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Decellularization of bone tissue in a supercritical carbon dioxide environment

Abstract. The use of pig bone tissue as a graft makes it possible to obtain high-quality, relatively cheap and biocompatible ECM. This is since the mineral composition of pork bone is most similar to the mineral composition of human bone. Decellularization in SC-CO_2 is a promising direction, since carbon dioxide easily diffuses into the depth of the cell and is a good solvent for lipids, it is non-flammable, non-toxic and chemically non-aggressive. In this paper, the ECM in SC-CO_2 was obtained in three ways: 1) in dynamic mode with large SC-CO_2 flows; 2) in static and dynamic modes with large SC-CO_2 flows; 3) in static and dynamic modes with large SC-CO_2 flows, with preliminary exposure in ethyl alcohol as a co-solvent. According to the histological examination, the removal of ICC by the first method is 55%. The use of a co-solvent before starting the decellularization process increases the percentage of ICC removal to 98%, which allows the use of ECM as a transplant.
Key words: decellularization, tissue engineering, transplantation, supercritical extraction, carbon dioxide, biomaterials, extracellular matrix, xenografts.

Introduction

Bone tissue is in second place in terms of transplantation after blood, in this regard, the need for high-quality and inexpensive bone grafts is increasing every year. It is related to an increase in the number of patients with diseases of the musculoskeletal system, victims of various accidents that lead to injuries and infections formation. However, common methods of treatment, such as tissue/organ transplantation, surgeries, use of various mechanical devices, face several unresolved issues and problems, which aroused great interest in tissue engineering [1, 2].

Various biomaterials are used to obtain transplants. By origin, materials can be divided into allogenic (donor is another person), autogenic (donor is the recipient), alloplastic (synthetic biomaterials), xenogenic (donor is an animal) [3-5].

The use of xenogenic materials is a promising direction. This is due to their maximum availability and cheapness, which makes it possible to almost recreate the damaged area completely. Most often, bovine and pig bones are used as xenogenic material. However, in the works [6-8], it was proved that the use of bovine bone tissue leads to further rejection of the graft and inability of colonization with stem cells. That is why pig bone grafts are now widely used. The trabecular pig bone is most similar in mineral composition to the mineral composition of human bone [9-11].

The most common method of draft obtaining is decellularization. Decellularization is a method in which all intracellular components (ICC) are removed under the action of various agents. The result of decellularization is the extracellular matrix (ECM), which during the process must not be destroyed and retain its composition, as well as mechanical and biological properties. Decellularization can be chemical, biological, physical and with the help of supercritical fluid technologies. Each method has a number of advantages and disadvantages.

Chemical decellularization includes treatment with acids and bases, detergents, hypotonic and hypertonic solutions [12]. Acids and bases in the process of decellularization cause or catalyze hydrolytic degradation of biomolecules, but at the same time reduce the strength of ECM [13,14]. Detergents such as SDS (sodium dodecyl sulfate)

and Triton X-100 completely remove ICC, however, they affect structure and composition of ECM, since together with ICC, glycosaminoglycans are washed out and eliminate growth factor. The use of hypotonic and hypertonic solutions has the least effect on ECM, however, the removal of ICC does not occur completely. Biological decellularization with the help of enzymes also has a minimal effect on ECM, however, complete removal of ICC by this treatment cannot be achieved. The removal of ICC during physical decellularization occurs due to physical forces influence, but they can also affect the ECM. Physical decellularization includes such methods as freezing-thawing, osmotic pressure, ultrasonic treatment, etc. [15].

