAF	iAF 35	P: C6/7
2 O > 2yrs	42 f	IV O-P	C5/6	NP	NP	oAF	oAF 17 iAF 9	P: C6/7
3 O >4yrs	42 f	IV O-P	C5/6	NP	NP	oAF	iAF 11 NP 20	
4 O >5yrs	52 m	IV O-P	C6/7	NP	NP	oAF	oAF 28	Pt: C5/6
5 O >6 m	57 f	IV O-P	C5/6			oAF	iAF 14	
6 O > 3yrs	59 f	IV O-P	C5/6		NP	oAF	iAF 33	P: C3/4 Pt: C6/7
7 O > 2yrs	42 f	IV O-Pt	C5/6		NP		iAF 11	Pt: C2/3, C3/4
8 O 4 m	44 f	IV O-Pt	C5/6		oAF-iAF	oAF	oAF 4 iAF 5	P: C6/7
9 O > 3yrs	45 f	IV O-Pt	C6/7	NP	NP	oAF	iAF 5	Pt: C5/6
10 O > 3yrs	46 f	IV O-Pt	C6/7	iAF-NP	iAF-NP		iAF 8	Pt: C4/5
11 O >12yrs	45 f	V O-Pt	C5/6	iAF-NP	iAF	oAF	NP3	P: C5/6 >12 yrs ago
12 O >10yrs	50 m	V O-Pt	C5/6	NP	iAF-NP	oAF	Ø	P: C6/7
13 O > 2yrs	52 f	V O-Pt	C6/7	iAF-NP	iAF-NP	oAF	iAF 9	Pt: C6/7
14 O > 3yrs	53 f	V O-Pt	C4/5	NP	iAF-NP	oAF	oAF 5 iAF 24	
15 O >4yrs	57 m	V O-Pt	C5/6		NP	oAF	Ø	Pt: C6/7 P: C5/6 3 yrs ago
16 O >7yrs	57 m	V O-Pt	C6/7	NP	iAF-NP	oAF	Ø	
17 O > 2yrs	52 f	V O	C4/5	iAF-NP	iAF-NP	LL, oAF	Ø	Pt: C6/7
18 O > 3yrs	57 f	V O	C6/7			oAF	Ø	Pt: C5/6
19 O 8 m	57 f	V O	C5/6	NP	NP		iAF 8	P: C6/7
20 O >8yrs	59 f	V O	C4/5		NP	oAF	Ø	Pt: C3/4

O osteochondrosis group, *P* prolapse, *Pt* protrusion, *DG* radiological degeneration grade

(a) *Necrotic cells* were identified when there was loss of integrity of the cell membrane and sometimes the nuclear membrane, together with swelling and vacuolisation of cell organelles and/or loss of organelle membranes [17, 18].

(b) *Apoptotic cells* were identified by shrinking of the cell and blebbing of the cell membrane. The nucleus showed DNA condensation, margination of chromatin and ruffling of the plasma membrane (budding), eventually breaking up into apoptotic bodies. These apoptotic bodies comprised cell organelles and/or nuclear material surrounded by an intact cell membrane [19–22].

(c) *Chondroptotic cells*, in contrast to the above, were defined as having patchy condensed chromatin. The Golgi apparatus and endoplasmic reticulum were increased and autophagic vacuoles were frequently present. Blebbing of cytoplasmatic materials/vesicles were present, but no true apoptotic bodies according to the definition of Kerr were apparent [10, 23].

(d) *"Balloon" cells* were defined by a rounded homogeneous nucleus (mostly euchromatin) surrounded by a sharp border, visible as an osmiophilic dark nuclear envelope. Frequently, a nucleolus was visible. Various amounts of rough endoplasmic reticulum as well as mitochondria were present in the cytoplasm of these cells. The Golgi apparatus was obvious in most cells and glycogen deposits were frequently observed [12].

Statistical analyses

Cell morphology characteristics were summarised as frequencies and percentages or with a mean, range, minimum and maximum values. The Kolmogorov–Smirnov test was used to test for normality. Subsequently, differences between unpaired groups were evaluated with an ANOVA

(if normally distributed) or a Kruskal–Wallis test (if not normally distributed) and, if necessary, further analyses were carried out with a Post hoc analyses (Sidak tests) or Mann–Whitney *U* tests. All reported *p* values were two-sided; a type I error level of 5% and a statistical power of 80% were employed. Calculations were performed using SPSS (version 16.0) software.

