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Review

DSC investigation of connective tissues treated by IR-laser radiation☆

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Abstract

The structural changes of intact and laser treated connective tissues were investigated by differential scanning calorimetry and thermomechanical analysis. Total collagen denaturation in the fibrous connective tissue was observed. Partial collagen denaturation in the cartilage tissue was proved. By means of enzymatic digestion it was shown that proteoglycans in the cartilage act as thermal stabilizers of the collagen, after their removal collagen denatures completely.

Keywords: Differential scanning calorimetry; IR laser; Cartilage; Connective tissue; Collagen

Contents

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1. Introduction

Local laser heating is a new approach in clinical surgery and used today in cartilage reshaping [1], thermal modification of capsular [2] or selective dermaplasty [3]. This method is based on local heating of tissues and organs by IR laser irradiation [4]. In spite of the fact that this method is widely used nowadays, there is a lack of background knowledge of its therapeutic value. Understanding of physical–chemical processes occurring in tissues under laser irradiation ensures safe and effective application of local laser heating in clinical practice. The goal of our work is to study physical–chemical changes in connective tissues irradiated by non-ablative IR laser energy.

The main protein in the matrix of connective tissues is collagen. Triple helical macromolecules of collagen are organized in fibrils, fibers and filaments. Heating results in collagen denaturation or transformation of the native triple helical structure into a more random (coiled) configuration [5]. The tertiary triple helical structure of fully hydrated fibers of collagen I melts near 60–70 °C. It is the so-called denaturation process which can be considered as a first-order phase transition (helix–coil transition). The enthalpy of this process amounts to $\Delta_m H = 65 \pm 5\,J\,g^{-1}$ collagen [6–10].

The matrix of the fibrous connective tissue consists mostly of collagen I (heterotrimer). Cartilage tissue is an essentially three-dimensional network of fibrils of collagen II (homotrimer) included in the gel formed by a proteoglycan (PG) network and proteoglycan aggregates with hyaluronic acid (PGA). Proteoglycans consist of glycosaminoglycans attached to a core protein [5,11].

We determined the conditions and the differences between collagen denaturation in connective tissues induced by laser radiation and by ordinary heating. Differential scanning calorimetry (DSC) was used to detect alterations of connective tissues induced by IR laser radiation.

2. Materials and methods

2.1. Sample preparation

Nasal septa cartilage and facial fibrous connective tissue (FCT) from a 12-month-old calf were carved post mortem. Cartilage samples were kept in ice and were used during 12 h after excision. FCT was subsequently washed in water and kept in acetone at 4 °C for 12 h, in diethyl ether at 20 °C for 4 h and dried at room temperature. Dry matter (dm) contents of samples were estimated after vacuum drying over P_2O_5 at room temperature for 7 days. The water content of the FCT samples was adjusted by rehydration in distillated water during 1 h at room temperature. The samples of cartilage and FCT were cut with a thickness of 1–1.2 mm.

2.2. Enzyme digestion

Cartilage was treated with α-chymotrypsin (αCT, Sigma-Aldrich, St Louis, MO, USA) to degrade proteoglycans. This enzymatic digestion of cartilage specimens (about 50 mg) was performed at an enzyme/substrate ratio of 1:20 (w/w) for 20 h at 37 °C in incubation buffer. The composition of the incubation buffer was 0.1 M Tris–HCl, 25mM EDTA, 5000 units penicillin and 5 mg streptomycin (per 1000 ml). All chemicals were of analytical grade (Sigma-Aldrich, St Louis, MO, USA).

Solid residuals and supernatants obtained after enzymatic digestion were analyzed to estimate their amino acids content.

2.3. Biochemical analysis

Specimens were hydrolyzed as described by Tsugita and Schaffler [12], and amino acid analysis was carried out by an amino acid analyzer (Hitachi-835, Hitachi, Ibaragi, Japan) in the standard way for protein hydrolysate analysis with cation-exchange separation and ninhydrin postcolumn derivatization. The collagen content was estimated assuming that hydroxyproline is a collagen specific amino acid and that it makes up 13.3% of the collagen molecule.

A spectrophotometric assay for the glycozaminoglycan content was performed using 1,9-dimethylmethylene blue dye reaction [13]. Duplicate 40 μl aliquots of each extraction solution were assayed with 10 ml DMMB dye. Absorbance at 540 nm was measured by a Varian Cary 3E UV/VIS spectrophotometer (Varian, Inc., Scientific Instruments, Mulgrave, Australia) using DMMB as a reference solution. Calibration standards were prepared using solutions of chondroitin sulfate A.