Supercritical fluid CO_2 extraction currently is of great interest to scientists. This is due to unique properties of CO_2 that it possesses under supercritical conditions. The main advantage of supercritical carbon dioxide (SC-CO_2) during the decellularization process is that it is a non-toxic, non-combustible, relatively inert gas and diffuses independently from bone tissue over time due to the transition to a gaseous state under normal conditions. Due to diffusing, more channels are formed inside the tissue. Due to the unique properties of diffusion, density and viscosity SC-CO_2 is used as a selective extraction solvent. It is especially suitable for extracting components from a solid microporous structure, including bone tissue. Due to the high transfer rate and high permeability possessed by the supercritical fluid, any endotoxins, allogeneic proteins and bio-loading are completely removed from the ECM, which leads to a decrease in the immunogenicity of the graft. Consequently, the decellularized tissue obtained by this method will be safe for the recipient after implantation, since SC-CO_2 treatment avoids the use of toxic organic solvents and does not leave a cytotoxic residue. From an economic point of view, use of SC-CO_2 is promising, since it is found in large quantities in nature, which significantly reduces its cost and increases availability. Cyclic installations used for decellularization under supercritical conditions allow carbon dioxide to be used repeatedly. The use of supercritical fluid in the decellularization process can completely or partially reduce the use of aggressive, toxic and expensive reagents. Due to this, as a result of decellularization in SC-CO_2, will be obtained commercially available, biochemically pure and structurally intact ECM [16].

In paper [17], a comparative analysis of the traditional process of decellularization using detergents and decellularization in SC-CO_2 was carried out. The obtained ECMs were examined using histological examination, scanning electron microscopy (SEM) and DNA and collagen were determined. The ECM analysis confirmed that decellularization in SC-CO_2 proceeds efficiently and does not have a significant effect on the matrix, unlike detergents. The traditional decellularization process takes on average from 3 to 14 days, whereas decellularization in SC-CO_2 at 23 ml/min flow rate took less than 3 days. In addition, in this method, the number of stages of decellularization is significantly reduced and there is no need to wash the ECM before introducing the graft into the body. While removal of ICC using SC-CO_2, there is no need to use high temperatures, since carbon dioxide goes into a supercritical state at T=31.1°C, due to which thermosensitive biomaterials can be subjected to this treatment [18].

Materials and methods

The pig trabecular bone tissue was used as a biomaterial. Sample preparation of the initial biomaterial was carried out, which included the removal of meat and fat residues using a scalpel, the production of sections using a band/saber saw, freeze drying and vacuuming.

The decellularization process using SC-CO_2 was carried out on an experimental semi-industrial supercritical installation. The installation diagram is shown in Figure 1.

Decellularization process in SC-CO_2 in dynamic mode with constant flow [19]. To carry out the decellularization process in SC-CO_2, bone sections were fixed in an extraction vessel so that the samples did not come into contact with the bottom of the vessel. The vessel was moved into the reactor, the reactor lid with a pressure gauge was tightly fixed and the outlet-underwater channels were closed. By turning on the medium-temperature refrigeration unit, the CO_2 in the condenser passes from the gaseous state to the liquid state and enters the expansion column with the help of a high-pressure pump, where the CO_2 passes into the supercritical fluid state and enters the reactor. The flow rate is 1500 g/min, the temperature is 37°C, the pressure is 190-200 atm. After the reactor, the CO_2 with extracted compounds passes into the cyclone separator, where its transition to gaseous state occurs again. Then the gas passes through the adsorber back into the condenser. Due to the cyclical nature of the installation, the CO_2 consumption is minimal. The conditions for decellularization in SC-CO_2 in a dynamic mode with a constant flow are given in Table 2.

