Spectral analysis of bone tissue of rats exposed to hyperthermia and mineral bone component injection

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Abstract. Using the method of Raman spectroscopy the study of bone tissue of rats has been made in this work after hyperthermia and injection of allogenic hydroxyapatite. It has been established that the process of thermal treatment of bone material up to 70°C for 15 minutes allows preserving organic components of biomaterials.

1. Introduction

The motor system diseases are one of the most widespread pathologies and often lead to disability, decline in the quality of life and untimely death. The progression of the diseases is based on imbalance of proportion of mineral and organic components of bone tissue, imbalance of phosphor-calcium proportion, micronutrient deficiency and hormonal imbalance of functioning of endocrine glands. In particular, the result of the changes is a bone resorption causing its density reduction, loss of strength and, finally, braking.

Finding the mechanisms of metabolic disorders causing bone resorption is an urgent task of practical medicine. The use of several models of bone resorption in experiments: glucocorticoid, hypoestrogenic, hyperthermal and others [1-4] is known at the preclinical stage for studying bone protective properties of different medicines, bioimplants, such as mineral bone component (MBC). The choice of the thermal model in this research was first of all due to high proven efficiency of bone resorption development, simplicity and convenience of setting the experiment. The previous experiments have also shown that hyperthermia leads to endogenous increase of the level of steroids causing bone resorption [1].

According to studies high temperature can influence metabolic processes in bone and cartilage tissues [5-6]. In view of the above it is necessary to evaluate the efficiency of using bone plasty materials of allogenic origin for correction of bone resorption. One of the methods for such assessment is a Raman spectroscopy method.

The spectroscopy methods are the newest methods for studying biotissues. One of the promising methods is a Raman spectroscopy [7-9]. It is a simple to use, noninvasive, nondestructive method.

The aim of this work is spectral analysis of bone tissue of rats after hyperthermia and MBC injection.
2. Materials and methods of research
The experiment was made on mature rats. The first group included control intact rats. The rats of the second group were daily exposed to short-term hyperthermia (70°C) for 28 days, ones, 12 minutes a day. The rats of the third group had the similar exposure with additional intramuscular injection of MBC suspension in a dose of 100 mg/kg body weight on the 14th day after thermal exposure. The forth group included the placebo rats (hyperthermia in the above described manner with injection of saline solution instead of MBC). The results of the placebo group were similar to the results of the group of thermal exposure. Therefore, these groups were further combined. The MBC (hydroxyapatite) was received on original patented method [10]. On the 28th day the rats were withdrawn from the experiment according to bioethical standards.

The materials of the study were the samples of rat humeri that were taken after decapitation, cleared from soft tissues and the spectroscopy of their surface was made.

The method of the study was Raman spectroscopy method, implemented using the stand described in details in [7-9].

3. Results
Figure 1 shows the typical average Raman spectra of the tree groups of bone tissue samples in the area of wave numbers of 750 –1800 cm⁻¹. The main differences are seen in the Raman lines of 956 cm⁻¹, 1068 - 1274 cm⁻¹, 1407 - 1443 cm⁻¹, 1557 cm⁻¹, 1660 cm⁻¹ and 1738 cm⁻¹. The analysis of the Raman spectra is shown in Table 1.

![Figure 1](image-url)

**Figure 1.** Average normalized Raman spectra of bone tissue samples: 1 –control, 2 – heating with oup HAp, 3 – heating with HAp.
Table 1. The table of Raman spectral lines, characteristic for bone tissue samples.

| Raman shift (cm\(^{-1}\)) | Assignments |
|---------------------------|-------------|
| 815 | Phosphodiester bands in RNA / DNA (C’',O-P-O-C, stretching) (α-form helix, Phosphpate) |
| 850, 870 | Benzene ring of proline and hydroxyproline (collagen assignment) (C-C stretching) |
| 956 | PO\(_3\)^{2-} (ν1) (P-O valence symmetrical) |
| 1000 | Aromatic ring breathing of phenylalanine ν(C–C), (collagen assignment) |
| 1031 | Phenylalanine (CH\(_2\)CH\(_3\) bending modes (protein assignment)) |
| 1068 | CO\(_3\)^{2-} (ν1) B-type substitution (C–O planar valence) |
| 1106 | CO\(_3\)^{2-} (ν1) A-type substitution (C–O planar valence) |
| 1138 | Phospholipids, fatty acid (protein assignment) |
| 1167 | GAGs, CSPGs |
| 1200 | Tyrosine (collagen assignment) |
| 1230-1280 | Amide III random coil (disordered) and α-helix |
| 1407 | CH\(_3\)in-phase deformation T, A, G of DNA |
| 1429 | Deoxyribose, (B.Z-marker) |
| 1443 | Lipids and fatty acids, CH\(_2\) scissoring & CH\(_3\) bending (collagen assignment) |
| 1557 | Amide II (Parallel / Antiparallel β-sheet structure) |
| 1666 | Amide I (C=O stretching) Unordered or random structure, Collagen IV, I |
| 1734 | Phospholipids, fattyacid (C=O ester group) |