Results

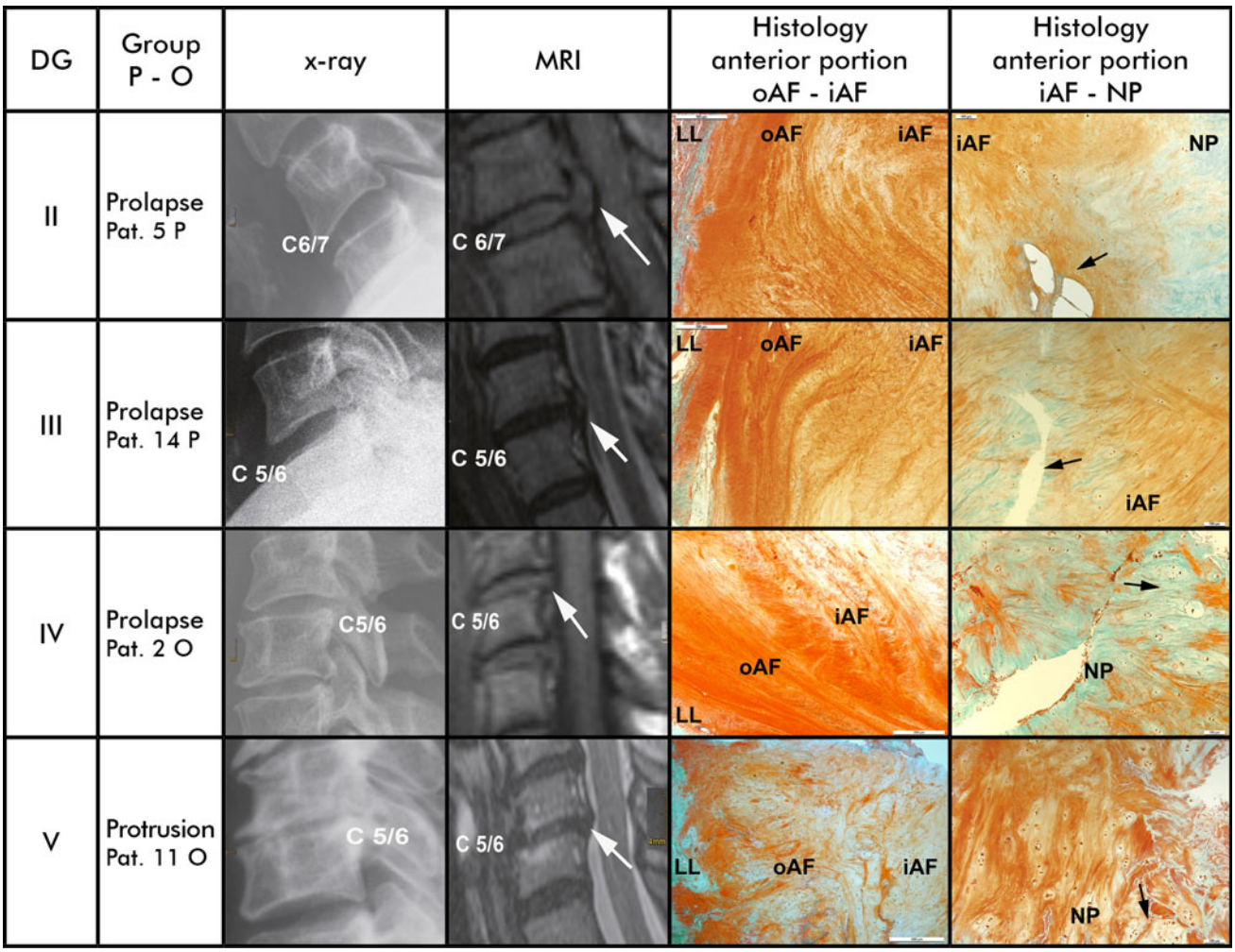
The mean age of the patients in the prolapse and osteochondrosis groups differed significantly ($p = 0.003$; P-Group (grade II, III) mean: 43.3 years; O-Group (grade IV, V) mean: 50.3 years). The time between onset of pain in patients of the prolapse group varied between 3 weeks to more than 2 years, whereas most of the patients of the osteochondrosis group stated experiencing pain for up to 12 years (Table 1).

Imaging

Degeneration grade II and III

Disc height loss was found ranging from 0–20%. The vertebral margins were rounded in grade II, whereas in grade III segments the margins were mostly pointed. Osteophytes were rare and, if seen, were smaller than 2 mm. There was no calcification observed in grade II and III segments, nor than sclerosis and the endplate shape was defined as normal and continuous (Table 2: Pat. 5 P DGII; Pat. 14 P DG III).

Table 2 Radiological and corresponding histological findings in the different degeneration grades (DG) in disc herniation



Standard radiographs in the lateral projection of the diseased segment. Corresponding MRI pictures: DGII and DGIV are T1 weighted; DGIII and DGV are T2 weighted. Retrolisthesis of the segments of DGIV and V. Subchondral edema is seen in DGV. Disc prolapse is obvious in the segments of DGII and III; protrusion in DGIV and V. Histological investigations in the different DG II-V show increasing disorganization of the different layers, cysts (DGII) and ruptures (DGIII-V) in the anterior portion of the disc. Hugh cluster formations are obvious in DGIV and DGV (*black arrow*)

CT and/or MRI scans revealed disc prolapse in all of the investigated segments. In this group, only one patient revealed Modic changes Type 1. The endplate was intact in all cases (Table 2: Pat. 5 P DGII; Pat. 14 P DG III).

Degeneration grade IV and V

Disc height loss of more than 20% was obvious and maximal with grade V degeneration. Nearly all patients presented with osteophytes either ventrally or dorsally. Intranuclear calcifications were absent. All patients presented a moderate (DG IV) to severe sclerosis (DG V) with irregular and sometimes disrupted endplates (Table 2: Pat. 1 O DGIV; Pat. 11 O DGV).

