Study of the processes of cleaning biological and chemical substances from implants of biological origin using supercritical CO₂

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Abstract. Chemical reagents used in standard technologies are significantly inferior in effectiveness to their intended use in the purification of a biological matrix by supercritical carbon dioxide due to several reasons: retention of residual solvent in the structure of the biological matrix, the presence of pronounced cytotoxicity, problems associated with capillary effects (limited penetration of the reagent to the cleaned surface), pronounced allergenicity of chemical reagents. Laboratory studies of the influence of pressure processing from 20 to 35 MPa of a two-stage bone matrix cleaning process at a holding temperature of 308, 313 K to the 60-minute static processing time and the 240-minute dynamic processing time were carried out, including subsequent biochemical analyzes to determine residual DNA, calcium protein in the obtained samples. The addition of a cosolvent - ethanol in an amount of 5% of the main solvent of supercritical CO₂ was studied. Pyrogenic testing was carried out. All samples are recognized not pyrogenic. Physical properties and surface morphology were determined using a Carl Zeiss AURIGA CrossBeam scanning electron microscope. Identification and quantification of potential degradation products using liquid chromatography was determined.

1. Introduction

Optimization of the healing process of bone defects using new technologies and materials is an urgent problem in medicine. Currently, restoration of bone defects remains a central concern, both in fundamental medical science and in clinical practice. An analysis of the literature makes it possible to conclude that at present, a wide selection of transplant materials of various composition, shape, and properties is offered to replace bone defects. Despite the wide range of materials for osteoplasty, today none of them meets all the requirements of modern reconstructive surgery, which makes it necessary to actively search for new and improve existing materials. The technology for the use of supercritical media has significant advantages in comparison with the existing standard methods for cleaning, modifying and sterilizing biological and synthetic matrices, based mainly on chemical, vacuum or ultrasonic cleaning methods. Purification of biological matrices in supercritical fluids allows one to obtain a biological product without residual harmful impurities of solvents and associated reagents, which significantly increases its biocompatibility and regenerative potential. The main objective of the study is to develop a technology for cleaning the extracellular matrix of various organs and tissues to obtain medical products for regenerative medicine.
2. Methods of conducting experiments and analyzes
The schematic diagram of the setup is shown in figure 1.

![Figure 1](image_url)

Figure 1. Scheme of the experimental setup: 1- cylinder with CO\(_2\); 2- filter drier; 7,11 - valve; 3- thermostat; 4- heat exchanger for cooling; 5- flow meter; 6- high pressure pump; 8- control unit; 9- high pressure manometer; 10 - extraction unit; 12- extract receiver.

The matrix is placed in the extraction unit 10, the temperature is maintained at 313 K, the pressure in the system is 30 MPa, the original experimental sample is processed by the static method for 60 minutes. Then the valve (11) opens for processing by the dynamic method for 120-480 minutes (120-minute step) with a flow of carbon dioxide of 3-4 g/min. The consumption of carbon dioxide is also recorded using the control unit (8). Pressure relief is carried out with the valve closed (7) and the valve open (8). The extract obtained at the end of the processing is collected in the extract receiver (12). The resulting samples are placed in a penicillin vial with a capacity of 10 ml. The modernization of the installation, used for cleaning bone matrixes, consists in changing the design of the extraction unit. The upgraded extractor has a more elongated shape and a smaller diameter. This design allows increasing the contact of supercritical carbon dioxide with the test material and increasing the degree of saturation of the supercritical solvent.

The technical result is achieved due to the fact that the bone matrix at the stage of degreasing and cleaning is subjected to processing with carbon dioxide in a supercritical state. Supercritical CO\(_2\) is able to penetrate micropores of solid matrices much better than any other liquids, and has good ability to dissolve lipids. At the same time, bone microporosity becomes much more accessible, which makes it possible to release native protein growth factors after the demineralization step, thereby increasing the osteoconductive properties of the osteoplastic material after implantation. In addition, the supercritical fluid cleaning method is safe and environmentally friendly because it does not use chemicals [1-2].