2.4. Laser treatment

The tissue samples were irradiated by an Er-glass fiber IR laser (LS-1.56–5, “IRE-Polyus”, Moscow, Russia) at a wavelength of 1.56 μm with a power output of 2 and 3.5 W. A waveguide delivered the laser beam to the vertically hold samples at an incidence angle of 45°. The power density at the sample surface amounted to $6\,W\,cm^{-2}$, the absorbed energy in the sample to $50\,J\,g^{-1}\,s^{-1}$. The end of the waveguide and the surface of the sample were 8 mm apart. The diameter of the exposed region was 6 mm. Exposure time of laser treatment was 10–50 s for cartilage and FCT. Real time temperature control of samples’ surface during irradiation process was performed by IR camera IRTIS 200 at scanning rate of 1 frame per second.

2.5. Differential scanning calorimetry (DSC)

The thermal behavior of connective tissue samples (10–20 mg) was studied by means of a differential scanning calorimeter (DSC 30, Mettler TA 4000, Schwerzenbach, Switzerland). Samples were sealed in standard aluminum pans (40 μl) and an empty pan was used as reference. Start temperature, end temperature and heating rate of the DSC

experiments for the cartilage and FCT were 25, 100 °C, 10 K min^{-1} and 0, 100 °C, 10 K min^{-1}, respectively.

2.6. Thermomechanical analysis

Thermomechanical properties of cartilage tissue were investigated with a thermomechanical analyzer cell (TMA 40, Mettler TA 4000, Schwerzenbach, Switzerland). Tissue samples in form of a round plate (4 mm diameter and 0.8 mm thickness) were analyzed between 25 and 100 °C at a heating rate of 10 K min^{-1} and at a load of 0.005 N.

3. Results and discussion

3.1. Laser heating

In the studied samples, IR radiation is mainly absorbed by water, whose absorption coefficient at 1.56 μm wavelength is 9.65 cm^{-1} [14]. Thus, the characteristic penetration depth of radiation is close to the thickness of the tissue sample. Thus we can assume that laser heating is homogeneous, i.e. surface and bulk temperatures of samples are approximately equal. The dynamics of the transient temperature in laser irradiated samples are given in Fig. 1.

3.2. Fibrous connective tissue (FCT)

Thermograms of intact and irradiated FCT are presented in Fig. 2. An endothermic peak with a maximum temperature of 66.4 ± 0.2 °C and a heat effect of 46 ± 2 J g^{-1} dm is observed in the thermogram of intact FCT (curve 1) with a collagen content of 71% (for dried FCT), and a heat of Q = 65 ± 3 J g^{-1} collagen. Temperature and heat effect of this process correspond to the denaturation of collagen fibers.

The thermograms of FCT samples heated up to 50 °C by laser radiation (Fig. 1, curve 1) were similar to those of intact ones (Fig. 2, curve 2). But the endothermic peak of collagen denaturation disappeared or became diffuse (Fig. 2, curves 3, 4) for FCT samples heated by laser radiation to 70 °C (Fig. 1, curves 2, 3). This means that laser heating of fibrous connective tissue till 70 °C and higher may cause total collagen denaturation (depends on exposure time) while laser heating till 50 °C remains ineffective.

We also observed an exothermic transition at 21 °C (6 J g^{-1} dm) and an endothermic process at 40 °C (4.5 J g^{-1} dm) for FCT samples heated to above 70 °C (Fig. 2, curve 3). We suppose that these peaks originate from partial renaturation of amorphous areas of the collagen network and its subsequent melting. Reconstruction of molecular structure of recovered collagen (which was previously denatured) was proved by X-ray diffraction [6]. During heating of recovered collagen endothermic peak was observed, but its heat effect and peak temperature were smaller then those found during first heating [6,7]. So a partial renaturation of amorphous collagen takes place after both heat and IR-laser treatments.

3.3. Cartilage

3.3.1. Collagen II denaturation in cartilage

α-Chymotrypsin (αCT) is a catalyst of intensive hydrolysis of peptide bonds of phenylalanine, tryptophane and leucine [15]. When αCT affects cartilage tissue the framework of non-damaged collagen fibrils remains intact [16].

