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Differential scanning calorimetry in the research of degenerative musculoskeletal disorders

P. Than [a], I. Domán [a], D. Lőrinczy [b,*]

[a] *Department of Orthopaedic Surgery, Medical and Health Sciences Centre, University of Pécs, Pécs, Hungary*
[b] *Faculty of Medicine, Institute of Biophysics, University of Pécs, Szigeti út 12, H-7643 Pécs, Hungary*

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Abstract

Degenerative musculoskeletal disorders are very frequent diseases in human beings; the pathology of these has been a subject of much research before, using a wide spectrum of examining methods. DSC is a well-established method for the investigations of thermal consequences of alterations in biological systems. With foregoing studies, the authors have demonstrated the feasibility of DSC in the investigation of the intact hyaline cartilage and intervertebral disc (IVD). The aim of this study was to establish the thermograms of hyaline cartilage and intervertebral disc degeneration according to different stages. The calorimetric experiments with osteoarthritic knee joint samples and degenerated discs repeatedly demonstrated that the method is feasible for the investigation of tissues of the musculoskeletal system. The studies clearly demonstrated thermal differences between various stages of osteoarthritis and intervertebral disc degeneration.

Keywords: DSC; Musculoskeletal disorders; Osteoarthritis; Hyaline cartilage; Intervertebral disc

1. Introduction

Degenerative musculoskeletal diseases are among the most frequent medical problems affecting large parts of the population. Due to these diseases, millions of people across the world are not able to carry out their work, spend their leisure time as they would like to. Recognizing this problem, the WHO has dedicated the first decade of the new century to the research of musculoskeletal diseases, calling it the “bone and joint decade”. The special meaning of the problem is underlined by the fact that in the United States more than 16 million people are affected by degenerative joint disorders and a total of 750,000 joint replacements are performed annually because of osteoarthritis of the hip and the knee [1,2]. Besides the weight-bearing major joints, the most affected part of the musculoskeletal system is the lumbar spine. According to the data of “The Bureau of National Health Data Statistics” the cost of the surgical treatment of degenerative lumbar spinal problems and its indirect costs represent an amount of 16 billion dollars each year [3].

The above facts also underline the importance of degenerative musculoskeletal disease research, out of which the research of the aforementioned has to be paramount. The growth of basic research data and the etiological exploration of arthritis makes it possible to prevent the progression of the disease thus easing the pain of patients and taking off the financial burden of endoprosthetic interventions from the medical healthcare system.

Several methods are possible for the basic research of osteoarthritis, the number of related publications in international literature is almost impossible to overview. With our preliminary studies, we could justify that calorimetry—which is a long-established method for the research of biological systems—is feasible for the investigation of tissues of the musculoskeletal systems, namely joint hyaline cartilage and intervertebral disc (IVD) [4,5].

The purpose of the given study was to assess if calorimetry is feasible for the detailed study of structural deformities caused by degenerative diseases and for the statement of fine differences according to stages. Our further aim was to compare the experienced calorimetric scans with histological examinations both regarding the hyaline cartilage and the intervertebral disc.

* Corresponding author. Tel.: +36-72-536-260; fax: +36-72-536-261.
E-mail address: denes.lorinczy@aok.pte.hu (D. Lőrinczy).

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2. Materials and methods

2.1. Examination of hyaline cartilage

Human samples considered pathological were derived from waste tissues of operations, such as femoral condyle and patella pieces removed during knee replacement. Selected patients (27) had clinically and radiologically proven osteoarthritis, both in patellofemoral and femorotibial joints and were operated on at the Department of Orthopedics. In all patients, uni- or total condylar knee replacement was carried out. Average age of the patients was 64 years (46–79). The stage of osteoarthritis was classified during operation according to Outerbridge [6]. Stage I was diagnosed in 2; stage II in 14; stage III in 11 patients. The samples were formed to final form under sterile circumstances and put into storage liquids. We especially focused on the calorimetric description of various stages of degenerative deformities, to maintain objectivity, additional histological examinations were carried out at the Institute of Pathology of the University.

Twenty-seven arthritic samples were examined, all were derived from different individuals. Gender ratios were approximately identical in each groups. In order to make measurements more objective, exclusively cartilage from the weight-bearing surface of the medial femoral condyle and from the medial facet of the patella were measured. From all the patients, two to three samples were taken from the same place, on one hand, to be able to repeat the measurement in case of any disturbance on the other hand, to test the reproducibility of the measurements with different samples taken from the same patient. Although the form of the sample does not influence the examination, our target was to standardize its size. Samples were prepared in a cylindrical form, measuring 3 mm in diameter and 15 mm in length.