Sterility was in accordance with GOST ISO 11737-2011. For this, the samples were extracted with 1 ml of a sterile solution of sodium chloride at a temperature of 310 K, after which 10 μl of the extract was placed on the surface of Müller Hinton broth (NICF, St. Petersburg) for selection of staphylococci, since the samples can be seeded with these microorganisms, such as autochthonous microflora and concomitant microflora. Seeding was incubated for 48 hours at a temperature of 410 K. To identify staphylococci, the colonies were transferred from agar to a glass slide and stained with Cresyl violet (BioOptica, Italy). Protein was determined by Kjeldahl method according to GOST 938.7-68 in the modification. DNA was isolated from the samples by the sorbent method and the phenol-chloroform extraction method according to Maniatis. Calcium content was determined according to GOST ISO 12081-2013. Pyrogenic testing was performed by NPP Kazan University Vivarium LLC. As equipment were used: mini-centrifuge-vortex Microspin. Helikon (Russia), WM STAT FAX 2100 enzyme-linked immunosorbent analyzer, endotoxin reagent assay kit lal-test/chromogenic test (manufacturer HBT, Netherlands) [3].

A Carl Zeiss AURIGA CrossBeam scanning electron microscope was used to study the determination of physical properties and surface morphology. Samples were analyzed by tandem-mass spectrometry method with the use of Agilent 1260 Infinity Binary System coupled to an AB Sciex TripleTOF 5600 high-resolution mass spectrometer equipped with a DuoSpray ionization source. For the study of the composition, 2 samples are presented - before processing (control) and after...
processing (experience). To isolate lipids, 100 mg of each sample was ground and poured into 2 ml of methanol and incubated for 24 hours at 37°C. Methanol extracts were analyzed by TLC in a solvent system (isopropanol: benzene) (70:30) on Merck silica gel plates. Sections of silica gel with a value of Rf 0.21 were extruded with methanol and 1 ml of extracts was transferred for analysis. DNA was isolated from 120 mg of the test sample and 33 mg of the control sample using the QIAamp DNA Investigator Kit (QIAGEN, Germany) for DNA extraction from bones of a forensic purpose. As a result, 1.62 μg in mg of bone was obtained in the original sample before processing (control), after processing with supercritical carbon dioxide (experience), 0.29 μg in mg of bone.

3. Experimental part

3.1. Experimental purification of bone matrix as a function of the pressure

The processing parameters of the extracellular bone matrix (BM) are presented in Table 1. The process parameters differ from those presented in previous works [4-5].

| Sample | Weight in mg | CFU/ml | CFU/ml | mg DNA/g of sample | mg of protein/g of sample |
|--------|--------------|--------|--------|-------------------|--------------------------|
| BM -original | 642,8       | 10     | 0      | 0,106 ± 0,02      | 2,1                      |
| BM -5  | 395          | 0      | 0      | 0                 | 0,473 ± 0,008            |
| BM -6  | 389          | 0      | 0      | 0                 | 2,32 ± 0,37              |
| BM -7  | 536,8        | 15     | 0      | 0                 | 0,8                      |
| BM -8  | 318          | 0      | 0      | 0                 | 1,24 ± 0,012             |
| BM -81 original | 735       | 0      | 0      | 25,5 ±0,4         | 0,32 ± 0,02              |
| BM -81 | 563          | 0      | 0      | 14,27 ±2,75       | 0,26 ± 0,06              |

The optimal pressure for carrying out cleaning processes revealed 30 MPa, this pressure allows one to penetrate the bone structure without disturbing the surface. The experimental solubility results of substances of various origins show an increase in solubility with increasing pressure and, accordingly, the density of the SC solvent. This tendency is consistent with the Poynting effect, which involves an increase in the pressure of saturated vapors of the condensed phase under conditions of applied external pressure.

Accordingly, with increasing pressure, the saturation of the solution increases, which allows a deeper purification of the target component from the original solution to the supercritical fluid phase. There is a slight change in the concentration of calcium in bone matrix samples.

3.2. Cosolvent experimental cleaning

In this work, ethyl alcohol in an amount of 5% of the main solvent SC CO₂ was used as a cosolvent. Experimental studies were carried out at an extractor temperature equal to T = 313 K and a pressure equal to P = 20-30 MPa, decalciﬁng bone matrix (DBM) was treated with supercritical CO₂ using the
60-minute static method, then the 240-480-minute dynamic method with a 120-minute step and a carbon dioxide consumption of 1 g/min.

