CHARACTERIZATION OF NORMAL AND DEGENERATED HUMAN HYALINE CARTILAGE WITH THERMAL ANALYSIS

By
Dr. Gellért Sohár

Supervisor:
Prof. Dr. Habil. Kálmán Tóth

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1. INTRODUCTION

Osteoarthritis (OA), the most prevalent joint disease, is characterized by the progressive loss of articular cartilage that leads to chronic pain and functional restrictions in affected joints. The prior notion of OA as a bland disease related to aging and “wear and tear” of the joint has given way to views of a dynamic system with multiple pathogenic contributors. Recent studies have elucidated the importance of local factors as well as crystals and inflammation in contributing to disease progression. Osteoarthritis represents a major therapeutic challenge to medical and health-care providers. In part, this is because osteoarthritis is a chronic condition in which symptoms evolve over long periods of time and in which symptomatic episodes are frequently separated by lengthy asymptomatic periods.

Over the past years, the pathophysiology of avascular necrosis (AVN) of the femoral head has not been completely elucidated. Whereas some cases of the disease clearly have a direct cause (trauma, radiation, or Caisson disease), the pathophysiology is uncertain for most cases. Multiple investigators have postulated vascular impairment, altered bone-cell physiology, and other theories.

An increasing number of publications have been published with the use of calorimetric techniques in the examination of human hyaline cartilage. Previously, thermoanalytical studies were used for the investigation of normal and degenerative human hyaline cartilage. They have concluded that structural manifestation of osteoarthritis appears as a remarkable change of thermal stability of hyaline cartilage samples. The healthy cartilage samples used in these studies were of cadaver origin as waste material, pathological cartilage was derived as intraoperative tissue fragments. The samples were washed in sterile phosphate-buffered saline and stored in complex solution containing fetal bovine serum, antibiotic, antifungal solution, and amino acids. The measurements were conducted in 48 hours of sample deriving. The reported data on the calorimetric enthalpy changes proved to be inconsistent. In severely affected osteoarthritis, the $\Delta H$ has increased almost twofold, while in an earlier study, enthalpy changes in the intact hyaline cartilage altered from higher to lower levels in some cases.

Prior studies have demonstrated the usefulness of calorimetric examination in the characterization of cartilage degeneration. We have extended the use of thermal analysis by introducing thermogravimetric investigations. Thereby new information on the physiochemical properties of normal, OA, and AVN tissues has been acquired.
2. AIM S OF THE INVESTIGATIONS

Based on previous studies, we hypothesized that

- thermodynamic findings clearly differentiate normal and degenerated human hyaline cartilage,
- physicochemical transformations may provide information on the role of water content in osteoarthritis and avascular necrosis, and
- enthalpy change of the process, initiated by the temperature change, might represent potential marker of the disease activity.

In order to get answer for the hypotheses above, patients with primary end-stage OA and AVN were chosen for our investigations.

The aim of this study was:

1. to investigate whether cartilage undergoes complex changes in matrix composition (water, proteoglycan, and collagen content) during the late stage of degeneration. These complex deviations develop from the normal matrix composition during the diseases OA and AVN are hypothesized to correlate with changes in thermal analysis;
2. to introduce thermogravimetric examination as part of thermal analysis alongside calorimetry, since water content of the cartilage has not been measured before by thermogravimetry;
3. to establish the kinetic character of the effect of water loss by heating;
4. to find correlation between the enthalpy changes and the severity of OA and AVN;
5. to establish a new protocol for sample extraction during live surgery; and
6. the further purpose of this study was to elucidate the importance of water content in contributing to disease progression.
3. PATIENTS AND METHODS

3.1 Patients

In order to conduct the thermoanalytical study, 35 samples were collected from live surgeries of OA patients between October 2005 and April 2006. During hip arthroplasty procedures performed at the Orthopedic Department, University of Szeged, 16 OA and 12 AVN human hyaline cartilage samples and normal cartilage from 7 knee were obtained. There was no clinical meaningful difference in age between OA patients (64 ± 5.2), AVN patients (59 ± 6.4) and controls (61 ± 4.2). There was no considerable sex differences between OA patients (75% females), AVN patients (60% females), and controls (70% females); Chi-square $P = 0.54$.

