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Differential sacnning calorimetric examination of the human intervertebral disc: establishment of calorimetric standards of different stages of degeneration

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Abstract

Intervertebral disc (IVD) degeneration is a common orthopaedic disorder with significant social and economic impact. The major pathological changes occur in the structure of anulus fibrosus (AF) and nucleus pulposus (NP). According to previous reports differential sacnning calorimetric (DSC) proved to be a suitable method for the demonstration of thermal consequences of local and global conformational changes in the structure of the human intervertebral discs. According to the present study, the DSC results clearly proved that definitive differences are present between the stages of disc degeneration in calorimetric measures. The structural differences between the stages could be also demonstrated by histology.

Keywords: DSC; Intervertebral disc; Degeneration; Anulus fibrosus; Nucleus pulposus

1. Introduction

From all the tissues of the musculoskeletal system cartilage is the most affected in the degenerative changes. The degeneration of the intervertebral disc (IVD) begins already in adolescent and progresses along life. Degeneration itself is part of the natural aging process, however, several additional factors may influence its development. Physical and mechanical shocks, microinjuries play pivotal role in changes of the mechanical and morphological characteristics of the IVD eventually leading to severe destruction [1–3].

Previously we reported the results of calorimetric characterization of normal and degenerated discs. We found that calorimetry is a suitable method to investigate the IVDs and can detect small structural changes resulting in altered thermodynamical features. First, we presented that the annulus fibrosus (AF) and nucleus pulposus (NP) show thermodynamically distinct behavior. The thermal denaturation of normal AF and NP is almost identical regarding the main transition temperatures, but completely different in the total calorimetric enthalpy changes. These results served as standards for further comparison. Second, we compared the calorimetric curves of the thermal denaturation of degenerated and healthy specimens and found significant differences; furthermore, significant differences were present between the AF and the NP of the degenerated discs as well [4].

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2. Hypothesis-objectives

Since we found differences between the two endpoints of the disc degeneration, we decided to explore and characterize the intermediate stages. We hypothesized that utilizing large number of specimens from patients of different ages and differently compromised motional segments we may comprehend the structural changes along the degenerative process.

The aims of the study were:

1. to establish the calorimetric standards of the different stages along the degeneration;
2. being aware of the biochemical and biomechanical changes in the length of degeneration, to find the elements, molecules that could be responsible for the thermodynamic outcomes;
3. to complete our investigation with histological analysis.

3. Materials and methods

3.1. Sample preparation

IVD specimens were obtained from L4-L5 segments of cadavers with age ranging from 14 to 86. According to the visual evaluation of macroscopic changes affecting the motion segments we enrolled the discs into different stages described by Thompson [5]. This morphologic evaluating system is based upon the macroscopic appearance of the motion segment elements (i.e. AF, NP, endplate, vertebral body) on the transsection cut sagittally 5 mm lateral to the mediansagittal plane (Table 1). Out of 40 samples, 6 were in stage I, 8 were in stage II, 8 were in stage III, 8 were in stage IV, and 10 were in stage V according to Thompson classification. The mean age in the different groups was as follows: stage I—20 years (17–24), stage II—33 years (19–40), stage III—46 years (39–68), stage IV—57 years (43–84), and stage V—78 years (70–88).

The samples were obtained by devices specially designed for this task, with standard methods and from the same anatomic regions. The shape of the sample was prismatic with 5 mm of length and 10 mm of width. All samples were indentical in sizes. Specimens were harvested only from cadavers lacking the anamnesis of any spinal disease, operation involving the spinal column, general connective tissue disorder, diabetes, or disease resulting in deposition of crystals in the tissues (e.g. gout, chondrocalcinosis). Furthermore, obese individuals were also excluded from the study (BMI $>$ 30). All tissues were yielded in accordance to legal regulation, international ethical concerns and relative consent.