Enzymatic treatment of cartilage specimens with αCT led to a 50% weight loss of dry matter. The number of amino acid residues of leucine, serine, and valine per 1000 amino acids in the supernatant were about two times higher than that in the residual tissue. The content of the mentioned amino acid residues is markedly higher in the core and link proteins than

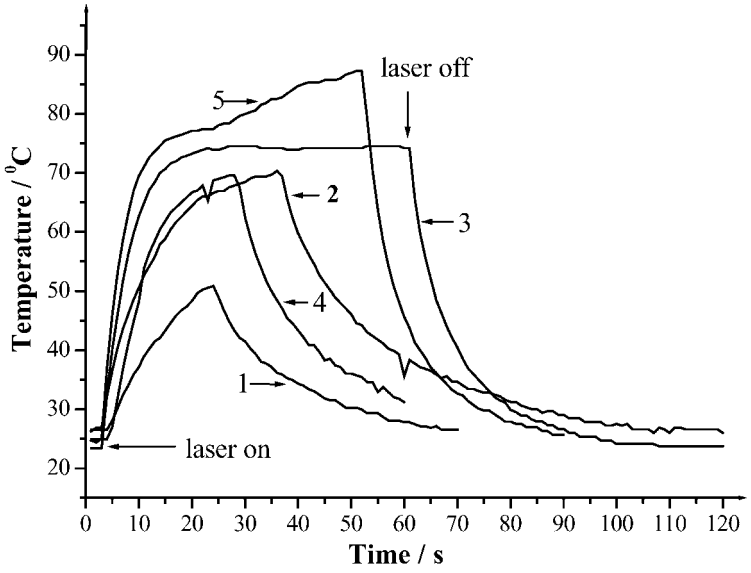

Fig. 1. Dynamics of transient temperature in laser irradiated samples: FCT (curves 1–3) and cartilage (curves 4, 5).

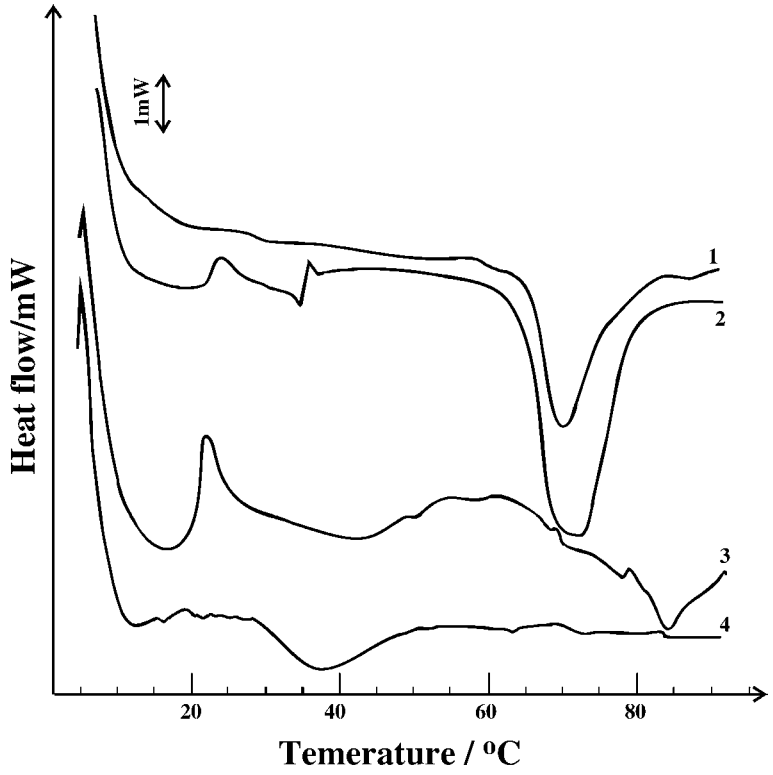

Fig. 2. Thermograms of intact (curve 1) and irradiated FCT samples (curve 2–4). Temperature dynamic during laser treatment is presented in Fig. 1 (curve 2: Fig. 1, curve 1; curve 3: Fig. 1, curve 2; curve 4: Fig. 1, curve 3).

in collagen [17]. Therefore, core proteins of proteoglycans and link proteins of proteoglycan aggregates are hydrolyzed by αCT a process accompanied by a washing out of oligopeptides and glycosaminoglycans. Analysis of the supernatant indicated that αCT had removed at least 75–80% of the glycosaminoglycans (measured in terms of chondroitin sulfate content) from the tissue, so that the collagen part in the residual tissue increased up to 0.7 g g^{-1} dm.