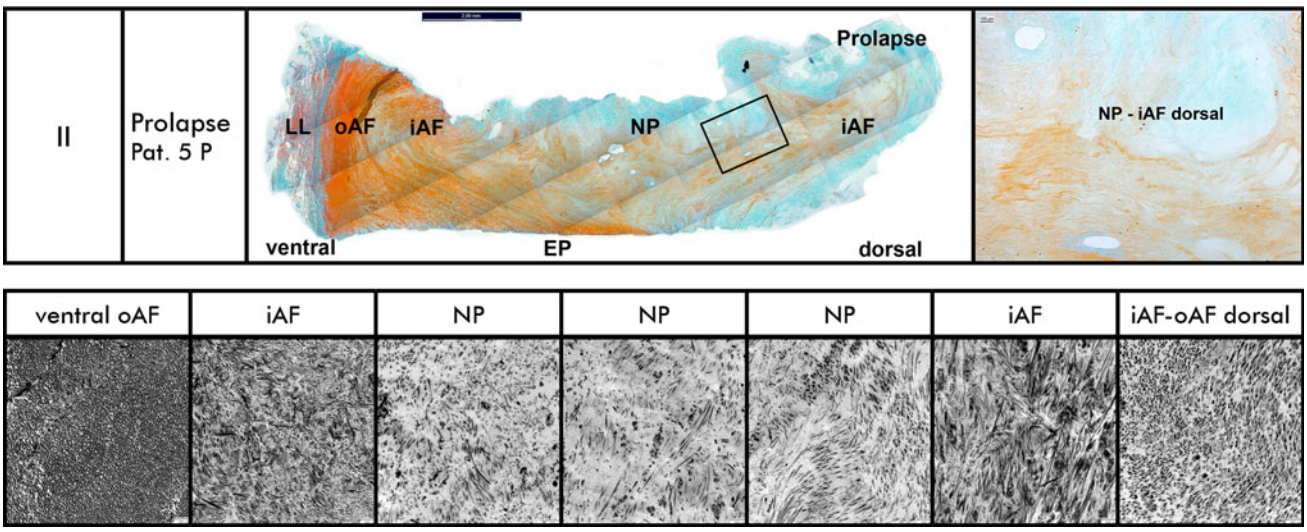
MRI scans revealed varied changes such as prolapse and protrusions. The nucleus was misshapen and in no case was rounded/oval. A few patients revealed Modic changes of Type I, but no Modic changes of Type II and III (Table 2: Pat. 1 O DGIV; Pat. 11 O DGV).

Histological observations

The architecture of the different layers within the oAF showed early signs of disorganization and due to soft disc herniation the anterior aspect was collapsed (Table 2: Pat. 5 P DGII; Pat. 14 P DG III). In the osteochondrosis group, single layers looked thinned, disorganized and could not be visually separated (Table 2: Pat. 1 O DGIV; Pat. 11 O DGV). Discs from both groups presented with blood vessel in-growths especially in the oAF, whereas cluster formation was observed frequently in both the iAF and the NP (Table 1). No marked fibrocystic cell invasion was seen around vessel in-growths that appeared to approach the oAF from the longitudinal ligament. The posterior portion was obviously more disorganized (Table 3: Pat. 5 P DGII) with more and larger cluster formations in the osteochondrosis group.

It was obvious that with increasing degree of degeneration, single cell proliferation was more prevalent in the oAF and the iAF, whereas cell proliferation clusters were found in the iAF and the NP (Table 1). There were significantly more cells/mm^2 in the oAF in grade V than in grade IV samples of the osteochondrosis group (DG IV < DGV: $p = 0.012$). Comparing the prolapse and the osteochondrosis group, a significant difference was obvious in all regions (oAF $p = 0.026$; iAF $p = 0.011$; NP $p = 0.011$) (Fig. 1). Cyst formations were observed mostly at the border of the oAF and iAF and were present in most specimens. Surprisingly, the NP was not the most affected area of degeneration and did not show the highest levels of cell death according to Trypan blue staining (Fig. 2) especially in those discs with higher degeneration grades. TUNEL analyses revealed the highest rates in the NP of the prolapse group, but only 5.6% cells were TUNEL positive. The outer and inner annulus showed even less TUNEL positivity. The osteochondrosis group presented less TUNEL positive cells in all regions than the prolapse group (Fig. 2). There was no significant difference either for Trypan blue or TUNEL investigations found between the prolapse or the osteochondrosis group.

Table 3 Histological mosaic picture and corresponding extracellular matrix (TEM) showing the anterior and posterior portion of the disc of Patient 5P (DGII)



Corresponding extracellular matrix variations of anterior and posterior regions is shown in detail throughout the disc in the different portions (oAF, iAF and NP) of the disc. The different fibre arrangements (oAF: cross-section of parallel fibres; iAF: network structure of fibres) of the different portions are visible (4,000×)

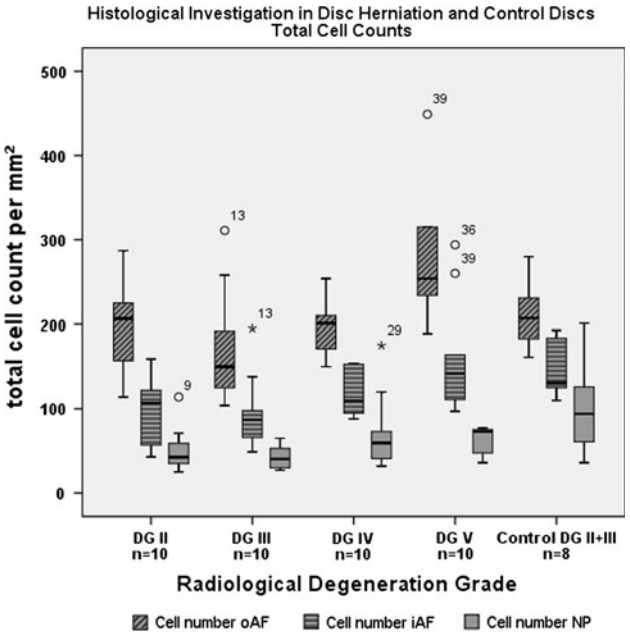


Fig. 1 *Box* and *whisker plots* of the incidence of total cell counts per mm^2 identified by light microscopic appearance in the outer and inner annulus fibrosus and nucleus pulposus of human discs with different degeneration grades (DG II–V) and for the control discs. The *boxes* show the 25 and 75% percentile and median (*horizontal line*) values. *Bars* show the upper and lower extremes

