The research of the composition of mineral component of compact bone tissue after flow delipidation using the optic method

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Abstract. The results of the research of bone mineral component (BMC) composition using the Raman spectroscopy method are presented in the work. The subjects of the research were the groups of BMC samples made of compact bone tissue of cows using "Lyoplast"® technology where vacuum delipidation of initial material was replaced by the flow one by washing it out in hydrogen peroxide and ether. The solution filtration after bone tissue demineralization was used as additional treatment. The results of the experiment show that the flow delipidation can be used for preparing this biomaterial (BMC), which is cost-effective compared to standard vacuum cleaning and additional filtration stage can be rejected.

1. Introduction
Full regeneration of bone tissue in the area of defective bone is one of the most topical issues of regenerative medicine despite the accumulated knowledge in this field and wide range of used biomaterials [1].

The increasing demand of finding biologically compatible materials that satisfy the requirements for active implantable medical devices causes necessity in evaluation of quality and quantity composition of bone mineral component (BMC) made using "Lyoplast"® technology (technical specification TU-9398-001-01963143-2004).

It is known that lipids take part in bone tissue mineralization where decrease of concentration of them in situ indicates active mineralization as lipids binds proteins and minerals [2], and the speed of reparative osteogenesis in the area of defective bone directly depends on the quality of BMC and its physical and chemical properties (composition, size and porosity of the particles, their ability to aggregate) [3].

On the other hand, forming toxic peroxide compounds, first of all hydroxyl radical OH⁻, as a result of lipid peroxidation induces the damage of cell structures, primarily of membranes. Therefore the process of vacuum delipidation reaches maximum extraction of lipids and free water from a bone.

It follows that successful prevention and treatment of diseases related to bone loss depends on allogenic properties of the material, lipid composition and BMC production quality control. A constant quality control of the material and evaluation of its organic and lipid components is necessary in the process of BMC production.
Bioimplants should correspond to the above technical specification. Relative concentration of lipids in bone tissue after treatment should be less that 5% (1.2 ± 1) %. According to the technical specification standard treatment includes vacuum delipidation of initial material, therefore one of the aims of this work was determining whether preliminary vacuum cleaning of bone tissue should be made, which requires expensive special equipment. Therefore, evaluation of composition of such bioimplants is a very important task.

One of the widespread rapid methods of evaluation of biomaterials made for different purposes is physical methods [4, 5]. For instance in the work [4] the samples of tooth mineral component were studied using X-ray diffraction method. Luminescent characteristics of the analogue of mineral compound of tooth enamel nanocrystalline carbonate substituted HAP of a calcium type B with defects on the surface of nanocrystals in the form of nanopores with sizes 2-5 nm have been studied in this work. The spectroscopy methods are effective methods of studying the BMC structure [5]. For example, in the work [6] the following results were received using the Raman spectroscopy: according to the Raman spectrometry structural features of human bone tissue were revealed in coxarthrosis, the amount of carbonate ions of B-type in the structure have reduced.

As shown previously [7], the Raman spectroscopy allows estimating the reduction of mineral components in the process of demineralization of hydroxyapatite taken from different donors and decomposition of organic matter under thermal action.

The aim of the work is studying the composition of mineral component of compact bone tissue after flow delipidation using the optic method.

2. Materials and Methods
The subjects of the study were the groups of BMC samples made of compact bone tissue of cows using "Lyoplast"® technology.

The samples were divided in two main groups: prepared of filtered and non-filtered solutions after demineralization of bone tissues of cows. Filtration of the solutions was made using the filters as "Red ribbon" with pore sizes 8-12 μm.

The main method of studying the BMC powder was the Raman spectroscopy method implemented by the high-resolution digital spectrometer AndorShamrock SR-303i providing spectral resolution of 0.15 nm, with the build in cooling camera DV420A-OE and fiber-optic probe for Raman spectroscopy RPB785 combined with the laser module LuxxMaster LML-785.0RB-04 (power up to 500 mW, wavelength of 785 nm) [8].

The spectra were taken in three different points and averaged using the software "Wolframmathematica" [9].

Biochemical analysis was made as an additional research method.

The amount of lipids in the BMC samples was determined using colorimetry with spectrophotometer "ShimadzuUV-1280" (Japan) by reaction with acetylacetone [10].

3. Analysis
Figure 1 shows the results of the research of the BMC samples. Minor changes are seen in the lines of 1000 cm⁻¹ and 1030 cm⁻¹ (Phenylalanine), and 1648 cm⁻¹ (Amide I). There are no significant changes in any other Raman lines.

These changes are caused by accumulation of macroscopic conglomerates of big organic inclusions on pores of the filter.

Minor changes in the line of 960 cm⁻¹ (ν PO₄³⁻) are caused by different demineralization grade of the studied samples [7].
To make the received Raman spectra more informative nonlinear regressive analysis of the spectra was made including their spectral line decomposition. Figure 2 shows the results of decomposition of spectral contour on the sum of distribution of the Gaussian lines.

The average value of the coefficient of determination of the result spectrum on the initial one in the range of 800-1780 cm\(^{-1}\) was R\(^2\) = 0.99, the relative error of spectral line intensity evaluation a was less than 7%, the average standard deviation of the coordinate of the line x\(_0\) was 0.9 cm\(^{-1}\), the average standard deviation of the width of the Gaussian line (HWHM) dx was 1.9 cm\(^{-1}\).
For relative quantitative analysis of component composition of BMC the ratios of the Raman lines intensities to Amide I line intensity were used.

The method of linear discriminant analysis in the software IBM SPSS Statistics was chosen for further analysis of spectral lines received after decomposition.

Figure 3. The chart of values of linear discriminant function of the BMC samples

Figure 3 shows the results of comparing LDA of the two groups of samples. 108 Raman spectra of BMC (54 filtered and 54 non-filtered) were analyzed. The discriminant function LD-1 describes the 100 % of dispersion. The positive values of LD-1 mostly characterize the Raman spectra of received filtered materials and vice versa the negative values characterize the Raman spectra of non-filtered materials. The areas of the groups have significant intersection in the range of LD-1 = {-1.25; 1.75}.

Figure 4. The values of factor structure coefficients

Figure 4 shows the coefficients of the factor structure matrix that have physical meaning of correlation between variables in the model and discriminating function. The higher the modulus value of LD-1 for variable, the more it defines the difference in the discriminant model between the groups of samples.
Specificity of the diagnostic model on the basis of discriminant analysis was 50%, sensitivity was 55.6%, which indicates that there is no statistically significant changes of the spectral composition between the groups of samples.

The biochemical analysis has provided credible differences between the BMC samples of filtered and non-filtered solutions. All the received numbers of lipids do not exceed the requirements of technical specifications of bone allogenic bioimplants. All samples were prepared without vacuum delipidation; therefore, this stage can be avoided in the process of BMC preparing.

4. Conclusions
The spectral changes of BMC samples prepared using "Lyoplast"® technology in the process of their cleaning and filtration were found during the research. Chemometric analysis of the Raman spectra of BMC samples prepared with the use of flow delipidation was made. It was found that insignificant differences between the researched samples are in the Raman lines of 1000 cm\(^{-1}\) and 1030 cm\(^{-1}\) (Phenylalanine), and 1648 cm\(^{-1}\) (Amide I). There are no significant changes in the other Raman lines, which indicate that flow delipidation of BMC is enough and is cost-effective in comparison with the use of standard vacuum cleaning.

The results of the research are confirmed with the biochemical analysis.

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