The relative coefficients were introduced for relative quantitative assessment of component composition of bioimplant surface. Amide II related to the wave number of 1557 cm\(^{-1}\) is a relatively constant component in the studied samples, therefore the amplitude of this divided line has been used as a denominator \(I_{1557}\) in the introduced coefficients \((k)\):

\[
k_i = \frac{I_i}{I_{1557}},
\]

where \(I_i\) – intensities at wave numbers of the analyzed components.

The mineral components of bone tissue related to the lines of 956 cm\(^{-1}\) and 1068 cm\(^{-1}\) provide the bone quality and strength [11]. Introducing the hydroxyapatite to bone tissue composition leads to improving its physico-mechanical properties: tensile strength in bending, modulus of elasticity and firmness increase. The line of 1068 cm\(^{-1}\) in Raman spectra corresponds to substitution of carbonate component of hydroxyapatite in the apatite lattice, known as B-type carbonate substitution [12]. The increase of the degree of B-type carbonate substitution in bone resorption causes the increase of bone fragility and vice versa its decrease characterizes younger bone tissue [13].

Amide III and amide I, presented by the main lines of 1238 cm\(^{-1}\) and 1272 cm\(^{-1}\) and 1666 cm\(^{-1}\), are correspondingly attributed to collagen structures that change significantly when comparing the samples of the first and the second group with the samples after HAp treatment.

The analysis of amplitudes of the divided lines normalized to the intensity of the line of 1557 cm\(^{-1}\) (Amide II) using the method of linear discriminant analysis (LDA) was made. This method was implemented in the software SPSS Statistics 23. The results of analysis of difference between the groups of samples of bone tissue are presented as a set of data: the chart of scores (Figure 2) and the chart of the most informative variables of the structural matrix (Figure 3).

The analysis of relationship between the groups of samples on the basis of thermal treatment of bone materials up to 70°C for 15 minutes is shown in Figure 2. It is shown that the main differences between the three groups of samples are described by discriminant function LD-1 (81,9 % of explained dispersion). A sampling is 42 Raman spectra. The positive values of LD-1 characterize mainly the Raman spectra of the samples after thermal treatment, and vice versa, the negative values...
characterize the Raman spectra of control samples without treatment. The remaining 18.1% of explained dispersion are described by the function LD-2, which negative values characterize the samples without additional dose of hydroxyapatite. The areas of the groups do not have significant intersections.

Figure 2. The chart of scores of linear discriminant functions of bone tissue samples.

Figure 3. The chart of loads for differentiation of the samples on the basis of influence of thermal treatment.
The most significant differences between the groups of samples are described by the Raman lines shown in Figure 3 and having the highest value of linear discriminat functions LD–1 and LD–2 on module.

The higher is the value LD-1 for a variable, the more it influences the observed difference of component composition, e.g. it can be seen in the value of coefficient k960, that corresponds to P-O symmetrical valence fluctuations of PO₄³⁻(ν₁) hydroxyapatite, which value is higher for the samples of the group after thermal treatment.

4. Conclusion
The comparative spectral analysis of the samples of humeri was made. Using the spectral analysis, we have shown that relative intensities of the lines of 957 and 1068 cm⁻¹, that correspond to mineral components of the studied samples, are higher in the samples after heating than in control group. We have also established the increase of relative intensity of the lines of 1666 cm⁻¹ (amide I) and the lines of 1000 and 1031 cm⁻¹ (phenylalanine) and decrease of relative intensities of the lines, corresponding to glycosaminoglycans, tyrosine and lipids in the process of thermal treatment.

Therefore, it was established that in the process of thermal treatment of bone materials up to 70°C for 15 minutes the organic components of biomaterials are preserved.

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