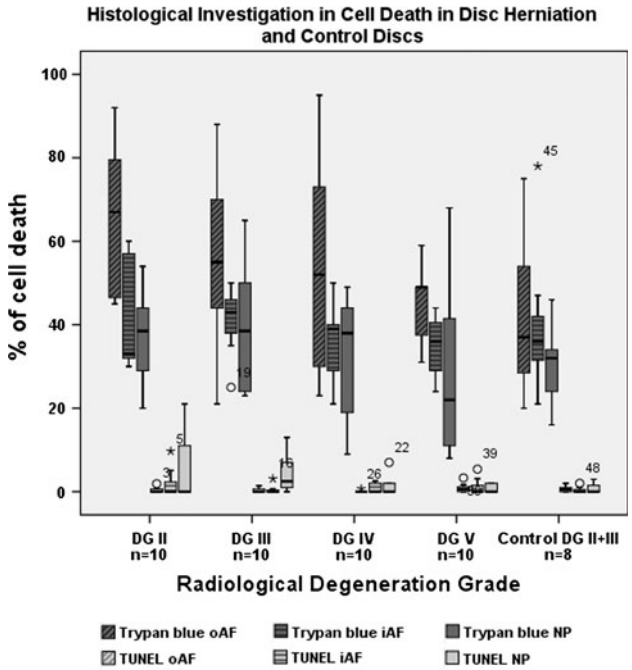


Fig. 2 *Box* and *whisker plots* of the incidence of cell death (trypan blue positive cells, TUNEL positive cells) identified by light microscopic appearance in the outer and inner annulus fibrosus and nucleus pulposus of human degenerated discs with different degeneration grades (DG II–V) and for the control discs. The incidence of trypan blue-positive cells and TUNEL positive cells identified by light microscopy, fluorescence microscopy, respectively, of the same groups is shown equally for the different disc regions. The boxes show the 25 and 75% percentile and median (horizontal line) values. *Bars* show upper and lower extremes

Ultrastructural observations

The different disc cell morphologies of the pathological discs are shown in Fig. 3. Statistical significances between the prolapse and osteochondrosis group (and between the different degeneration grades (II:III; IV:V)) are presented in Table 4.

Cell and matrix

There were only few micro-ruptures in the intervertebral disc matrix (oAF and iAF) in some samples of both groups. The fibre structure seemed to be mostly unaffected but in some patients appeared thinned. In the inner AF and outer AF of the posterior portion fibre structure looked relatively thickened when compared with the anterior portion (Table 3: Pat. 5 P DGII). Cells from the outer regions were mostly fibrocyte-like and were aligned along collagen fibres, in contrast to the iAF and NP where cells looked more rounded and possessed an obvious lacuna. All patients of the osteochondrosis group with grade V presented with healthy-appearing cells containing glycogen storage granules, whereas in patients with prolapsed discs (DG II and DG III) and patients with grade IV, these cells were often seen but with varying frequency. The prolapse group presented in 65% of the discs healthy-appearing cells with no glycogen deposits. In contrast, only 45% of the investigated discs of the group O presented healthy-appearing cells with no glycogen deposits, but in a small number. Clusters of healthy disc cells were found in all of the degeneration grades (Fig. 4a, b). Significant differences between the groups were only seen in the iAF and NP, where the prolapse group displayed the least number of healthy-appearing cells (mean 45%) and the osteochondrosis group the highest number (mean 57%) in the iAF (iAF $p < 0.001$; NP: $p = 0.009$). In the prolapse group, a significant difference between grade II and III was seen in the oAF ($p = 0.009$) and the NP ($p = 0.015$), whereas no difference in the osteochondrosis group was observed between grade IV and V.

Necrosis

Necrosis presented with swollen cell organelles, loss of ribosomal ER, ruptured cell membranes and a complete loss of cell integrity, with no obvious osmiophilic structures. In some patients, the nucleus seemed to be more involved in the necrotic changes; there was a thickened nuclear membrane instead of the nuclear double layer

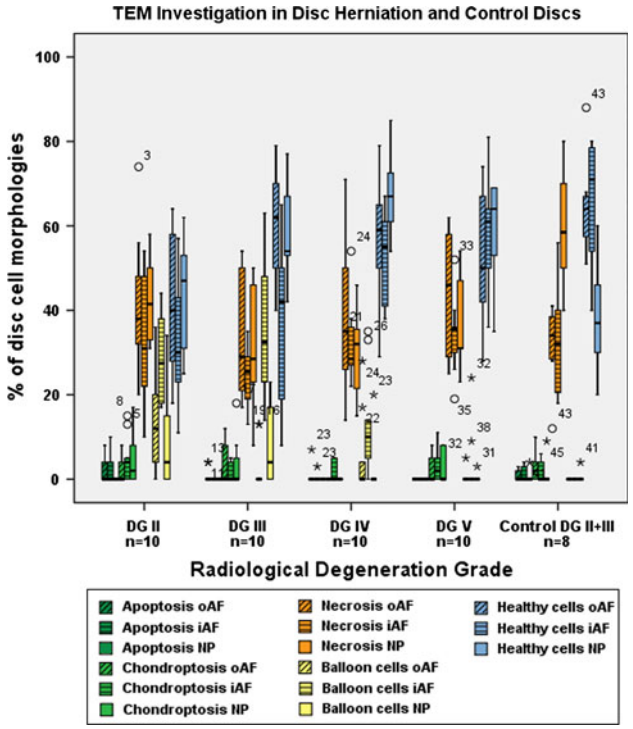


Fig. 3 *Box* and *whisker plots* of the incidence of different cell morphologies identified by ultrastructural appearance in the outer and inner annulus fibrosus and nucleus pulposus of human degenerated discs with different degeneration grades (DG II–V) and for the control discs. The incidence of different disc cell morphologies of the same groups is shown equally for the different disc regions. The boxes show the 25 and 75% percentile and median (*horizontal line*) values. *Bars* show the upper and lower extremes

membrane, together with a reduction in the perinuclear cytoplasm (Fig. 4c). grade V discs frequently revealed this type of necrosis. Between 8 and 74% of cells were necrotic (mean 35%) in all regions of the IVD, with no significant differences between the groups or the different degeneration grades.