Samples were washed three times in PBS (sterile phosphate-buffer saline, pH 7.4) in order to eliminate tissue remnants, than the NP was separated from the anulus fibrosus in all specimens. Samples were then put into RPMI-1640 solutions (Sigma) containing 10% fetal bovine serum (Hyclone laboratory), antibiotic,

Table 1
Description of the morphologic grades of intervertebral disc degeneration according to Thompson

Stage	Nucleus pulposus	Anulus fibrosus	End-plate	Vertebral body
I	Bulging gel	Discrete fibrous lamellas	Hyalin, uniformly thick	Margins rounded
II	White fibrous tissue peripherally	Mucinous material between lamellas	Thickness irregular	Margins pointed
III	Consolidated fibrous tissue	Extensive mucinous infiltrations; loss of anular-nuclear demarcation	Focal defects in cartilage	Early chondrophytes or osteophytes at margins
IV	Horizontal clefts parallel to end-plate	Focal disruptions	Fibrocartilage extending from subchondral bone; irregularity and focal sclerosis in subchondral bone	Osteophystes <2 mm
V	Clefts extend through nucleus and anulus		Diffuse sclerosis	Osteophystes >2 mm

antimycotic solution (1 U/ml penicillin, streptomycin and fungisone, Gibco laboratory) non-essential amino acids (Gibco laboratory) and sodium carbonate. All the individual samples were stored separately at 4 °C, no longer than 12 h, then subjected to calorimetric measurements.

3.2. Differential sacnning calorimetric (DSC) measurements

The thermal unfolding of IVD was monitored by a SETARAM Micro DSC-II calorimeter (SETARAM, France). All the experiments were performed between 0 and 100 °C with a scanning rate of 0.3 K/min. Conventional hastelloy batch vessels were used during the denaturation experiments with 850 μl sample volume in average. RPMI-1640 buffer was used as reference sample. The sample and reference vessels were equilibrated with a precision of ±0.1 mg. There was no need to do any correction from the heat capacity point of view between the sample and reference vessels. The samples were irreversibly denatured during each cycle.

3.3. Evaluation of DSC scans

The repeated scan of denatured sample was used as a baseline reference, which was subtracted from the original DSC scan. Calorimetric enthalpy was calculated from the area under the heat absorption curves using two points setting SETARAM peak integration.

3.4. Statistical analysis

Paired Student's *t*-test were used for statistical analysis with a significance level of 0.05.

3.5. Histological evaluation

The samples from different stages of Thompson categories were examined histologically. In addition to hematoxylin–eosin staining, we also performed PAS and Giemsa staining, as well as picrosirius staining of the collagen fibers. PAS stains certain fibers and the extracellular cartilage matrix (containing sugar components in high quantity) purple. Giemsa stains proteoglycans purple due to metachromasia, while picrosirius stains collagen fibers red, allowing the detection and analysis of the collagen fibers when using polarized light [6].

4. Results

The results of the calorimetric measurements of degenerated IVDs were highly diverse (Table 2). Comparing the consecutive stages, highly significant differences were found between stages I, III and V in both the main transition temperature and the total calorimetric enthalpy changes ($P < 0.05$).

When we examined these samples histologically, the structure, organization, and the density of fibers of the AF after staining were definitely dissimilar. In

Table 2
Calorimetric results of IVDs from different stages of Thompson evaluation system

Stage	Number of samples	Average of age (year)	Anulus fibrosus	Nucleus pulposus
I	6	20	T_m (°C): 60.5 ± 0.3 ΔH (J/g): 0.87 ± 0.04	T_m (°C): 60.7 ± 0.4 ΔH (J/g): 0.45 ± 0.07
II	8	33	T_m (°C): 60.6 ± 0.4 ΔH (J/g): 0.80 ± 0.1	T_m (°C): 60.4 ± 0.2 ΔH (J/g): 0.43 ± 0.07
III	8	46	T_m (°C): 61.1 ± 0.4 ΔH (J/g): 0.62 ± 0.07	T_m (°C): 59.5 ± 0.2 ΔH (J/g): 0.37 ± 0.09
IV	8	53	T_m (°C): 62.5 ± 0.3 ΔH (J/g): 0.48 ± 0.09	T_m (°C): 58.9 ± 0.3 ΔH (J/g): 0.30 ± 0.05
V	10	76	T_m (°C): 62.7 ± 0.3 ΔH (J/g): 0.42 ± 0.05	T_m (°C): 58.6 ± 0.2 ΔH (J/g): 0.29 ± 0.04

T_m, transition temperature; ΔH, calorimetric enthalpy; both are presented with average and standard deviation.