### Table 3. Bone matrix processing parameters for cosolvent cleaning.

| No.   | T, K | P, MPa | CO₂ consumption, g/min | τ<sub>stat</sub>, min | τ<sub>din</sub>, min |
|-------|------|--------|-------------------------|-----------------------|---------------------|
| DBM-1 | 313  | 30     | 1                       | 60                    | 120                 |
| DBM-2 | 313  | 20     | 1                       | 60                    | 240                 |
| DBM-3 | 313  | 20     | 1                       | 60                    | 360                 |
| DBM-4 | 313  | 20     | 1                       | 60                    | 480                 |
| DBM-5 | 313  | 20     | 1                       | 60                    | 480                 |

The amount of extract discharged is determined by the mass difference before and after the experiment. The results of the experimental study are presented in table 4.

### Table 4. The results of an experimental study by the weight method.

| No.   | Sample weight before the experiment, m, g | Sample weight after the experiment, m, g | Extract weight, m, g |
|-------|------------------------------------------|------------------------------------------|----------------------|
| DBM-1 | 0,8085                                   | 0,6024                                   | 0,2061               |
| DBM-2 | 1,5455                                   | 1,3678                                   | 0,1777               |
| DBM-3 | 1,7835                                   | 1,538                                    | 0,2455               |
| DBM-4 | 1,2820                                   | 1,2061                                   | 0,0759               |

As a result of the experimental study, visual purification of the sample is observed, which is confirmed by the weight method.

### Table 5. Content of Staphylococcus sp. in samples (CFU) of the colony of the forming units, DNA and protein per 1 g of the sample.

| Sample | Weight, mg | CFU/ml Staphylococcus sp. | µg DNA/g sample | µg protein/g sample | WCa in the sample |
|--------|------------|---------------------------|-----------------|--------------------|-------------------|
| DBM original | 673 | 0 | 14.10 ± 0.56 | 0.45 ± 0.02 | 1.47 |
| DBM-1 | 1830 | 0 | 11.3 | 0.28 | 0 |
| DBM-2 | 1640 | 0 | 11.6 | 0.24 | 0 |
| DBM-3 | 1817 | 0 | 8.25 | 0.41 | 0 |
| DBM-4 | 1450 | 0 | 12.6 | 0.38 | 0 |
| DBM-5 | 940 | 0 | 14.1 | 0.21 | 0 |

Due to the effects of the cosolvent, it does not lead to the necessary DNA purification, the protein is almost halved in almost all samples, and the dynamic processing time is optimal - 240 minutes. It may be necessary to select another cosolvent for this process or to change the concentration. Since all the images are decalcified, then calcium is not contained, which was confirmed by analyzes.

### 4. Test results

4.1. Pyrogenic testing (LAL test protocol)
The analyzed samples were presented in the form of three sets exposed to CO₂ at high pressure and temperature. However, it is known that endotoxin may remain active under such conditions. According to the constructed calibration line, pyrogenic samples in this LAL test are samples whose absorption increases 0.15 Eu/ml. The analysis of the presented samples showed that the absorption of the reaction mixtures with the chromogen and endotoxin antibodies at 405 nanometers, taking into account the controls, did not exceed 0.15. Thus, all samples are recognized as not pyrogenic.

4.2. Physical properties and surface morphology using electron microscopy analysis
An analysis of the surface of the crude bone matrix was carried out on sample BM-10 and after exposure of carbon dioxide with parameters P = 30 MPa, T = 313, exposure time = 480 min, without cosolvent.

Figure 2. Image of the original sample (500x magnification).

Figure 3. Image of the processed sample (500x magnification).

The bone cleavage surface is a porous structure consisting of micropores with a size of 0.5-1 microns, which, intertwined, form a microporous network; pores with a dimension of 10–20 μm, and large cavity formations (50–100 μm) are also visible. Pores permeate the entire thickness of the sample. Contamination in the pores is not observed. There are no pores on the outer surface; a slit-like and dense structure of the material is visible. When comparing the original sample and the purified sample by external components, it can be said that the pores were completely cleaned, the total pore volume did not change, but the pore size itself increased.