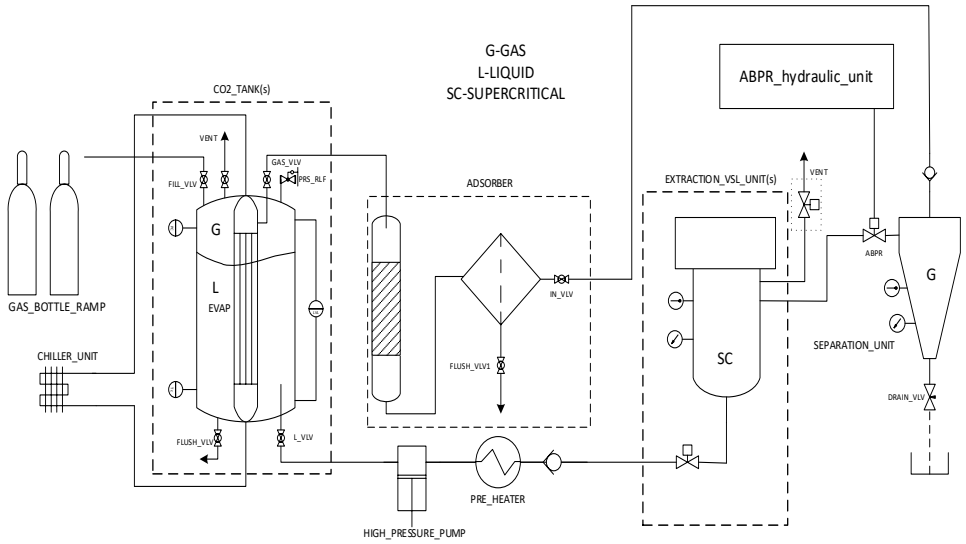

Figure 1 – Diagram of an experimental semi-industrial supercritical installation

Table 1 – Main components of an experimental semi-industrial supercritical installation

Name	On the diagram	Specifications
High pressure pump V4	HIGH_PRESSURE_PUMP	Inlet pressure: 10…80 bar, outlet pressure: 15...450 bar, CO_2 flow: 0,2…2 l/min
Condenser	CO2_TANK(s)	Working pressure: 70 bar, volume: 30 L
Medium-temperature refrigeration unit	CHILLER_UNIT	Refrigerant: R404a, cooling capacity: 10 kW at -15 °C
Cyclone separator	SEPARATION_UNIT	Working volume: 2 L, working pressure: 100 bar
Back pressure regulator	BPR	Working pressure: 30…500 bar, conditional pass: 6 mm
Hydraulic station	Not specified in the diagram	Hydraulic fluid flow: 1...10 l/min, working pressure: 160 bar
Heater block	Not specified in the diagram	Number of circuit: 2, working temperature: up to 100 °C, circuit power: 6 kW
Heat exchanger	PRE_HEATER	Shell and tube, working pressure: 450 bar

Table 2 – Conditions for decellularization in SC-CO_2 in dynamic mode with constant flow

Sample, #	Decellularization time, hours
1	0,5
2	1
3	2
4	4
5	6
6	8

Decellularization process in SC-CO_2 in static (with exposure in SC-CO_2) and dynamic (with constant flow) modes. To carry out the decellularization process under these conditions, the same preparatory operations are carried out that are given for the decellularization process in SC-CO_2 in dynamic mode, the difference is the alternation of static and dynamic modes. To carry out decellularization in static mode, SC-CO_2 is converted to a liquid state, using a high-pressure pump, the pressure in the reactor is pumped up to 200-230 atm and then the pump is turned off.

The process in dynamic mode proceeds according to the method described above. The flow rate is 1500 g/min, the temperature is 37°C, the pressure is 190-200 atm. The conditions for decellularization under supercritical conditions in static and dynamic modes are presented in Table 3.

Table 3 – Conditions for decellularization in SC-CO_2 in static and dynamic modes

Sample, #	Decellularization time in static mode, hours	Decellularization time in dynamic mode, hours
1	0,25	0,25
2	0,5	0,5
3	1	1
4	2	2
5	4	4
6	6	6
7	8	8

Decellularization process in SC-CO_2 in static (exposure in SC-CO_2) and in dynamic (at constant flow) modes with preliminary exposure in 96% ethyl alcohol. According to paper [20], the use of a co-solvent during decellularization in SC-CO_2 increases the percentage of ICC removal. 96% ethyl alcohol was used as a co-solvent. The samples were previously soaked in ethyl alcohol before the process and after the process was carried out for 6 hours in static mode and 6 hours in dynamic mode. The flow rate is 1500 g/min, the temperature is 37 °C, the pressure is 190-200 atm. The preliminary exposure time of samples in 96% ethyl alcohol is shown in Table 4.