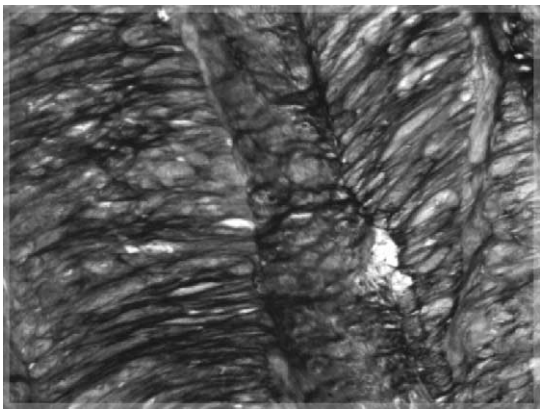

Fig. 1. AF of stage I sample (picrosirius stained section at 200× magnification).

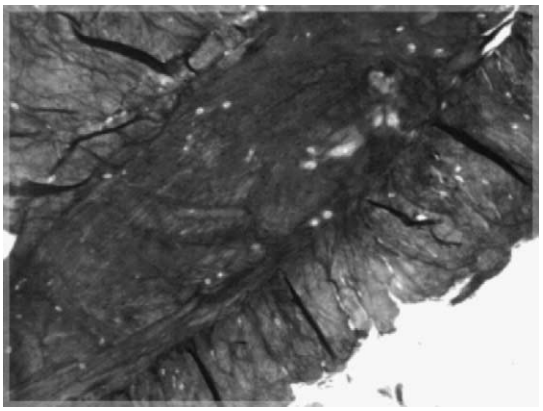

Fig. 3. AF of stage V sample (picrosirius stained section at 200× magnification).

stage I, the fibers are heading to different directions quite randomly, yet holding repetitively similar angles with the neighboring fibers and composing relatively wide and organized bundles. The individual fibers are distinct and recognizable, forming an even, finely structured network lacking irregularity in the overall density (Fig. 1). In stage III, the bundles of fibers are various in size and density. The individual fibers are very difficult to distinguish, the fine structure becomes more irregular, fibers conglomerate (Fig. 2). In stage V fibers become confluent, denser, and no fine structure is present. The main direction of the fibers is not recognizable, the whole structure becomes homogenous (Fig. 3).

The histology of the samples from the NP also adequately correlates with the results gained from the calorimetric experiments. In stage I (originating from young healthy cadavers) the NP appears as a tissue dominated by cells, yet stained very light, and morphologically the specimens seemed soft and gelatinous. Histologically, the residue of the dorsal chord is still recognizable—shows as bright groups of cells in the central area (Fig. 4). In stage III, the tissue is less rich of cells, the extracellular matrix is more compact with the signs of degeneration in the central region and macroscopically curious denaturized granular substance is seen (Fig. 5). In stage V (samples from cadaver at advanced age) the tissue contains

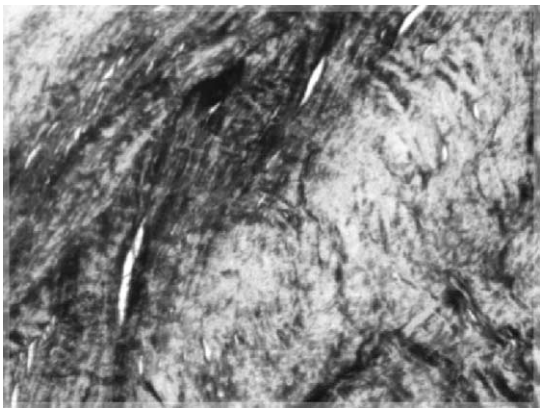

Fig. 2. AF of stage III sample (picrosirius stained section at 200× magnification).

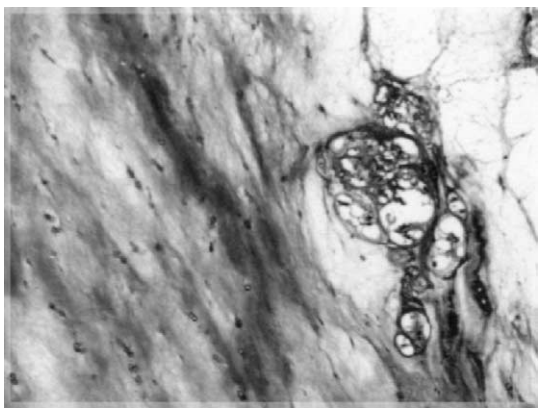

Fig. 4. NP of stage I sample (Giemsa stained section at 200× magnification).

