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Thermostability of bone tissue after immobilization induced osteopenia in a rat model

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Abstract: Immobilization of load-bearing bones results in imbalance of bone turnover followed by bone loss and impairment of its mechanical function. The question is whether immobilization induced bone loss is accompanied by deterioration of properties of the bone tissue components. Thermally induced transformations of collagen reflect the overall condition of the structure and cross-links in collagen network. The aim of the present study was to investigate whether immobilization induced osteopenia effects stability of collagen in bone tissue. Bone loss was developed by unilateral hindlimb immobilization in adult rats. Effects of unloading on cortical tissue from tibiae were studied after three weeks of unloading (I3R0) and four weeks after remobilization and free convalescence (I3R4) in both tibiae. Thermodynamic parameters of collagen degradation in bone were determined from differential scanning calorimetry (DSC) analysis of partially dehydrated cortical bone samples from tibiae in the range of temperatures from 60°C up to 300°C. All bone samples were thermally very stable showing first clear endothermal process with a peak temperature within a range from 150°C to 169°C, for different samples. The next endotherm, wider and flatter, was observed between 245-298°C with a peak at 255°C - 260°C. There were significant side-to-side (right to left) differences for both endothermal processes in tibiae samples from experimental groups: I3R0 and I3R4. Immobilization of load-bearing bones influences stability of collagen in bone tissue. Free remobilization was not sufficient for recovery of thermal parameters of bone.

Key words: Bone collagen - DSC - Immobilization osteopenia

Introduction

Cyclic mechanical loading is essential for normal metabolism in load bearing bones. Immobilization results in imbalance of bone turnover followed by bone loss and impairment of its mechanical function. Moreover, complete and permanent recovery of bone from immobilization-induced osteopenia without any treatment is questionable [1-4]. Despite a large number of studies concerning the role of mechanical loads in bone turnover, only a few referred to the effects of immobilization on the structure and physicochemical properties of main components of bone tissue [5-8].

Bone is a composite of apatite crystals deposited in an organic matrix of collagen fibres with highly hierarchical structure. The main component of the organic matrix is type I collagen. The primary sequence of collagen molecule is identical in bones and in other connective tissues, but bone collagen has a specific cross-link profile that influences its structure and physical properties [9]. There are studies indicating that collagen cross-links influence strength of bone [10-12]. Moreover, the role of collagen is crucial for energy absorbed during deformation of bone tissue [12,13].

Thermally induced transformations of collagen reflect the overall condition of the structure and cross-links in the collagen network. Thermal stability of collagen was proved to be valuable in assessing the efficiency of native and artificially induced intermolecular cross-links in a number of tissues [12,14-17] and as an indicator of bone turnover and changes in bone tissue during ageing [12,16]. Thermal techniques, in particular, differential scanning calorimetry (DSC) provides a powerful method for examining conditions in which the stabilization of molecules breaks down (i.e. the helix-coil transition) [14,15,17-19], and was proved to be sensitive to amount of covalent cross-links [14].

Considering bone collagen, it is presumable that its thermostability is dependent not only on the molecular integrity of collagen itself, but also on the degree of mineralization. There are experimental evidences that mineralization increases dramatically the thermal stability of the collagen molecules [15]. DSC studies quoted above showed that thermal denaturation of col-

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lagen triple helix into a random chain, occurs at approximately 60-70°C for fully hydrated collagen molecules [14,18] and increases dramatically both in dehydrated collagen [18] and in mineralized collagen in bone tissue [15,19].

The aim of the present study was to investigate whether immobilization induced disorganization of bone turnover effects thermal stability of collagen in bone tissue.

Material and methods

Animals. 24 weeks old Wistar male rats with mean body weight 480 g (SD=30 g) were used in the study. Rats were randomly divided into two study groups: I3R0 (n=6), I3R4 (n=8) and one control group C (n=4). Right hindlimbs of the study animals were immobilized against the abdomen using bandages and padded tape as previously described [2]. All animals were housed in typical wire cages, 3 - 4 animals per cage and were fed standard laboratory chow and water *ad libitum*. After three weeks rats from the group I3R0 were sacrificed by cardiac puncture under anesthesia. Rats from the group I3R4 were released from tapes and bandages and allowed moving freely for next four weeks. One day after the remobilization they used all legs while moving. After four week rats from the groups I3R4 and C were killed. All rats were killed by cardiac puncture under ether anesthesia.

Material samples. Both tibiae from experimental rats and right tibiae from the control were removed and outer surfaces of the bones were cleaned mechanically from soft tissues and frozen in -15°C. Before the calorimetric measurements bones were thawed in room temperature. Both epiphyses of each tibia were cut off to obtain bone shafts composed of cortical tissue. Defatted in alcohol bone samples were dried in air, milled in an agate mortar and sieved to obtain bone powder of diameter ≤0.2 mm. Taking into account a strong dependence of enthalpy and temperature of denaturation of collagen on water content [18,19], all samples were carefully pre-heated in the same conditions (2 h in 40°C) and weighted with accuracy of 10-4g just before calorimetric measurements.