Table 4 – Preliminary storage time of samples in 96% ethyl alcohol

Sample, #	Preliminary storage time of samples in ethyl alcohol, hours
1	0,5
2	1
3	2
4	4
5	8
6	12
7	24
8	48
9	72
10	96
11	120
12	144

Histological examination. Histological examination includes several stages: fixation, decalcification, dehydration, paraffin impregnation, paraffin filling, obtaining histological sections, deparaffinization, staining, microscopic analysis. Formalin was chosen as the fixing fluid, in which the bone tissue was kept for 24 hours. After fixation, to remove formalin residues, the samples are placed in ethyl alcohol with an increasing concentration (70, 80, 96%) for 12 hours for each solution. 5% nitric acid was used for decalcification. The samples were placed in the solution for 24 hours with constant stirring (175 rpm) using a universal orbital shaker (LOIP LS-110, РФ). The nitric acid solution must be replaced 3 times with a fresh one within 24 hours. After decalcification, the samples are crushed with a scalpel to a size of no more than 1 cm in length, 1 cm in width and 0.3 cm in thickness. After the obtained rectangular-shaped materials are subjected to dehydration in ethyl alcohol with increasing concentration (50, 80, 96, 100%). In each solution, they are kept for 24 hours. At the end of dehydration, the samples are placed in a mixture of chloroform:ethyl alcohol = 1:1 and kept for 3 hours, changing the solution to a fresh one every hour, then transferred to a pure chloroform solution for 3 hours. Then the samples are placed in a saturated solution of paraffin in chloroform for 12 hours and placed in an electric dry-air thermostat (ТС-1/80 СПУ, РФ) at 37 °C and constant stirring. After the time has elapsed, they are transferred to a pure paraffin solution for 3-4 hours and thermostated at 56-57 °C with constant stirring. Then the samples are placed in homemade paper boats and filled with paraffin and left until the

paraffin completely solidifies for 12-24 hours. After using a manual microtome (MS 2 TU 64-16, РФ), sections of bone tissue 5-7 microns thick are obtained from the obtained paraffin blocks and transferred to a slide. For further staining, it is necessary to remove the remnants of paraffin: for this, slides with samples are placed sequentially for 15 minutes in chloroform solutions (changing the solution 3 times), in solutions of ethyl alcohol with a descending concentration (100, 90, 80%) and in distilled water. After that, staining with eosin and Mayer's hematoxylin is carried out, placing samples for 10 minutes in each solution. After that, the dehydration process is carried out by placing the samples in solutions of ethyl alcohol with an ascending concentration and then in a solution of o-xylene. After that, the slices are enclosed in Canada balsam, dried in a thermostat for 12 hours at 37 ℃ and microscopic analysis of the obtained slices is performed [21].

Study of bone tissue surface morphology using optical microscopy. Optical microscopy was used to control changes in surface morphology during the decellularization process. The study of the morphology of the bone tissue surface was carried out on a trinocular microscope (MX-300, РФ) at a magnification of 40 times in the " Open Type National Nanotechnology Laboratory" at the al-Farabi Kazakh National University and on optical microscope (BRESSER ADVANCE ICD, China) with magnification of 20 times in the Center of Physics and-chemical methods of research and analysis (CFChMA) in the laboratory of sorption and catalytic processes.