Apoptosis and chondroptosis

In general, apoptotic and chondroptotic cells (Fig. 4d) presented with less necrotic features in the cytoplasm. In the prolapse group, only six patients presented with apoptotic cells in the oAF (8%) and two patients in the iAF (4%). Apoptosis was not seen in the NP. In these groups, cells of oAF and iAF showed slightly higher levels of chondroptosis (up to 17%) (Fig. 3).

Apoptotic cells were not seen in the grade V osteochondrosis group, and few chondroptotic cells were present (oAF mean 1%; iAF mean 3%; NP mean 6%). No significant difference was between the prolapse and the osteochondrosis group or in between the different grades of those groups.

'Balloon' cells

"Balloon" cells were obvious in all patients of the prolapse group (DG II + DG III), particularly in the iAF. In those cases where the posterior portion was present, a similar number of "balloon" cells could be counted in the dorsal

Table 4 Incidence of cell morphologies, identified by TEM, in human discs from the different degeneration degree in the prolapse and osteochondrosis group

	Prolapse DG II	Prolapse DG III	p DGII <> DGIII	Osteo-chondrosis DG IV	Osteo-chondrosis DG V	p DGIV <> DGV	p P (DDII/III) <> O(DDIV/V)	p P (DDII/III) <> Control (DDII/III)
Apoptosis and chondroptosis								
oAF	5/9 56%	5/10 50%	Ø	1/10 10%	3/10 30%	Ø	Ø	Ø
iAF	8/10 80%	3/10 30%	Ø	5/10 50%	5/10 50%	Ø	Ø	Ø
NP	5/10 50%	3/10 30%	Ø	0/7 0%	3/9 33%	Ø	Ø	Ø
Necrosis								
oAF	9/9 100%	10/10 100%	Ø	10/10 100%	10/10 100%	Ø	Ø	Ø
iAF	10/10 100%	10/10 100%	Ø	10/10 100%	10/10 100%	Ø	Ø	Ø
NP	10/10 100%	10/10 100%	Ø	7/7 100%	9/9 100%	Ø	Ø	0.002
Ballooned								
oAF	7/9 78%	2/10 20%	0.017	3/10 30%	1/10 10%	Ø	Ø	0.023
iAF	10/10 100%	10/10 100%	Ø	9/10 90%	3/10 30%	0.008	<0.001	<0.001
NP	5/10 50%	5/10 50%	Ø	1/7 14%	1/9 11%	Ø	0.015	0.043
Healthy cells								
oAF	9/9 100%	10/10 100%	0,009	10/10 100%	10/10 100%	Ø	Ø	Ø
iAF	10/10 100%	10/10 100%	Ø	10/10 100%	10/10 100%	Ø	<0.001	<0.001
NP	10/10 100%	10/10 100%	0.015	7/7 100%	9/9 100%	Ø	0.009	0.028
Age			Ø			0.013	0.003	<0.001