Calorimetric measurements were performed using a differential scanning calorimeter (Unipan 605M, Poland) with 35-38 mg samples of powdered bone. Samples were contained in high-pressure steel capsules. Heating was performed from 60°C to 300°C with an empty capsule as a reference with the rate of heating 1.2°C/min. The peak temperature (T_m) and enthalpy (Q) were determined numerically from thermograms for each endothermal process. The peak temperature was calculated as the temperature at minimum value of calorimetric signal within the endotherm. The enthalpy was determined from the area between endotherm and a baseline which was constructed by extrapolating to the scan beyond the endotherm. Before calculations the calorimeter was calibrated with indium as a standard.

The energy of activation (E) for the process of collagen denaturation was calculated using the dependence of the heat evolved with temperature [20]:

$$ln\left(ln\frac{Q_t}{Q_t - Q}\right) = \frac{E}{R}\left(\frac{1}{T_m} - \frac{1}{T}\right)$$

where, Q_t is the total heat of the process, Q - heat evolved at a given temperature T, T_m is temperature at the peak of the endotherm, and R is universal gas constant. Q_t and Q were obtained from the area under the endotherm. A plot of ln[ln[Q_t/v-Q]] versus 1/T should give a straight line with the slope being -E/R.

Ethical issues. The experiment was approved by the committee of ethics for animal experiments at the Medical University of Lublin.

Results

All bone samples were thermally very stable showing first clear endothermal process starting above 140°C with a peak temperature ($T_{m,1}$) within a range from 150°C to 169°C, for different samples. The next endotherm, wider and flatter, was observed between 245-298°C with a peak ($T_{m,2}$) at 255°C - 260°C. In the Figure 1 typical DSC recordings are presented. The scans were obtained during heating of bone samples from both femora of one rat from experimental group I3R4 and one bone sample from the control group (C). In each bone sample there was also a single exotherm following the first endothermal process. The exotherm reflects an oxidation process as a small amount of air was always closed in the capsule with the sample.

Processes described in this study were irreversible what was proved for both endotherms in few samples. No signs of any process were observed during rescanning after reaching the end of the endotherm and cooling the sample without opening the pan. Few samples were examined visually just after completion of successive endotherms. When a sample pan was opened after the first endotherm, bone powder was white and showed no evidence of changes. A sample of bone powder examined just after the second endotherm (just above 280°C) was brown indicating that decomposition of organic phase started.

Average values of thermal parameters obtained for bone samples are given in the Table 1. Analysis of differences between right (immobilized) and left (loaded) bone (Table 2) shows that there were significant side-to-side differences for both endothermal processes in both experimental groups: after three weeks of immobilization (I3R0) and after immobilization and four weeks of free remobilisation (I3R4). There were also significant differences between the right tibiae in the control group and right and left tibiae in the group I3R4.

Activation energy was calculated only for the first endotherm as the plots of ln[ln[Qt/Qt-Q]] versus 1/T for the second endothermal process did not give a straight line in any sample.

Discussion

The results of the present study demonstrated that bone tissue was thermally very stable both in the control and experimental samples and confirm earlier reports that bone collagen is much more stable than nonmineralised collagen [15,19].

The process of thermal activation of type I collagen involves rupture of hydrogen bonds coupling the three α-chains and a rearrangement of the triple helix into a

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Table 1. Thermal parameters for bone samples from rat tibiae after three weeks of immobilization of the right hindlimb (I3R0), after three weeks of immobilization followed by four weeks of free remobilization (I3R4) and from the control group (C).

		Q_1 (J/g)	T_{m1}(°C)	E (kJ/mol)	Q_2 (J/g)	T_{m2} (°C)
I3R0; n=6	P:	5.0 (0.9)	157.7 (2.1)	544 (73)*	15.4 (0.9)	256.1 (1.3)
	L:	4.4 (1.1)	155.2 (1.8)	592 (69)*	16.9 (1.5)	255.5 (1.2)
I3R4; n=8	P:	4.7 (1.4)	158.1 (3.1)	516 (52)	17.4 (2.1)	257.5 (1.4)
	L:	4.1 (1.0)	155.0 (2.0)	551 (64)	14.8 (2.3)	256.5 (1.0)
C; n=4	P:	4.7 (0.6)	151.5 (1.0)	445 (32)	15.4 (1.1)	255.1 (1.1)

*activation energy was calculated in four right and five left bone samples as plots of $\ln[\ln[Q_t/Q_t\text{-}Q]]$ versus 1/T for three bone samples from I3R0 did not give a straight line

Table 2. Significance of differences (*p-values*) for thermal parameters between groups of bone samples; P vs L - nonparametric Wilcoxon test for pairs: right bone - left bone after immobilization (I3R0) and after convalescence (I3R4); K.-W. - nonparametric Kruskal-Wallis test for differences between groups: right and left bones in I3R4 and the control group (C).