Preoperatively, the diagnosis of the patients were established on basis of the patient history, clinical signs, laboratory tests, and radiological findings. The state of the hyaline cartilage was determined intraoperatively. All patients in the osteoarthritic group were considered to be Osteoarthritis Research Society International (OARSI) grade 5-6 articular surface degeneration. All patients in the AVN group were classified as Ficat stage 4. Samples were considered to be normal when hyaline articular cartilage was uninvolved with OA (OARSI Grade 0).

Usually, in OA of both medial and lateral knee compartments, total knee arthroplasty is performed. When only one compartment is affected and ligamental stability is intact, unicondylar prosthesis is implanted. We were able to obtain normal cartilage samples from those patients where one compartment of the same knee was degenerated, and the other one was normal. Therefore, the unaffected femoral condyle had to be sacrificed for the procedure because ligamental instability was the indication for total knee arthroplasty.

3.2 Sample preparation for thermal analysis

After the operation, a disc (5mm in diameter) was removed from the unhealthy and healthy cartilage surfaces. The samples were taken under sterile conditions, excess bone was removed, and only the remaining full thickness cartilage was used. The disc was first washed in sterile saline, then stored in 20 ml saline for transportation at room temperature. Mean storage time was 6 hours (min: 1 hour, max: 26 hour), 29 samples out of 35 were studied.
within four hours of preparation. Six samples were stored over-night at 5 °C. Preemptive control examinations did not show any change in the calorimetric and thermogravimetric properties after storage for 26 hours at 5 °C.

3.3 Thermal measurements

The success of the thermal experiments depends on the careful preparation of samples and the judicious selection of the appropriate experimental conditions (such as scanning rate and sample size). For accurate quantitative work the thermal mass of the sample and reference pans were matched.

All the thermal measurements were conducted at the Department of Pharmaceutical Technology, University of Szeged. The calorimetric properties of samples were determined by DSC method (Mettler-Toledo DSC 821e apparatus, Mettler-Toledo GmbH, Switzerland). Samples were heated from 0 to 80 °C. The heating rate was 0.3 °C/min. Conventional Hastelloy batch vessels were used with 40 µl sample volume. All the DSC measurements were preceded in Ar atmosphere, and the flow rate was 100 ml/min. From the DSC curves, the decomposition temperature (onset temperature), the transition temperature range (endset temperature), and the total calorimetric enthalpy change were calculated. Well-defined standards and calibration procedures are particularly important, therefore high care was taken in calibrating the instrument as close to the transition temperatures of interest as possible.

The thermogravimetric analysis was performed with the use of a MOM Derivatograph (MOM, Budapest, Hungary), and the TG, DTG, and DTA curves were determined. The temperature (T) curve shows the linear increase of temperature during the process. DTG curve represents the first derivative of the mass change curve. The DTA curve shows the same picture as a Differential Scanning Calorimetry examination, in which the temperature change of a sample is compared with the temperature of a thermally inert material in order to give information about the endothermic or exothermic enthalpic transition or other reaction. The heating was linear from 25 to 150 °C and the rate of heating was 5 °C/min. Al₂O₃ was used as reference material. In the first step, the total water loss and kinetic parameters were calculated. The kinetic parameters calculated by the software are the following: the reaction order (n), the activation energy (Ea), and the pre-exponential factor (A).
3.4 Statistical analysis

Fisher LSD method by the Statistica for Windows statistical program was used to compare enthalpy changes in the different groups.

Data are presented as mean±SD. Statistical significance was assessed by the Student t test and the Kruskal-Wallis one-way ANOVA on ranks. The results were considered significant, if p < 0.05.

3.5 Ethics

All tissues were yielded in accordance to legal regulation, international ethical concerns, and patients’ consent. The Human Investigation Review Board of the University of Szeged has decided (2006.09.18.) that the experiments comply with the ethics of research and the declaration of the Medical World Federation.

4. RESULTS

4.1 Thermogravimetry

All samples were examined with derivatograph. It was found, that the average total water content of intact (normal) cartilage is 81%, which was probably the interstitial water, and the difference was supposedly bound to the surface. To remove the cartilage extracellular water content, 52 kJ/M energy was needed. Total water content of the OA samples was 87%, and 73 kJ/M energy was used for the removal of the fluid content. Cartilage obtained from necrotic femoral head had a higher water content of 88% than the normal samples. Extraction of the cartilage fluid content needed 70 kJ/M energy (Table 1).