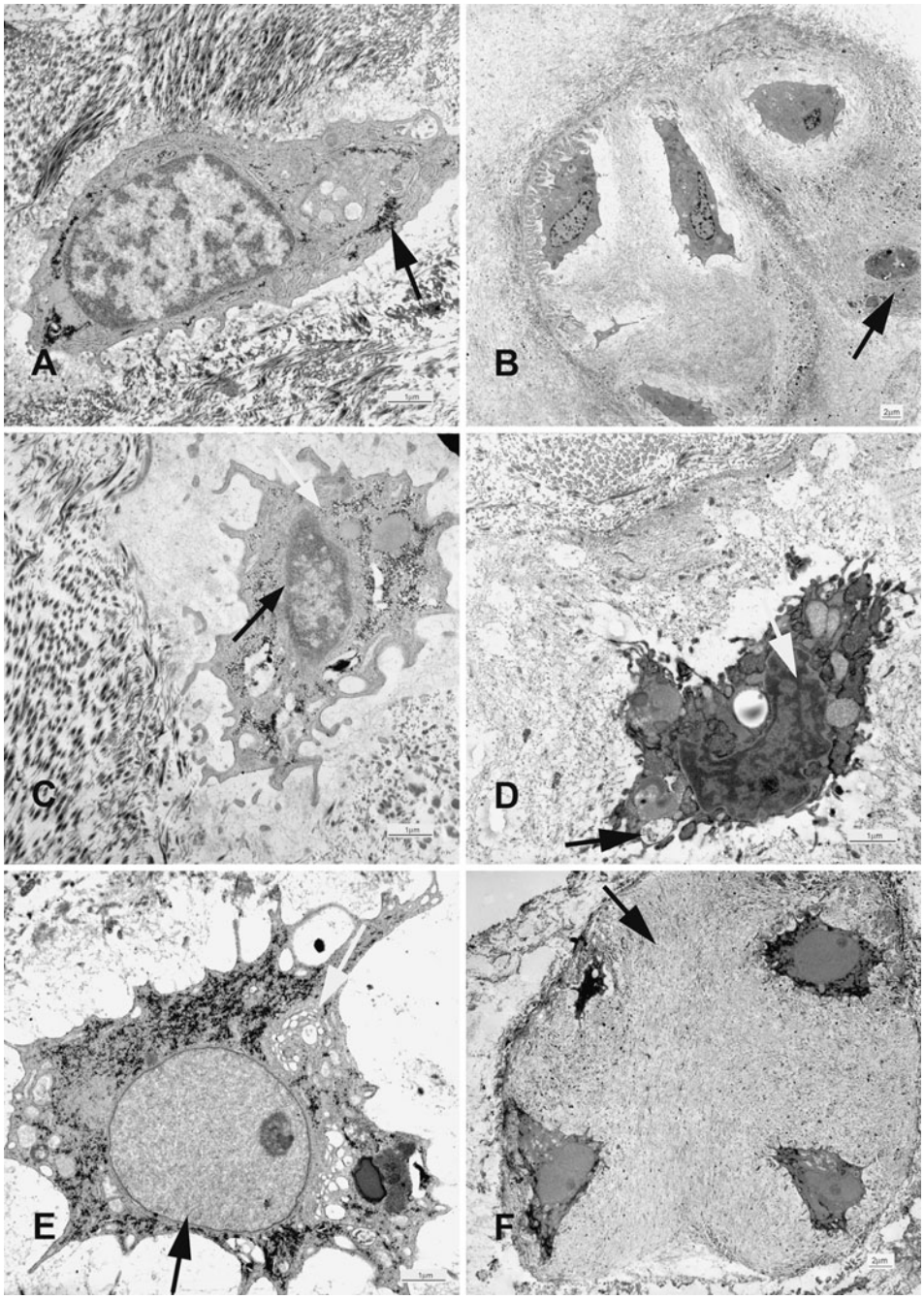


Fig. 4 **a** A healthy cell in the iAF at a magnification of 8,000×. Patient (No 18 P) and DG III: Note the intact cell membranes of rough endoplasmic reticulum. Evidence of glycogen storage (*black arrow*). Intact disc matrix where the cell is aligned in between the collagen bundles. **b** A cross-section of a healthy cell cluster in the iAF at a magnification of 1,600×. Patient (No 2 O) aged 47 years with severe osteochondrosis/DG IV (C5/6). No obvious glycogen storage in those healthy-appearing cells. Note the fine collagenous fibres of inner AF next to the lacuna. Cell residuum is pointed with a *black arrow*. **c** Necrosis in a patient of the osteochondrosis group at a magnification 8,000×. Patient (No 10 O) aged 46 years DG IV (C6/7) oAF. The nucleus without a visible double layer membrane surrounded by necrotic cell cytoplasm (*black arrow*). Note also dissolution of the matrix. **d** A chondroptotic cell at a magnification 8,000×. Cell from the iAF. Patient (No 20 P) aged 42 years DG III (C6/7). A dense cell matrix with osmiophilic stained organelles with some vacuolization. The nucleus shows many areas of condensed darkly staining heterochromatin (*white arrow*). **e** A 'balloon' cell in a patient of the prolapse group at a magnification of 8,000×. Patient (No 9 P) aged 55 years DG II (C5/6) in the iAF. The nuclear matrix stains entirely homogeneously with evidence of euchromatin (*black arrow*). Next to the nucleus the Golgi Apparatus (*white arrow*). There is evidence of organelle structure and heavy osmophilic cytoplasmic inclusions that may be glycogenic. **f** A 'balloon' cell cluster in the NP at a magnification of 2,000×: Patient (No 3 P) aged 36 years with disc prolapse DG II (C6/7). Again, the nucleus stains homogeneously. Also here evidence of glycogen storage. Note the huge lacuna with thin collagen fibres (*black arrow*)

iAF. In contrast, less balloon cells were found in dorsal oAF than in the ventral portion of the oAF (Patient 5P: ventral oAF 17%; iAF 34%; Np 15%; dorsal iAF 33%; oAF 6%). Generally, the cytoplasm was of a healthy appearance, but most cells displayed glycogen storage granules in their cytoplasm. Cell cluster formation occurred, comprised solely of "balloon" cells. The matrix around "balloon" cells in iAF and NP (but not in the oAF), was for the most, densely packed with thin collagen fibres. Cells seemed to be pressed towards the boarder of the lacuna in clusters (Fig. 4e, f). In the oAF hypertrophic "balloon" cells were found to contain a huge amount of rough endoplasmic reticulum in their cytoplasm. Highest numbers of "balloon" cells were observed in patients with prolapsed discs and a grade of III in the iAF (mean 36%). Patients with a radiological degeneration grade of II with herniation presented with similar cells but at a lower frequency (mean 28%).

All patients with a radiological degeneration grade of IV and prolapse or protrusions presented with "balloon" cells mostly in the oAF and/or iAF (range 4–35%). "Balloon" cells were observed in three patients with protrusions of the osteochondrosis group (DG V) with either 9 and 24% in the iAF or 3% in the NP. Another patient (DG V) with just osteochondrosis and disc prolapse in the adjacent segment with a grade IV had 8% "balloon" cells in iAF, but none were found in the other regions.

The prolapse and the osteochondrosis group revealed significant differences in the iAF ($p < 0.001$) and the NP ($p = 0{,}015$). A significant difference was obvious between grades II and III in the prolapse group in oAF ($p = 0.017$) and between the grades IV and V in the iAF ($p = 0.008$) of the osteochondrosis group.

Control group ($n = 8$)

Gross morphology showed normal or slightly diminished disc height, rare antero-lateral (<2 mm) osteophytes and none to mild sclerotic changes at the endplates (Table 6).

Histologically the layers in the outer and inner AF seem to be unaffected. In DG III ($n = 5$) discs some ruptures with surrounding cluster formations were obvious at the border between the outer and inner AF or in the NP (Tables 5, 6). Trypan blue investigations showed a slightly decreased rate of cell death compared with the pathological discs of DGII and III. Only some TUNEL positive cells were found (Fig. 2).

With respect to a post-mortem study, the ultra-structural investigations showed less necrosis in the outer regions. From outer regions towards the NP more necrosis was found. In contrast, there had been more healthy-appearing cells in the oAF. Glycogen deposits were rarely found in those cells (<5%). Apoptotic and chondroptotic cells were rare in all compartments. "Balloon" cells were not seen in DGII, whereas a few "balloon" cells were found in one disc of DGIII. Those "balloon" cells presented a diminished cytoplasm with rare ER and mitochondria. The different disc cell morphologies of the control discs are shown in Fig. 3.