Thermograms of intact and enzyme treated cartilage are presented in Fig. 3. The endothermic process at 65–80 °C in thermograms of intact tissue discussed above becomes evident (curve 1). The peak temperature of this process corresponded to the temperature of collagen denaturation but the heat effect (10 ± 5 J g^{-1} collagen) was substantially smaller than the enthalpy change of collagen denaturation. The endothermic peak dramatically rose after the enzymatic treatment and amounted to 58 ± 2 J g^{-1} collagen (close to the enthalpy change of collagen denaturation). This endothermic process can be assumed as the sum of three endothermic peaks at 60, 65 and 75 °C (pronounced shoulder), respectively. Melting processes of three collagen populations may be responsible for these three peaks. The first one concerns the most labile collagen (without cross-links), the second one is bound to sheaths at the peripheries of collagen fibrils and the third one takes place in the core of the fibril [18]. It should be mentioned that the fine structure of the cartilage thermograms changes from one sample to another, but that the heat effect of the endothermic process remains constant.

The thermomechanical behavior of the intact and enzymatically digested cartilage is presented in Fig. 4. In the temperature range from 45 to 65 °C all tissue samples behaved identically, i.e. gradually lost their stiffness. Beyond

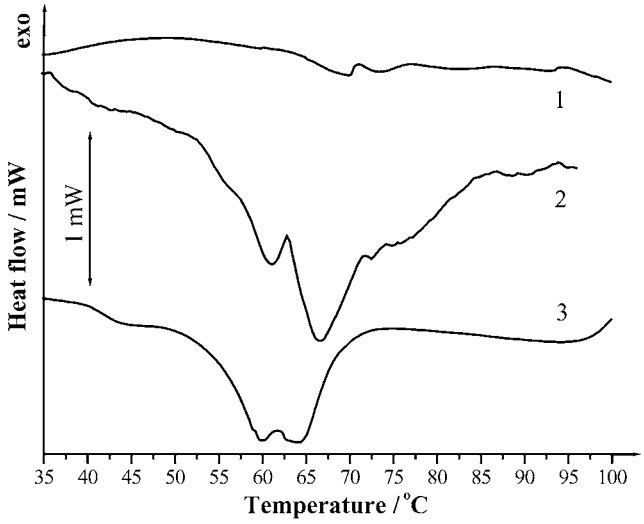

Fig. 3. Thermograms of intact (curve 1), digested by αCT (curve 2) and digested by αCT after substantial heating up to 100 °C in the DSC cell (curve 3) cartilage samples.

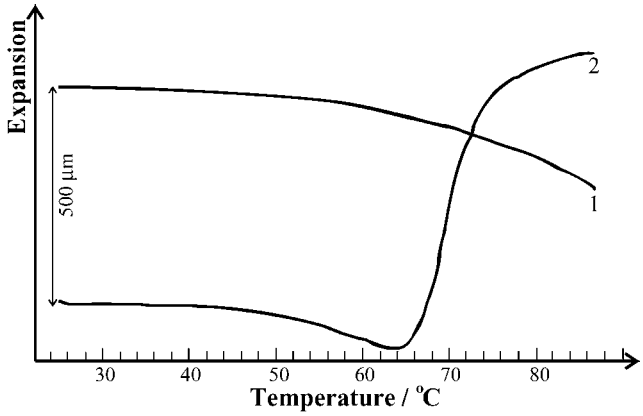

Fig. 4. Thermomechanical curves of intact (curve 1) and digested by αCT cartilage samples (curve 2).

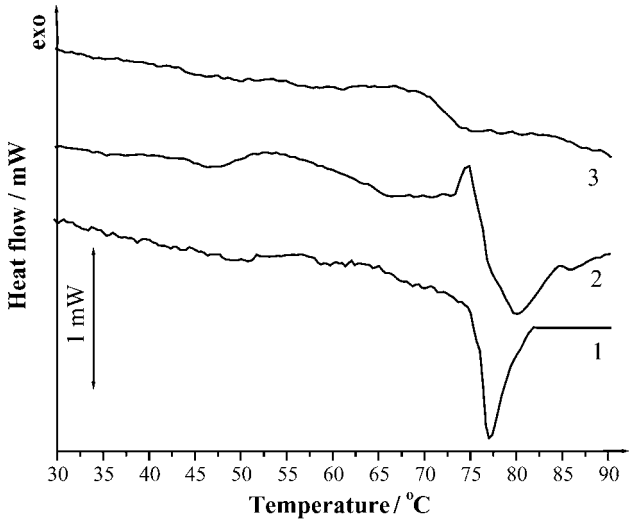

Fig. 5. Thermograms of intact (curve 1) and irradiated at (curves 2, 3) cartilage samples. Temperature dynamic during laser treatment is presented in Fig. 1 (curve 2: Fig. 1, curve 4; curve 3: Fig. 1, curve 5).

65 °C the thermomechanical properties of digested cartilage changed significantly, shrinkage in one direction accompanied by an expansion in another took place, whereas intact sample continued softening.