4.3. Identification and quantification of potential degradation products using liquid chromatography
The analysis of mass spectrometric data showed the presence of O and K substances in the samples that form ions with m/z during ionization: 415.213, 432.24, 437.195, 258.28, and 515.55 Da. It was found that ions 415.213, 432.24, 437.195 are adduct ions of [M + H] +, [M + NH₄] + and [M + Na] + of one chemical substance. An analysis of the isotope distribution and the fragmentation spectrum revealed that the mass spectrometric peaks with m/z 415.213, 432.24, 437.195 are due to the presence of nanoethylene glycol in the samples. Ions with m/z 258.28 and 515.55 are the adduct ions of the [M + H] +, [2M + H] + chemical compound C₁₆H₃₅NO. It is not possible to determine the exact composition from the fragmentation spectrum due to the lack of data for ions with m/z data in the Metlin, HMDB, and MassBank spectral libraries. However, by the nature of the fragmentation, it can be assumed that this substance belongs to the class of amino alcohols. Also, a series of chemicals is
detected in the samples, which are recorded in the spectra in the form of intense \([M + H] +\) ions, forming a distribution with a difference of \(m/z\) 44 Da. In the fragmentation spectra, fragment ions of ethylene glycol condensation products with \(m/z\) 133, 177, 121, and 247 are recorded, which indicates that this group of compounds is ethylene glycol oligomers, which explains the observed distribution of mass spectrometric signals. This conclusion is consistent with the results of an analysis of the isotope distribution recorded in panoramic scanning spectra. A thorough analysis of the samples did not allow the detection of substances with a lipid structure, which could be due to two reasons: a low analyte concentration and the presence of a significant concentration of polyethylene glycol in the samples. A high concentration of SAS leads to suppression of ionization and, as a result, the absence of an analytical signal.

5. Conclusions
The laboratory bench was modernized to study the processing the extracellular matrix of various organs using supercritical fluid technologies at pressures up to 45 MPa and temperatures up to 100°C. Laboratory studies of the influence of processing pressure from 20 to 35 MPa of a two-stage bone matrix cleaning process at a holding temperature of 308, 313 K for 60-minute static processing time and 240-minute dynamic processing were carried out, including, including subsequent biochemical analyzes to determine residual DNA, calcium protein in the obtained samples. The addition of ethyl alcohol as a cosolvent in an amount of 5% of the main solvent of supercritical CO\(_2\) was studied. Experimental studies were carried out at an extractor temperature equal to \(T = 313\) K and a pressure equal to \(P = 20-30\) MPa, decalcified bone matrix (DBM) was processed with supercritical CO\(_2\) using the 60-minute static method, then the 240-480 minute dynamic method with 120-minute step and a carbon dioxide flow rate of 1 g/min.

The optimal parameters for the cleaning process were identified: temperature in the system 313 K, pressure in the system 30 MPa, static processing time - 60 minutes, dynamic processing time - 240 minutes. The absence of a liquid phase in supercritical CO\(_2\) under normal conditions makes it possible, upon completion of the technological process of biological tissue modification, to transfer supercritical carbon dioxide from a dense supercritical medium to a rarefied gas, excluding the formation of a liquid phase, and the formation of the effect of “drying liquid drops”. In systems containing adjoining phases of practically immiscible solvents, which include the “contaminant – supercritical fluid” system, knowledge of the basic laws governing the distribution of solutes between a solvent and taking into account the synergistic effect are required. A quantitative analysis of the solubility of low-volatiles in supercritical fluids shows that the absolute solubility values do not exceed, as a rule, several hundredths of a mole fraction. The neutralization or denaturation of proteins makes it possible to transfer the mineral component of the bone together with the collagen matrix from animal to human without the development of undesirable reactions.

Further work will be devoted to studying the influence of thermodynamic parameters and the conditions of the experiment on the impregnation pharmaceutical preparations and recombinant protein growth factors (rhBMP) in the porous structure of a purified biological bone matrix.

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