2.2. Examination of intervertebral discs

Intervertebral disc specimens ($n = 40$) were harvested from L4–L5 segments of cadavers with age ranging from 14 to 86. All samples were obtained during autopsy with standard methods and from the same anatomic regions within 24 h post-mortem. The shape of the sample was prismatic with 5 mm of length and 10 mm of width. All samples were identical in size. 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 (body-mass index (BMI) >30). All tissues were yielded in accordance to legal regulation, international ethical concerns and relative consent. The applicability of the intervertebral discs taken from cadavers for IVD research is accepted. According to the visual evaluation of macroscopic changes affecting the motion segments, we enrolled the discs into different stages described by Thompson et al. [7]. This morphologic evaluating system is based upon the macroscopic appearance of the motion segment elements (i.e. anulus fibrosus (AF), nucleus pulposus (NP), endplate, vertebral body) on the trans-section cut sagittally 5 mm lateral to the mediansagittal plane. While stage I sample displays no degeneration, stage V sample shows severe degeneration. Samples of stages II, III and IV represent increasing degree of degeneration. According to Thompson classification, out of 40 samples, 6 were in stage I, 8 in stage II, 8 in stage III, 8 in stage IV, and 10 were in stage V. The mean age in 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); stage V 78 years (70–88).

2.3. Methods

Samples were washed three times in sterile phosphate-buffer saline (PBS), pH 7.4) in order to eliminate tissue remnants, so than in case of IVD samples, the NP was separated from the anulus fibrosus in all specimens. The samples were then put into RPMI-1640 solutions (SIGMA) containing 10% fetal bovine serum (HYCLONE Lab.), antibiotic, antimycotic solution (1 U/ml penicillin, streptomycin and fungisone, GIBCO Lab.) non-essential amino acids (GIBCO Lab.) and sodium carbonate. All the individual samples were stored separately at 4 °C, no longer than 12 h and then were subjected to calorimetric measurements.

The thermal denaturation of different parts of human hyaline cartilage and DSC samples was monitored by a SETARAM Micro DSC-II calorimeter. All the experiments were performed between 0 and 100 °C. The heating rate was 0.3 K/min. Conventional Hastelloy closed batch vessels were used during the denaturation process with 850 μl sample volume, on average without any purge gas. The pieces of different cartilages were stored in RPMI-1640 solution and used for sample, while the pure RPMI-1640 solution served as reference. The sample and reference vessels were equilibrated with a precision of ±0.1 mg. There was no need to do any correction from the point of view of heat capacity between the sample and reference vessels. The samples were irreversibly denaturated during each cycle.

The samples of hyaline cartilage and IVD from different stages of degeneration were also examined histologically. In addition to haematoxilin eosin staining, PAS and Giemsa staining, as well as picrosirius staining of the collagen fibers were performed [8].

3. Results

3.1. Examination of hyaline cartilage

Curves of the arthritic samples showed basic differences to the intact cartilage—published earlier [4]—both in terms of the patella and the femur. Independent of the differences

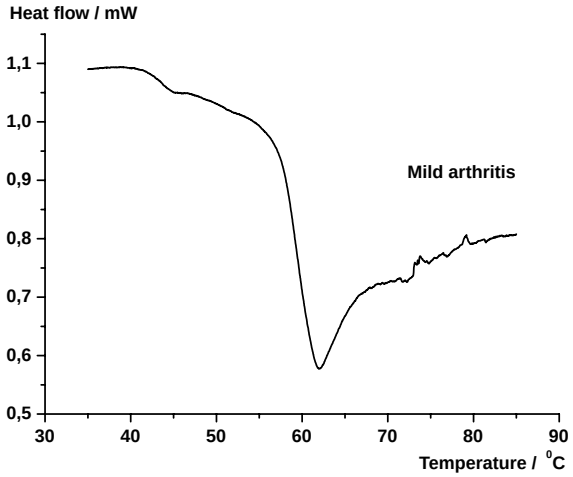

Fig. 1. Thermal denaturation of a hyaline cartilage with slight osteoarthritis. Endothermic transitions are directed downwards.

between samples, a characteristic endothermic reaction could be observed in the range of 60–70 °C with each arthritic sample. This thermodynamic effect occurred both in samples harvested from the femoral condyle and the patella. Even in samples with slight osteoarthritis (Fig. 1), the endothermic reaction at around 60 °C could be observed in all cases. The advanced cases (Fig. 2) also showed this thermodynamic effect between 60 and 70 °C, but two important differences could be observed in all samples.