Isolation of DNA from bone tissue followed by spectrophotometric detection. To isolate DNA from bone tissue, 400 mg of the sample is ground into powder using a mortar, 1 ml of Degestion buffer is added (4.7 ml EDTA +50 ml proteinase K +250 ml 10% N-Laurylsarcosyl) and incubated in a thermostat for 30 minutes at T = 50℃. After the sample is centrifuged at 10000 rpm for 15 minutes, the resulting supernatant is removed. Then another 1 ml of Degestion buffer is added to the precipitate, conduct incubation in thermostat at T = 50 ℃ for 24 hours and centrifugation at 13000 rpm for 15 minutes. The resulting supernatant is transferred to a test tube and 4 ml of Binding buffer is added (118.2 g of guanidinium thiocyanate, 10 ml of 1M tris (hydroxymethyl) is placed in a 200 ml volumetric flaskaminomethane, 8 ml 0.5 M EDTA, 1 g of N-Laurylsarcosyl and distilled water to the label), thoroughly mixed, 20 µl of silicon dioxide was added and incubated at T = 50 ℃, for 3 hours. At the end of the time, they are centrifuged for 15 minutes at 10000 rpm. The supernatant is removed, and 1 ml of cooled 80% ethyl alcohol is added to the resulting precipitate, centrifuged at 10000 rpm for 15 minutes and the supernatant is removed. 50 ml of TE buffer is added to the precipitate, incubated for 3 minutes at room temperature and centrifuged at 13000 rpm for 15 minutes. The resulting supernatant containing DNA is analyzed using a spectrophotometer (NanoDrop 2000/2000c, USA) at 260 nm wavelength [22].

Results and discussion

Histological examination is the most common method of determining the completeness of the decellularization process. Hematoxylin – stains the nuclei of cells, and eosin – stains the cytoplasm. The complete or partial absence of stained nuclei and cytoplasm characterizes the removal of ICC. At the end of the histological examination, the following microscopic images at x200 magnification were obtained for samples obtained by decellularization in SC-CO_2 in a dynamic mode with a constant flow (Figure 2). Based on the obtained results, it can be concluded that the time of decellularization directly affects the removal of ICC, which characterizes decrease in the number of brightly colored nuclei over time. However, according to the obtained images, it can be said that after 8 hours of the decellularization process, a small number of colored nuclei are observed in SC-CO_2, which characterizes the incomplete removal of ICC.

At the end of the histological examination, the following microscopic images at x200 magnification were obtained for samples obtained by decellularization in SC-CO_2 in static and dynamic modes (Figure 3).

Decellularization process time increase and addition of a static mode leads to a more complete removal of the ICC.

At the end of the histological examination, the following microscopic images at x200 magnification were obtained for samples obtained by decellularization in SC-CO_2 in static and dynamic modes with preliminary exposure in 96% ethyl alcohol (Figure 4).

Usage of 96% ethyl alcohol as a co-solvent significantly affects the decellularization process. ICC removal by this method proceeds more fully.

Results of microscopic analysis of samples obtained by decellularization in SC-CO_2 in dynamic mode with constant flow are shown in Figure 5.