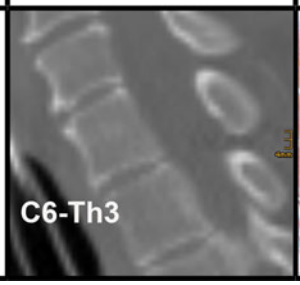
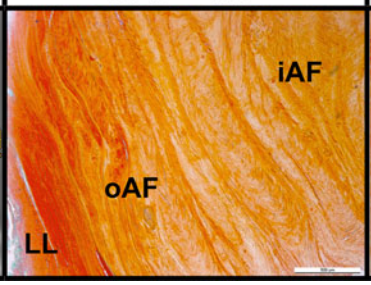
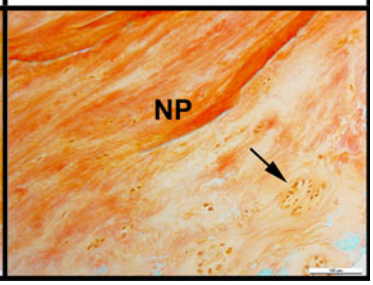
Discussion

In this paper, we examined 40 anterior discs with DDD of degeneration grade II-V in relation to cell morphology changes at EM level. As controls, eight discs without an

Table 5 Control group (C)—morphological investigations

Patients number	Age/sex	Pain onset	rDG	Level	Cluster >10	Cluster 4–10	Cluster <4	Vessel ingrowths	Ballon cells in %
1 C	70 f	No pain	II	C6/7	oAF–iAF	oAF–iAF	oAF–iAF	LL, oAF	Ø
2 C	67 f	No pain	II	C4/5		iAF	iAF	LL	NP 4
3 C	67 f	No pain	III	C5/6		iAF–NP		oAF	Ø
4 C	67 f	No pain	III	C6/7	NP		oAF–iAF	LL, oAF	Ø
5 C	72 f	No pain	III	C4/5			NP		Ø
6 C	72 f	No pain	III	C5/6			iAF–NP		Ø
7 C	74 f	No pain	III	C5/6			NP	LL, oAF	Ø
8 C	74 f	No pain	II	Th1/2			NP	oAF	Ø

Table 6 CT scans of the control patient (No 4C) 67 years DGIII (C5/6) shows minor ventral osteophytes in the adjacent segment C6/7

DG	Control Group	CT-Scan	Histology anterior portion oAF - iAF	Histology anterior portion iAF - NP
III	Control Pat. 4 C			

Histological investigations revealed intact layers in the oAF and iAF, as well as some cluster formations in the NP (black arrow) in the investigated segment C5/6

obvious evidence of DDD were studied. We found a significant change in proportion of "balloon" cells at the different degeneration grades in patients with DDD, but surprisingly, the proportion of necrotic cells did not change with increasing degeneration grades, but remained around 35% at all stages. The proportion of healthy-appearing cells increased with degeneration, especially in the NP of the most degenerated discs. In contrast, control discs presented less cell death in the outer regions and there were no "balloon" cells in DGII. Although the control group was not age-matched, some degenerative signs such as small cluster formations were obvious, but with significant differences to the prolapsed and osteochondrosis group. Fornasier et al. described in 80% of his autopsy analyses degenerative changes in NP such as cloning of chondrocytes, clefting and mucoid or fibrinoid degeneration. Moreover, none of our control discs showed any vacuum phenomenon in CT scans [24]. Other post-mortem studies have shown that lumbar discs do not narrow with age. We were able to confirm this with CT scans of the lumbar spine of the controls [25]. Roberts et al. published that changes seen with ageing are similar to those seen in degenerative disc disease and that this cause discussion as to whether ageing and degeneration are same processes or have to be separated [6].

As the largest avascular tissue, the healthy mature discs contains only limited blood vessels and nerves in the outer AF and is mainly dependent on the nutrient supply through the endplate by diffusion [7, 26]. Horner et al. stated that because the extracellular matrix is synthesized and maintained by the cells of the disc, the profiles of extracellular matrix composition across the disc presumably arise from differences in cellular activity. Different mechanical signals have been found to have a powerful influence on matrix synthesis and turnover. The metabolite concentration also varies across the disc, being highest in the NP. They found that different regions of the disc are populated by cells that are apparently distinct, with different developmental origins. They described that the matrix composition across the disc could arise because of different cell types. Their study shows clear morphological and synthetic differences among disc cells from the nucleus, inner annulus and outer annulus [27].

With increasing age, mild microscopic degenerative changes are known to occur and include alterations in cell density in the NP. In degenerative disc disease, enhanced disc cell death occurs but also cell proliferation with cluster formation [6]. Kokubo et al. described structural changes such as vessel in-growth and macrophage infiltration into the outer layers of the AF in herniated discs and Risbud et al. [28] report evidence for progenitor cells in cervical discs. In herniations, fragmented hyaline cartilage was observed [29]. Others were able to show at the light microscopic level, small cysts and fissures that, due to shear stress, form clefts in the posterior portion of the cervical disc [30]. Specifically in the outer layers of the IVD, structural changes in the herniated disc were more profound than in spondylotic discs. With increasing degeneration, spondylotic discs showed more advanced changes in the iAF. Kokubo et al. hypothesised that the differences indicated a different degeneration process in herniated and spondylotic intervertebral disc [31].

In the present study, we investigated the anterior portion of the disc. In this area, ruptures were rare and when present, they occurred in the NP and/or iAF, but there were some cyst formations present in nearly all the discs examined. Histological examination revealed varying degrees of cluster formation and vessel in-growth in different degenerative grades but with a tendency to an increased amount in the more degenerate discs.