1. On one hand, with severe arthritis there was no significant difference between the thermal capacities of the starting and ending condition. The underlying reason could be that the thermal capacity of any biologic system is basically dependent on the amount of water tied. In a tissue showing little water content at the start (which is the case in advanced osteoarthritis), the heat capacity of starting and ending stage does not differ significantly. Contrarily, as can be seen in Fig. 1, the less affected cartilage showing larger water content will have differences in heat capacities, since during denaturation it will gradually loose its water content, the graph stabilizes at a lower value at the end of the heating process.
2. The other evident difference between Figs. 1 and 2 is the fact that the endothermic reaction at 60 °C shows a narrower “peak” in case of the less affected cartilage. The reaction takes place quickly, while in advanced osteoarthritis the peak is wider, the thermal effect resulting presumably from collagen denaturation is significantly slower. This can theoretically be due to the reduced quantity of collagen, its abnormal structure or the deviations of the surrounding basic material.

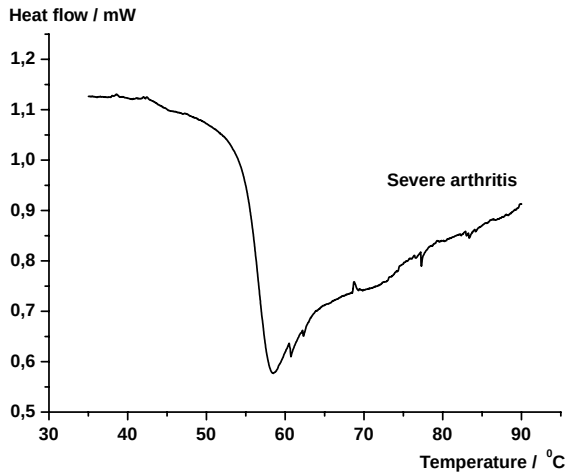

Fig. 2. DSC curve of a hyaline cartilage with advanced case.

The hyaline cartilage showing calorimetric graphs of Figs. 1 and 2 underwent histological examination as well. The assumptions stated above, regarding the differences of the calorimetric graphs have clearly been proven by histological examinations. Even by simple haematoxylin–eosin method, clear differences were evident. In slight osteoarthritis cases, some irregularities could be observed in the basic material. Cartilage cells were typical, no cell destruction could be observed. In the arthritic sample massive destruction of the cartilage could be observed, moreover cartilage cells disappeared in large areas, in other places they formed groups as a sign of regeneration. There were also signs for the multiplication of inorganic material, the picture showed signs of advanced cartilage damage.

3.2. Examination of intervertebral discs

The measurements could reliably be reproduced in different samples in several respects. The measurements from the same disc gave same results for both AN and NP. The measurements of samples of different stages from different cadavers did not differ significantly and displayed the following differences.

The thermal denaturation of healthy cadaver samples (stage I) demonstrated no significant differences between the AF and NP from the main transition temperatures (Table 1). This fact implies that both the two tissues are highly hydrated and retain a very integrated structure. The relatively narrow range of temperature for thermal denaturation and the almost symmetrical shape of the curve suggest this. These facts hint at the strong cooperation between these components. The endothermic peak at about 60 °C proposes the presence of stabile biological macromolecules and this phenomenon is probably due to the denaturation of the collagen and proteoglycan molecules of the disc. Differences were found in the total calorimetric enthalpy (Table 1, Fig. 3) which 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 compact structure significantly

Table 1
Calorimetric results of intervertebral discs (IVD) from different stages of Thompson evaluation system

Stage	No. of samples	Average age (years)	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 stands for the maximum transition temperature and ΔH is the calorimetric enthalpy change.

more energy is needed, thus it results in significantly higher enthalpy changes.

The thermal denaturation of severely degenerated cadaver samples (stage V) clearly demonstrated the differences between AF and NP (Fig. 4). This appeared in the main transition temperatures as well as in the total calorimetric enthalpy changes (Table 1). The elevated main transition temperature is a consequence of the mechanical overload in the degenerated AFs, which lead to secondary bindings (intra- and intermolecular hydrogen bridges) between the enzymatically disintegrated collagen fibres. The entire structure thus becomes more tightly 'packed'. The structural phase transformations, which begin at a higher temperature, represent the extra energy necessary for the disintegration of this compact structure. The decrease of the enthalpy of this degenerated structure is attributed to the loss of bound water and thermal cooperation of the components. The widening of the thermal transition period and the asymmetry of the curve itself also suggest this. 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 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 thermal cooperation in the degenerated NPs in comparison with the control (healthy) specimens.