DSC and TMA data are an evidence of the high thermal stability of collagen II in the cartilage. Only a partial collagen II denaturation in native cartilage occurs. To prove this proteoglycans were enzymatically removed from previously heated tissue sample (in the DSC cell, 10 K min^{-1}, 20–100 °C) and then it was analyzed by DSC again, Fig. 3, curve 3. Endothermic effect of collagen denaturation (Q = 60 J g^{-1} collagen) was observed. Therefore, total collagen II denaturation in the cartilage takes place only after the removal of proteoglycans (PG).

The stabilization of collagen structure in the cartilage framework is the result of an interaction between the collagen network and the proteoglycan macromolecules. Disruption of the labile PG network results in a dramatic change of the material properties of cartilage. The tensile stiffness, the permeability, the magnitude of the equilibrium compressive modulus, the dynamic shear modulus and the tensile fracture stress were greatly reduced when PGs were degraded [19–23]. There are some models for the stabilization of cartilage structure organization. The most abundant concept is presented by Mow and co-workers [21,22]. The main idea of his concept is that the frictional forces between collagen and proteoglycans are large enough to retard collagen pull out and realignment. Maroudas and co-workers consider that a high osmotic pressure (4 atm) arising from polyanionic proteoglycans results in tensile stress of the collagen network [22]. It should be noted that tensile stress gives rise to an increased denaturation temperature of collagen [24]. Collagen fiber denaturation (helix–coil transition) can be regarded as a first-order phase transition in accordance with the Flory model [9,24]. Transition (melting) temperature T_m is equal to $\Delta_m H/\Delta_m S$. The number of permissible configurations for collagen chains interacting with PG macromolecules is much smaller than the number of permissible configurations in a PG-free sample. Thus the entropy of fiber collagen and denaturated collagen is reduced and $\Delta_m S$ is reduced also and T_m increases ("Polymer-in-a-box" theory [25]).

3.3.2. *Laser-induced changes of collagen II in the cartilage*

Laser heating up to 70 °C (Fig. 1, curve 4) does not change cartilage thermograms to a great extent in comparison with the thermogram of intact cartilage (Fig. 5, curves 1, 2). At the same time, the endothermic peak disappears (Fig. 5, curve 3) after laser heating of cartilage up to 70 °C and higher for 10 s or more (Fig. 1, curve 5).

Additionally cartilage samples heated by laser radiation up to 70 °C and exposed at this temperature for a several time were subjected to enzymatic digestion. The endothermic peak (46 ± 3 J g^{-1} collagen) corresponding to collagen

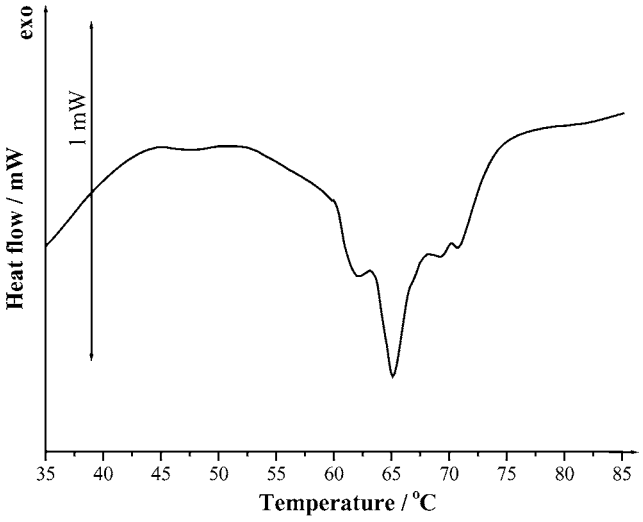

Fig. 6. Thermogram of a cartilage sample heated by laser radiation up to 70 °C and exposed at this temperature during 20 s and then digested by αCT.

II denaturation was detected in DSC thermograms (Fig. 6). This fact proves that IR laser treatment of the cartilage at a moderate intensity does not lead to a total collagen II denaturation, but that a detectable part remains in the natural state.

4. Conclusion

In fibrous connective tissue total collagen denaturation takes place during laser heating at the same temperature as under traditional "thermal" heating. Only a partial collagen denaturation occurs in the cartilage tissue which is proved by the absence of tissue shrinkage at 70 °C and a small heat effect of the endothermic process at the same temperature. However, a total collagen denaturation takes place in the cartilage after proteoglycan removal. Summing up, we can say that proteoglycans in the cartilage act as thermostabilizer of collagen fibrils molecules.

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