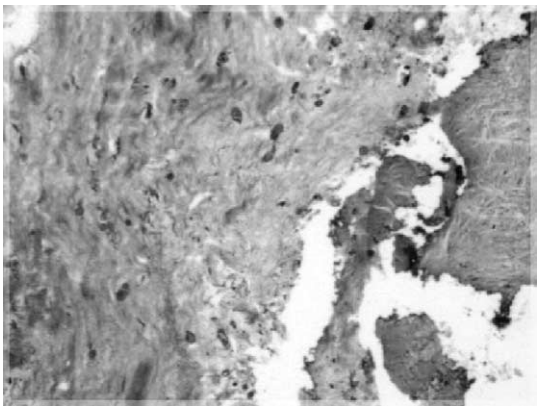

Fig. 5. NP of stage III sample (Giemsa stained section at 200× magnification).

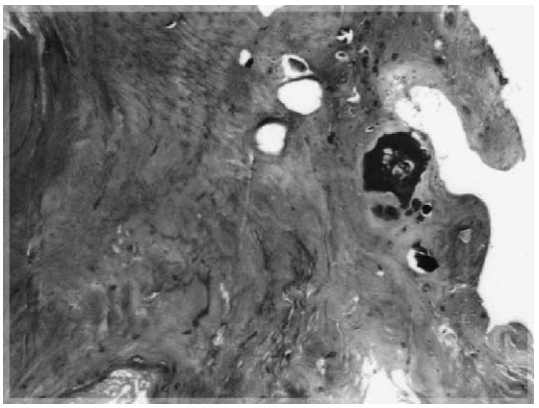

Fig. 6. NP of stage V sample (Giemsa stained section at 200× magnification).

fibers in much higher density, in a distinct, yet homogenous pattern, and focal calcification is present (Fig. 6).

5. Discussion

The normal (healthy) NP is a gelatinous tissue with high water content entrapped by negatively charged proteoglycan network connected and fortified by the collagen fibers. The water content of the NP can be as high as 80–90% of the total weight. Of the non-water constituents proteoglycans represent 65%, collagens represent 20%, while the remaining 15% is made up of elastin and several other components. In the healthy AF the collagen fibers are strictly organized running around the NF finally merging into the cartilaginous end plate, connecting the adjacent vertebral bodies. Crossing each others, the collagen fibers form a three-dimensional structure. The non-water constituents of the AF are composed of collagen (60%), proteoglycans (20%) and, in small quantities, various elastic fibers. AF is quite less hydrated than the NP, with a total water content of about 60–70% [7–9].

In the young IVD, the two major parts are distinct and easy to distinguish, even macroscopically. The healthy IVD fulfills all the requirements that are present during the normal physiological function of the spine: forms a very flexible structure for all kind of motions, absorbs most of the shocks arising during normal daily activity. The intact structure of AF and NP is inevitable to execute this sophisticated function of the spine. Being a highly hydrated tissue, the NP exerts an immensely high pressuring effect on the AF. At the same time the arising forces in the NP are transferred into all surrounding tissues in all three dimensions (Fig. 7). Ultimately, this peculiar situation results in the attempt to push the end plates apart and protrude the fibers of the AF in a radial direction [10,11].

According to our results, in the group of discs in stage I no significant difference was found regarding the main transient temperature between the AF and NP. This fact implies that the two tissues are both highly hydrated and retain a very integrated structure. It was suggested by the relatively narrow range of temperature for thermal denaturation and the almost symmetrical shape of the curves. These facts hint at the strong cooperation between these components. The endothermic peak at about 60 °C proposes the presence of stable biological macromolecules and this phenomenon is probably due to the denaturation of the collagen and proteoglycan molecules of the disc. The differences in the total calorimetric enthalpy is supposedly due to the different ratio of the two main components (collagen: AF: 60%, NP: 20%; proteoglycan: AF: 20%, NP: 65%) and their structure (NP: gelatinous with high water content, AF: concentrically organized, more than 60 distinct collagen fibril layers running in alternating directions). The AF is more complex and in order to decompose its more