Loss of water content in all three groups are presented with a sharp step on the TG curve, starting on average temperature of 37 °C and ending at 116 °C. Linear part of the TG curve begun at around 57 °C and ended at around 104 °C. Placing a line on this portion of the curve, the slope of the curve can be calculated which represents the speed of the water content loss (Table 2). The slope of the linear region correlated in all three groups.
Table 1. Average mass loss and activation energy of normal and degenerated samples.

In case of the normal hyaline cartilage, 0.196 mg of fluid content release was observed (average mass of the normal samples was 15.48 mg) with increase of temperature by 1 °C, therefore 1.3% °C⁻¹ loss was detected. In the osteoarthritis samples (average mass: 17.02 mg), 0.242 mg decrease was measured which represents 1.4% °C⁻¹ mass reduction. Necrotic samples (average mass: 15.51 mg) released 0.262 mg of water with the same increase of temperature, so 1.7% °C⁻¹ decrease in mass was observed. The resulting amount of mass lost in the linear region was recounted from these results (Table 2).

Table 2. Reaction kinetic parameters of normal and degenerated samples.

<table>
<thead>
<tr>
<th>Sample Group</th>
<th>Sample number</th>
<th>TG step linear region (°C)</th>
<th>Mass loss (%)</th>
<th>Reaction order (n)</th>
<th>Slope of linear region</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>7</td>
<td>62.67-102.25</td>
<td>-51.45</td>
<td>1</td>
<td>-0.039</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1 SD: 0.203</td>
<td></td>
</tr>
<tr>
<td>OA</td>
<td>16</td>
<td>58.0-104.6</td>
<td>-65.24</td>
<td>1.03</td>
<td>-0.048</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1.03 SD: 0.27</td>
<td></td>
</tr>
<tr>
<td>AVN</td>
<td>12</td>
<td>57.6-102.0</td>
<td>-75.48</td>
<td>1.03</td>
<td>-0.042</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1.03 SD: 0.32</td>
<td></td>
</tr>
</tbody>
</table>
4.2 Calorimetry

With the rise of temperature, an endothermic reaction was observed in all of the cases. The enthalpy change of the process initiated by the temperature change showed marked difference between the normal and pathological groups (Table 3).

<table>
<thead>
<tr>
<th>Sample group</th>
<th>Sample number</th>
<th>ΔH (J/g) (-)</th>
<th>DSC peak (°C)</th>
<th>Beginning (°C)</th>
<th>Ending (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>7</td>
<td>788.346</td>
<td>50.18</td>
<td>≈32.5</td>
<td>57.09</td>
</tr>
<tr>
<td></td>
<td></td>
<td>SD: 83.181</td>
<td>SD: 3.31</td>
<td>SD: 3.45</td>
<td>SD: 5.35</td>
</tr>
<tr>
<td>OA</td>
<td>16</td>
<td>543.838</td>
<td>50.34</td>
<td>≈33.8</td>
<td>≈33.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>SD: 88.572</td>
<td>SD: 2.937</td>
<td>SD: 4.3</td>
<td>SD: 4.3</td>
</tr>
<tr>
<td>AVN</td>
<td>12</td>
<td>567.083</td>
<td>48.93</td>
<td>≈34.16</td>
<td>53.38</td>
</tr>
<tr>
<td></td>
<td></td>
<td>SD: 120.17</td>
<td>SD: 5.93</td>
<td>SD: 4.37</td>
<td>SD: 4.28</td>
</tr>
</tbody>
</table>

Table 3. Thermal parameters of denaturation (mean ±SD) of normal and degenerated samples.