The results of the calorimetric measurements of degenerated IVDs were highly diverse (Table 1). Comparing the consecutive stages, such as stages I and II or IV and V, we found no significant differences. In contrast, when stages II and III, or III and IV were compared, we found significant differences in both the main transition temperature and the total calorimetric enthalpy changes. Thus, in contrast to the five stages, defined morphologically, we were only establishing three distinct stages when comparing the results from thermal analysis: the two marginal categories (stages I and V), and stage III in accordance to the Thompson evaluation system. This could be explained by the fact, that calorimetry measures more complex changes affecting the biochemical

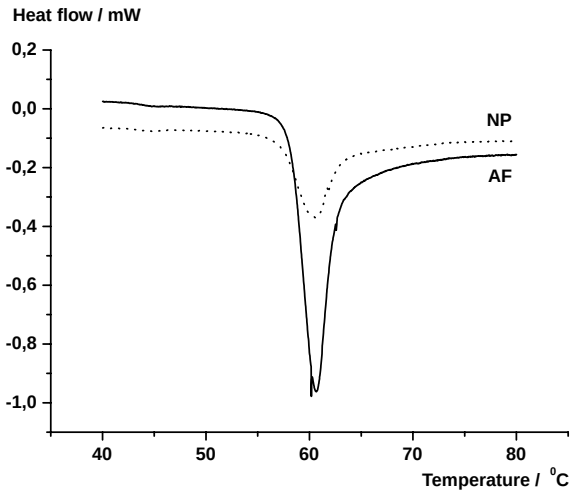

Fig. 3. DSC scan of a healthy anulus fibrosus (AF) and nucleus pulposus (NP).

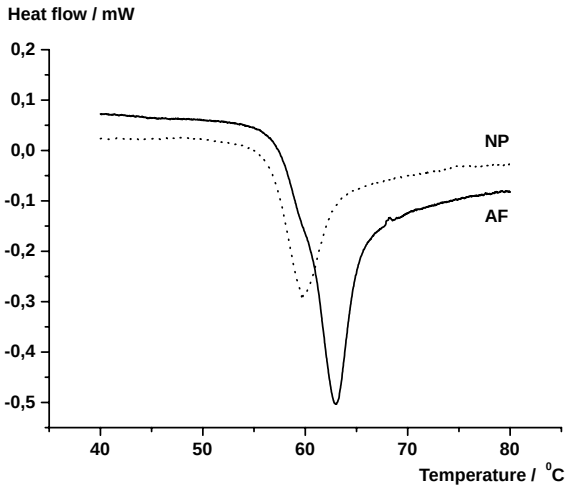

Fig. 4. Thermal denaturation of severely degenerated cadaver samples.

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 cannot be distinguished when looked into in a completely different, peculiar thermal aspect.

Samples from stages I, III, and V were also examined histologically. The structure, the organization, and the density of fibers of the AF after staining were definitely dissimilar. In 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. In stage III, the bundles of fibers are in various size and density. The individual fibers are very difficult to distinguish, the fine structure becomes more irregular, fibers conglomerate. 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. The histology of the samples from the NP adequately correlates with the results gained from the calorimetric experiments. In stage I, 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—shown as bright groups of cells in the central area. 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 present. In stage V, the tissue contains fibers in much higher density, in a distinct yet homogenous pattern, and focal calcification is present.

4. Discussion

Since calorimetry has not been applied for this purpose, our measurements cannot be compared to former publications, only our own results can be discussed. The most important achievement in our opinion is the fact that it could be proven: calorimetry is applicable to the examination of human organs of locomotion, namely the hyaline cartilage and the intervertebral disc. It could be verified that intact tissues have a characteristic and reproducible measurement result, which can serve as standard and reference value for further studies.

Our studies proved that the calorimetric scans of arthritic and healthy samples of both hyaline cartilage and IVD differ significantly; the thermal consequences of degeneration could be verified. Before discussing the possible causes of calorimetric differences, we should state that the biochemical and histological processes developing during osteoarthritis and IVD degeneration are extraordinarily complex. Therefore, it is almost impossible to explain the exact background of thermodynamic deviations observed with calorimetry when the cartilage is studied as a complex unit, only indirect conclusions can be made. The reaction observed in both tissues at 60 °C, falls into the range of stable macromolecules, the effect probably takes place because of the denaturation of the collagen or proteoglycan molecules of the cartilage and IVD. Since no deviation could be seen in healthy samples in this domain, we assume that collagen loses its thermodynamic stability in osteoarthritis and IVD degeneration, but it is too early to draw related conclusions.

Calorimetry itself is not suitable for structural studies, but together with histological examination the possible causes for the effects can be listed. The measurements carried out in the second study verified that morphologic differences between various stages of osteoarthritis and IVD degeneration can be demonstrated in an indirect way, even if not based on classic clinical categories.

We are convinced that a number of questions have to be clarified which arise as a consequence of the studies carried out so far. The most recent problem is the question, which components of the tissue can be made responsible for the deviations measured with DSC, and if the method is sensitive enough to study these. To answer these questions the main components have to be separately examined by calorimetry. The values gained this way can be compared to the values of the complete structure.

Acknowledgements

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