When comparing disc cell morphologies in the anterior portion of the disc, in disc prolapse and severe

osteochondrosis, quantitative and qualitative differences were obvious. Necrosis was evident in all tissues examined with no significant differences between the different radiological grades of degeneration. It has been reported that in aged tissue, up to 80% of cell death is necrotic [32].

However, there were varying degrees of non-necrotic cell death evident that involved both apoptosis and a specialised form known as chondroptosis, both of which were within the pathological IVD. The latter form of cell death was first described in chondrocytes and was suggested to result from the lack of phagocytic and other immune-related cells within cartilage [10, 33].

The effect of compressive load and/or mechanical damage on articular cartilage and the thoracolumbar disc has been described in several publications. Apoptosis was detected by TUNEL, immunohistochemical demonstration of caspase-3 production and by TEM [34–38]. It was shown that with increasing compressive load, apoptosis rates increased [39]. Despite, apoptosis has been suggested not to be a widespread phenomenon in vivo in osteoarthritic knees [8, 40]. We have been able to show similar results for osteochondrotic discs. In studies on herniated disc material, apoptosis was previously shown to play a major role [41]. In our study, the opposite side of the disc of the herniation was analysed. Decreased apoptosis rates, similar to that found for TUNEL rates, were seen in patients with higher degeneration grades. However, previous studies did not delineate between apoptosis and chondroptosis. Nevertheless, our data revealed similar findings for degenerative disc diseases in the IVD and we would argue that chondroptosis was more frequently observed than classical apoptosis in all investigated pathologies.

Recently, our group observed a new disc cell morphology in fractured discs involving compression. We have called this new cell morphology the "balloon" cell. In trauma patients, these "balloon" cells seem to be independent of age, gender or degenerative score. The most traumatized "balloon" cells displayed differences in their cytoplasm, varying from mainly intact membrane structures of organelles to slight vacuolization to severe necrotic changes [12]. Similar changes could be observed in degenerated discs, but at a considerably lower frequency. In disc prolapse, most of these cells appear living, sometimes hypertrophic and with the ability to form clusters. Furthermore, in all patients with a soft disc herniation, "balloon" cells were present. What the significance of this cell type is not known. It is clear that the cells show a variety of degenerative features and yet may be metabolically active. However, at the same time, the nuclear structure is striking in that there appears to be little if any heterochromatin but mostly euchromatin lending further credence to their metabolic status. In osteoarthritic articular cartilage, it is well known that cell proliferation accompanies cell death. In relation to the above, the pattern of homogeneous euchromatin is also similar to that seen in many tissues of highest activity [13].

Baba and colleagues studied the posterior portion of cervical herniated disc and were able to show that the presence of herniated discs correlated with degeneration of cartilaginous EP [42]. Particularly, the middle and posterior sections were found separated from the subchondral bone. Additionally, vertical and horizontal tears were more marked in the posterior regions. Others have speculated that in the degenerative process in ageing, changes in cartilaginous EP occur first and may cause changes in NP [11]. According to Vernon Roberts there are two potentially weak points: the cartilage endplate and the posterior and posterolateral segments of the annulus, which not only are thinner than the anterior and lateral segments but are also less firmly attached to the bone [43]. Tsuji et al. observed a very complex structure in the posterior middle annulus, which was found in ten foetal, one child and one adult disc. He found that disc rupture may be influenced by these anatomical variations [44]. Furthermore, as a consequence of the physiological position (lordosis) of the cervical spine more loads are found in the posterior regions.

In ageing and degeneration, structural disorganisation of the cartilage endplate with cracks as well as bone sclerosis occurs. First, age-related changes are described to occur at the end of the first decade of life [45]. Surprisingly, we found in our study a significantly higher number in total cells/mm^2 and significantly higher number of healthy-appearing cells in the more degenerated discs (DGIV and V), which presented radiologically a severe reduction in disc height and a marked sclerosis of the EP, compared with segments with disc prolapse (DGII and III). Furthermore, the observed glycogen deposits especially in disc cells of higher degeneration grades indicate a change to a more anaerobic cell metabolism [46]. Moreover, rare glycogen deposits were seen in the control specimens presenting a low radiological degeneration grade in the aged control group. On the other hand, it is known that EP sclerosis causes a decrease in nutrient supply and, thus, advanced changes in the IVD [7, 26, 47]. Only half of the control discs, but nearly all of the herniated discs showed an in-growth of vascular vessels from the longitudinal ligament inwards the outer AF. Surprisingly, with this change of nutrition supply the disc cell density increased especially in the outer regions, but the "balloon" cell morphology decreases with increasing DG.

Spontaneous regression changes of herniations in the lumbar spine are well known but less frequently seen in the cervical spine. Furthermore, conservative therapies are less responsive to cervical disc herniations, and the surgical gold standard for disc herniation is still decompression by

discectomy and fusion or total disc replacement by implants potentially preserving the spinal motion. Surgical procedures are necessary if conservative treatments fail and pain persists or neurological deficits are obvious. Recently, a new minimal invasive technique has been presented. Percutaneous PDD (plasma disc decompression) might offer a disc-preserving method resolving pain in patients with a painful disc herniation, where conservative treatments failed. This method might also offer a possibility to preserve the different disc cell morphologies in the disc [48].

This paper shows that the different disc cell morphologies changes significantly with increasing radiological degeneration grade. Furthermore, we could demonstrate the "balloon" cells are present with highest rates at the time of soft disc herniation. The evidence of endogenous progenitor cells in human degenerated discs was suggested to orchestrate the repair of those intervertebral discs. Currently, it is impossible to say if this change in morphology of disc cells being "balloon" cell is an additional part of a repair strategy or cause of herniation. Further investigations are needed to establish the role of the "balloon" cell in disc herniation.

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Conflict of interest None.

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