	Q_1	T_{m1}	E_1	Q_2	T_{m2}
P vs L (I3R0)	n.s.	0.043	n.s *	n.s.	0.046
P vs L (I3R4)	n.s.	0.033	0.028	0.043	0.018
K.-W.	n.s.	0.001	0.014	n.s.	0.055

* four pairs of bones

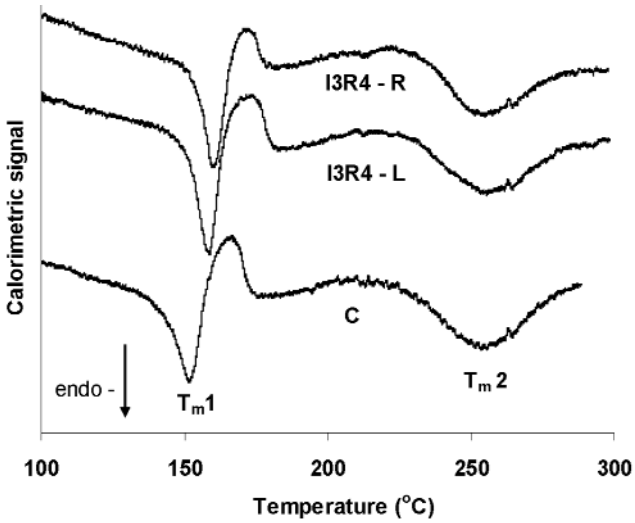

Fig. 1. Thermograms of a pair of tibiae samples (Right and Left) from the experimental group I3R4 after three weeks of immobilization and four weeks of remobilization of a right hindlimb and one right tibia from the control group (C).

random chain configuration [14,17,18]. In previous studies *in situ* denaturation temperature of collagen in fully mineralized bone was found to be 155-165°C and the temperature was dependent on degree of mineralization [15,19]. That increase in stability comparing with soft tissue collagen is attributed to the presence of a large amount of mineral that makes difficult conformational changes of collagen molecules as well as to interactions between mineral crystals and collagen molecules. The high thermal stability of collagen in bone results also from the complicated internal structure of the collagen fibres and variety of covalent intra- and intermolecular cross-links.

Results of the present study showed that thermal activity of collagen in bone was not limited to the denaturation of the triple helix reflected by the first endotherm seen in calorimetric scans. In all bone samples there was also at least one endothermal process between 245°C and 300°C (Fig. 1). It is presumable, that process of unfolding of such a large and complex molecule occurs in many stages. When the protein is thermally denaturated, at first weak bonds between three helices are broken but the covalent bonds inside and between molecules stay intact. We suppose, that endothermal processes observed in this study at higher temperatures are related to breaking of different covalent cross-links resulting in melting of the structure.

Existence of a second area of thermal activity of collagen in temperatures above 200°C was demonstrated in only few experiments [19,21,22]. Two separate areas found in thermograms of mineralised turkey tendons, Knott *et al.* [21] attributed to unfolding of collagen helix in two, thermally different, populations of collagen in the heterogenic tissue. However, in our previous study on trabecular and cortical bovine bone and nonmineralised tendon collagen [19] we proposed that thermal processes in temperatures over 200°C could result from breaking of different inter- and intramolecular cross-links in collagen microfibers that results in melting of collagen molecule. As a result of investigations of both mineralized and demineralised bone and nonmineralised tendon collagen we suggested that at the temperature close to 200°C melting of collagen begins and then in 260°C decomposition of organic phase and separation of smaller side chains form carbon backbone of the macromolecule. An irreversible melting of demineralised human bone collagen in 215°C was also reported by Fois *et al.* [22].

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Conclusions

On the basis of results of the present paper it can be concluded that immobilization of load-bearing bones influences stability of collagen in bone tissue. Free remobilisation was not sufficient for recovery of thermal parameters of bone. Immobilization triggered change in metabolic activity of bone tissue induces some structural changes at a level of collagen molecule. Peak temperatures of both endothermal processes, as well as activation energy of collagen unfolding in all experimental groups were higher than in the control. Moreover, there were significant side-to-side differences both after immobilization and after free convalescence. Considering results of the investigations quoted above [19] it can be presumed, that immobilized and loaded bones were different as concerns kind and/or amount of covalent cross-links stabilizing structure of bone collagen.

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