Greater change in the enthalpy was observed in normal cartilage: 788.346 J/g (SD = 83.18). In case of osteoarthritis 543.838 J/g (SD = 88.57), while in the necrotic samples 567.083 J/g (SD = 120.17) was measured (Table 3). Therefore, denaturation caused by heating was largest in the normal human hyaline cartilage. Consequently these samples required the largest amount of energy for decomposition. Statistical tests proved these calculations to be significant (Fisher LSD method, p<0.05). Denaturation peak in normal cartilage was at 50.18 °C (SD = 3.31), in necrotic samples it was lower at 48.93 °C (SD = 5.93) however in osteoarthritis 50.34 °C (SD = 2.93) was similar to control samples which had normal hyaline cartilage.
5. DISCUSSION

Though the comprehension about OA has grown enormously over the last years, there is still need to extend our knowledge about the basic context of OA genesis and development. Molecular pathology of osteoarthritis is under intense investigation since biomechanical factors result in chemical alteration within the joint. Rearrangements of intra- and intermolecular bonds in collagen molecule and disaggregation of proteoglycans and their elimination from OA cartilage found to be responsible for water accumulation. The data up to date show, however, that OA is a very complex disease procedure, and it can be speculated, that the context leading to the progressive process is not finally resolved.

We observed increase in water content of the cartilage matrix in all cases of the investigated degenerative cartilages. Based on our results, it can be stated that water content is higher in impaired samples, meanwhile water interstitial bonding was stronger in the OA and AVN cases. Rise in water adherence was well distinguishable since higher energy was needed for removal. Activation energy correlated considerably with water content in the samples. Denaturation caused by heating was larger in the normal cartilage than in the diseased ones, therefore normal samples required the largest amount of energy for decomposition.

The purpose of our study was also to clarify the previously reported studies in the literature. By acquiring normal cartilage from live surgery and by performing the investigation in a relatively short period of time compared to the earlier reports, similar sample environment was provided as with the degenerative samples. This way, we minimized the extracorporeal degeneration. All samples we used showed a clear denaturation peak on the calorimetric curve, therefore volume of the curve was easily calculated giving the enthalpy change of the sample. These changes correlated with the water content of the samples. Due to the increased number of samples acquired for our studies, the results were much better reproducible than results in the literature, and the difference between the normal and necrotic samples was significant.

The newly established thermogravimetric protocol that we used was sufficient for compositional thermoanalytical study of normal and degenerative human hyaline cartilage. Water content elevation contributing to disease progression was observed in both OA and AVN. Previously, this method has not been used for this type of investigations. The main goal of the thermogravimetric measurements was to identify the nature and quantity of water
molecules in the investigated samples. Water molecules’ binding mode may have an important consequence in pharmacokinetics. The reaction order turned out to be approximately 1 in all three cases (normal, OA, and AVN), and the standard deviation was low (Table 2). The TG curve’s slope of the linear region showed, that the rate of water loss depends on the water amount remaining in the tissue. Comparing the data in the presented tables (Tables 1, 2) (Total mass loss: normal: 80.79%, OA: 86.71%, and AVN: 87.80%), it can be concluded that the higher water content in the degenerative samples bound stronger to the matrix. However, the reaction order and the slope of the linear region correlated in all three groups. This first order kinetic means that the rate of water loss depends on the water amount remaining in the tissue, namely if the amount of water decreases in the tissue, the rate of loss also decreases.

DSC as part of thermal analysis was a reliable method for differentiating normal hyaline cartilage from degenerated samples. The available calorimeter proved to be adequate for these measurements.

Our study has had several limitations, as many other studies on OA and AVN. First, the sample size was not large enough to arrive at definitive conclusions. Additional measurements are needed to affirm the results of our study. Secondly, we investigated those patients for normal cartilage samples of the knee, who underwent surgery for the other compartment OA. This was the only ethical and technical way of acquiring normal tissues from living persons for our experiments. Previous thermoanalytical studies used cadaver samples for the investigation as normal human hyaline cartilage. All samples that were extracted for our studies were obtained during live surgeries and were macroscopically intact. There is no previous report in the literature of examining normal cartilage from live surgery. Only full thickness cartilage was used for the normal analysis. Prior results indicate that early OA is primarily characterized by the changes in collagen orientation and proteoglycan content only in the superficial zone, while collagen content does not change until OA has progressed to its late stage. A new protocol had to be established before the detailed investigation of human tissues could be performed. Most of the known changes in the extracellular matrix in OA come from animal models in the literature since human samples for investigation are not widely available for experiments.