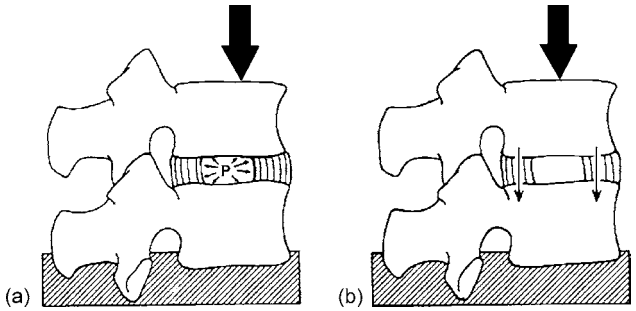

Fig. 7. Biomechanics of compression load transfer in healthy (a) and (b) degenerated discs.

compact structure significantly more energy is needed, thus it results in significantly higher enthalpy changes ($P < 0.05$).

As a result of the degenerative process characteristic changes can be observed in the structure and function of the IVD. By the third decade of life the definitive margin between the AF and NP is absent, since the latter gradually becomes fibrotic. This morphological change is accompanied by the decline of the concentration of proteoglycans, water and other non-collagenous proteins and the increase of the ratio of collagen. The decrease of proteoglycans and their ability to aggregate, as well as the loss of water is greater in the NP along the time [8]. The number of collagen fibrils (preferably type I collagen) and their size in diameter increases along the life span in every part of the IVD [12]. Repeating mechanical strain, probably via the increased production of proteolytic enzymes (especially matrix metallo proteinases), results in the degradation of the original structure of collagens and proteoglycans. As a consequence, the IVD starts to disintegrate and several different morphological changes develop (e.g. fissures, narrowing of the intervertebral gap, herniation, etc.) [4,8,13]. The degenerated NP, loosing its water content, becomes unable to complete its physiological function and to change its shape in a highly flexible manner. The ability of the IVD to transmit load becomes highly compromised. In contrast to the normal situation where the central region of the endplates are mostly loaded, the peripheral regions of the end plate get loaded, therefore considerable part of the compressive forces are transmitted to the neighboring vertebra through the AF [2,14] (Fig. 7).

The elevated main transition temperature is a consequence of the overload in the degenerated AFs. In this structure, disintegrated mostly by the matrix metalloproteinases, due to the mechanical overload secondary bindings may develop (intra- and intermolecular hydrogen bridges), the entire structure becomes more tightly ‘packed’. For the disintegration of this compact structure extra energy was necessary, thus the structural phase transformation began at a higher temperature. These changes reduce the ability of the originally elastic annulus to change its shape (elastic trait is decreased), but the stability of the structure increases. The decrease of the enthalpy of this degenerated structure is attributed to the loss of bound water and thermal cooperation of the components. It was also suggested by the widening of the thermal transition period and the asymmetry of the curves themselves. The drop in the main transient temperature in the degenerated NP is mostly due to the loss of the immensely hydrated proteoglycans. The fragmentation of this structure results in the decrease of bound water clusters, and so consequently the decrease of the thermal capacity (ability to store heat energy). In calorimetry, the significantly lower thermal capacity is an important sign of the loss of water clusters, resulting in a greater baseline shift when compared to the native stage. The consequence is the significantly smaller changes in the enthalpy and less thermical cooperation ($P < 0.05$) in the degenerated NPs in comparison with the control (healthy) specimens.

The DSC results clearly proved that definitive differences are present between the stages of disc degeneration in calorimetric measures. The structural differences between the stages were undoubtedly

demonstrated by histology. In contrast to the five stages defined morphologically, we were able to establish only three distinct stages when comparing the results from thermical analysis: the two marginal categories (stages I and V), and stage III in accordance to the Thompson evaluation system. It could be explained with the fact, that calorimetry measures more complex changes affecting the biochemical and biophysical structure as a whole. IVD degeneration is a continuous process rather than a disease with distinct stages, and stages established by morphological and histological means can not be distinguished when looked into in a completely different, peculiar thermical aspect. We believe that further studies are necessary to identify the exact roles of the biochemical components of the IVD, strongly influencing the thermal character.

Acknowledgements

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