Characterization of the altered metabolism in cartilage that promotes disease progression should lead to future treatment options that can prevent structural damage. With better understanding the exact amount of matrix water content and its binding characteristics, preventive measures can be developed. These therapeutic steps can be adequately tested and
monitored with thermogravimetric measurements. The use of this method can also determine the effectiveness of currently used medications (Glucosamin, Chondroitin) for resolving cartilage matrix degeneration.

Further understanding of the initiating events in cartilage destruction, the relationship between the different pathologic influences, and the role of the chondrocyte in maintaining extracellular matrix homeostasis will be necessary to reveal potential targets of therapy. Clinical trials are currently underway for a number of potential disease modifying agents that may significantly change the treatment approach for OA. With the possibility of disease-modifying OA drugs (DMOADS), the necessity for instruments that are sensitive to changes has become very apparent in clinical trials.

The promise of biomarkers has yet to be fulfilled in OA. Type II collagen, cartilage oligomeric matrix protein, hyaluronan, and aggrecan have been some of the many biomarkers investigated so far. Although numerous clinical studies have suggested that specific biomarkers or their combinations can have predictive value in terms of the presence and severity of the disease. The wide variability in these values limits their use for individual patients. Whereas, the use of thermal analysis could be a simple and effective method for controlling the relationship between these markers and disease progression. The revised protocol for sample taking during live surgeries eliminates the presence of disturbing substances during the examination. A detailed thermal examination is also needed on the same joint surface with samples taken from different grades of degeneration within the same joint.

6. SUMMARY AND CONCLUSIONS

In summary, we examined the water content of human hyaline cartilage of normal origin and in patients with OA and AVN. We were the first ones who used normal samples that were extracted from live surgeries for the investigations.

A newly established thermogravimetric protocol was used for our experiments. This method proved to be suitable for compositional thermoanalytical study of normal and degenerative human hyaline cartilage.

Our results showed clear evidence that:

- complex deviations from the normal matrix composition during the late stage of degeneration correlated significantly with changes in thermal properties;
• patients with primary OA and AVN had significantly higher levels of water content in the degenerative samples and water bound stronger to the matrix than in controls;
• the kinetic character of the effect of water loss by heating was established, the reaction order was approximately 1 in all cases;
• correlation was found between the enthalpy changes and the severity of OA and AVN;
• a new protocol was established for sample extraction during live surgery using simple saline solution instead of the previously used phosphate buffer;
• this new method proved to be suitable for the thermoanalytical investigations; and
• the introduction of thermogravimetric examination as part of thermal analysis alongside calorimetry might be a useful method for determining the severity of OA and AVN.

One of the possibilities of getting fast information is the use of thermoanalytical methods. The simple new protocol we established might also be used for gaining information about the healthy or sick state of other human tissues.

Additional investigation will be needed to fully understand how water content affect cartilage degradation. Further studies are in progress to elucidate the contribution of physicochemical properties of water in cartilage to the pathogenesis of the degenerative process of OA.

We need to strive to develop these methods and make them available in the clinical settings since without the means to monitor the effectiveness of DMOADs, we will never know if we can control and perhaps even prevent osteoarthritis.

From the work described in this thesis, a model can be proposed whereby the level of injury to cartilage within the joint can be monitored by a simple thermoanalytical method. A deeper knowledge of the pathways in the development of degenerative cartilage diseases might lead to the development of newer therapies for arthritis in the future.

We hope that our data provide further evidence for the importance of cartilage physicochemical properties in developing cartilage degeneration.
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PUBLICATIONS

List of full papers directly related to the subject of the Thesis


List of abstracts directly related to the subject of the Thesis


List of papers related to the subject of the Thesis


List of papers not related to the subject of the Thesis


List of abstracts not related to the subject of the Thesis

1) Sohár G, Anna P, Kopasz N, Tajti L, Meszáros T, Tóth K: Clinical results of screening and management of hip dysplasia at our Department. *Hip International* 2006; Vol. 16, 2, pp. 159. IF: 0.19


List of congress presentations related to the subject of the Thesis

1) Sohár G, Pallagi E, Tóth K, Szabó-Révész P: Thermogravimetric investigation of normal and damaged human hyaline cartilage. OARSI World Congress on Osteoarthritis, Prague, 2006. 12.7-10