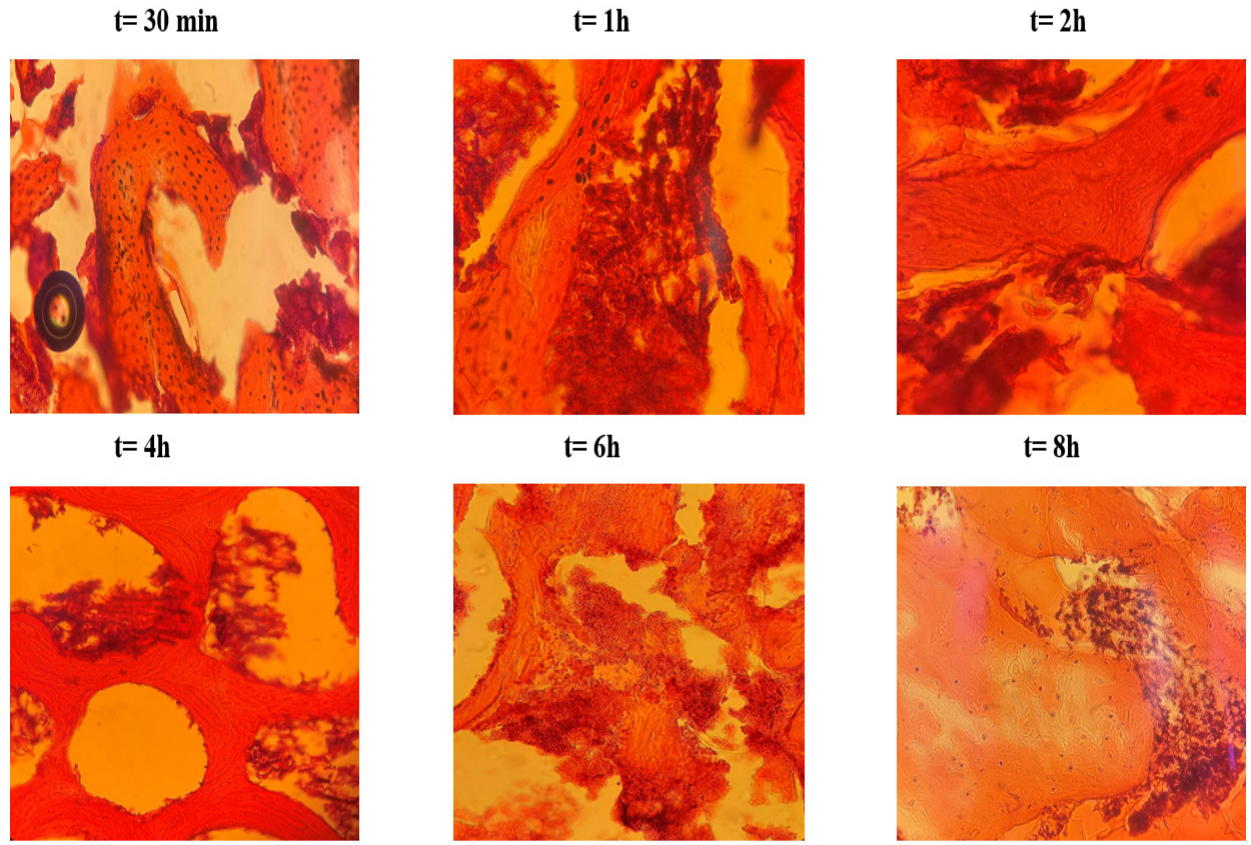

Figure 2 – Changes in the histostructure of pig bone during decellularization

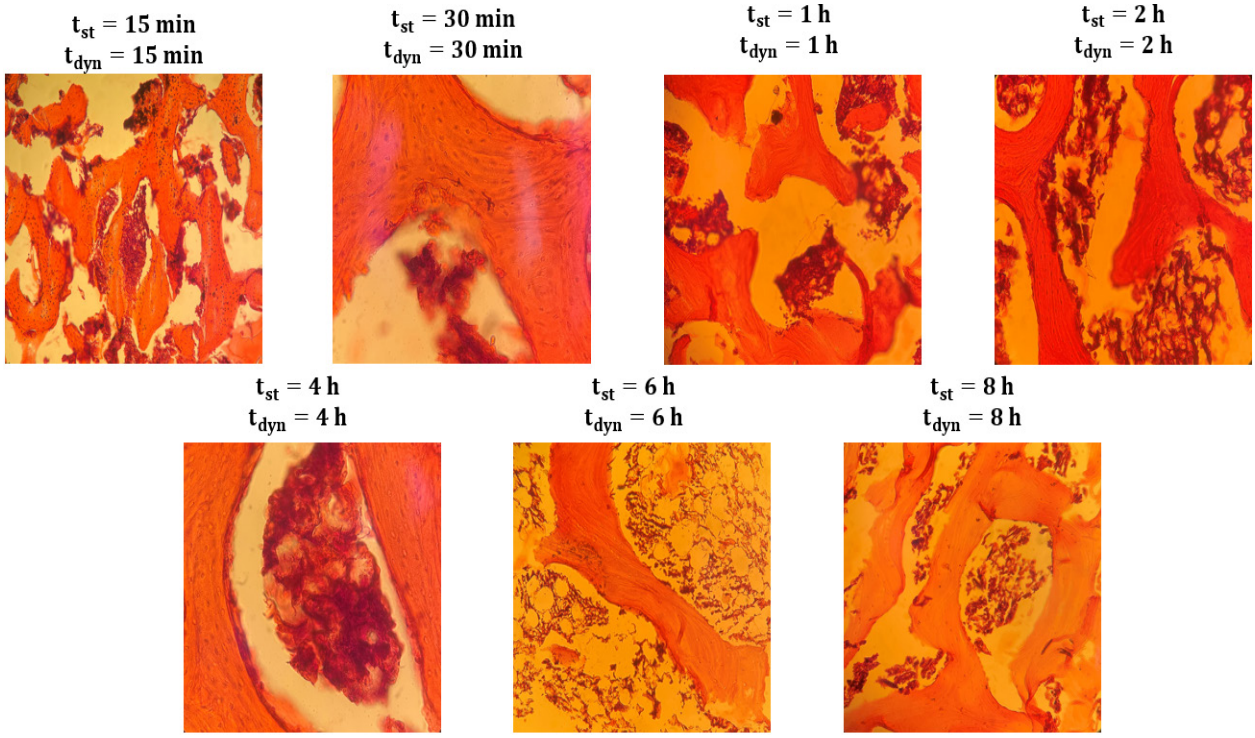

Figure 3 – Changes in the histostructure of pig bone during decellularization

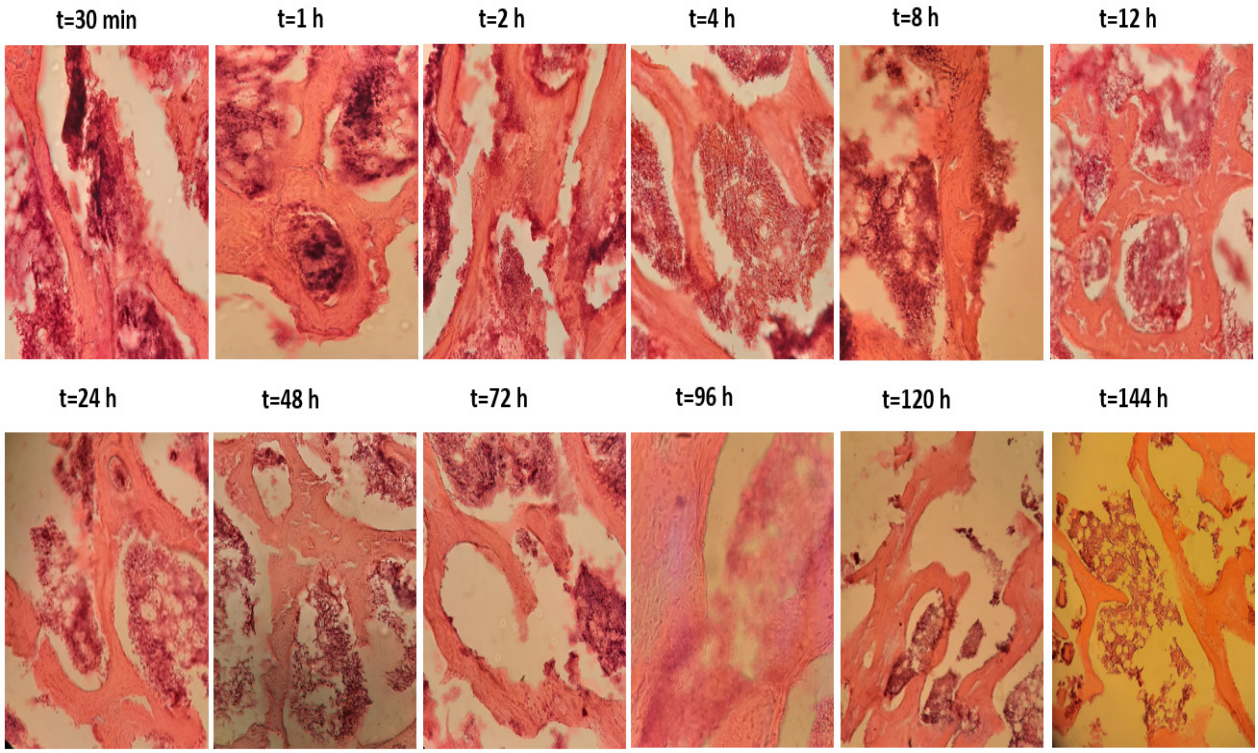

Figure 4 – Changes in the histostructure of pig bone during decellularization

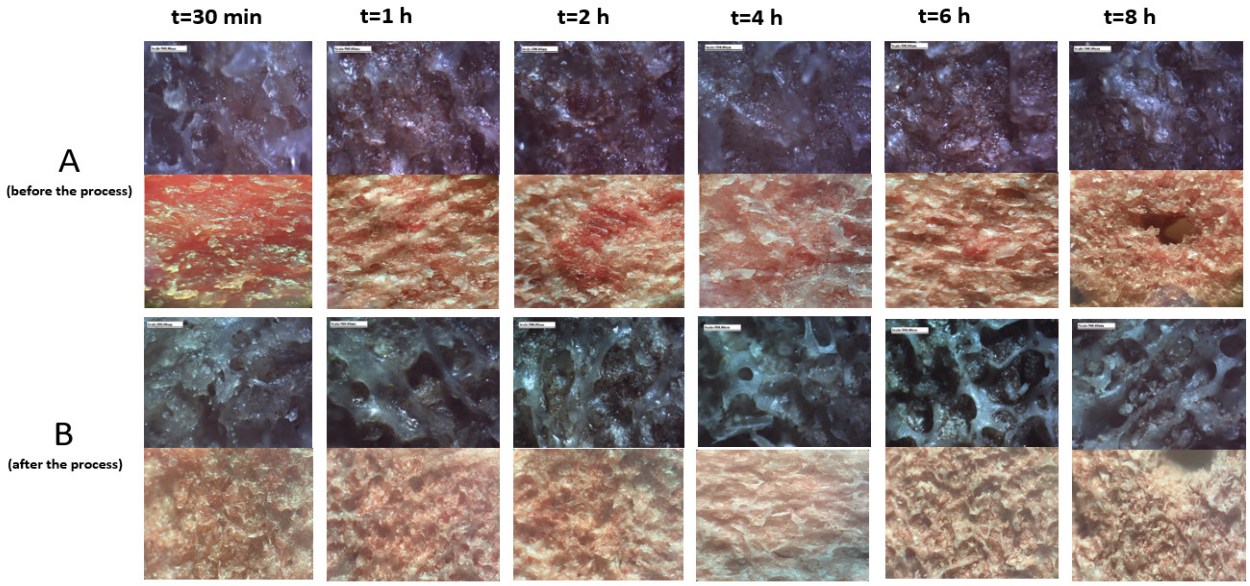

Figure 5 – Microscopic images of bone tissue surface morphology

Based on the obtained images of decellularization process, it can be concluded that the process directly depends on time. However, when decellularization of bone tissue in the SC-CO_2 medium with a constant flow after 8 hours, the presence of ICC is observed, which characterizes the incompleteness of decellularization and use of graft obtained by this method is doubtful.

The results of microscopic analysis of samples obtained by decellularization in SC-CO_2 in static and dynamic modes are shown in Figure 6.

When decellularization is carried out in SC-CO_2 medium in static and dynamic modes, a significant increase in the porosity of the ECM and the visual absence of ICC is observed, which characterizes the completeness of the decellularization process

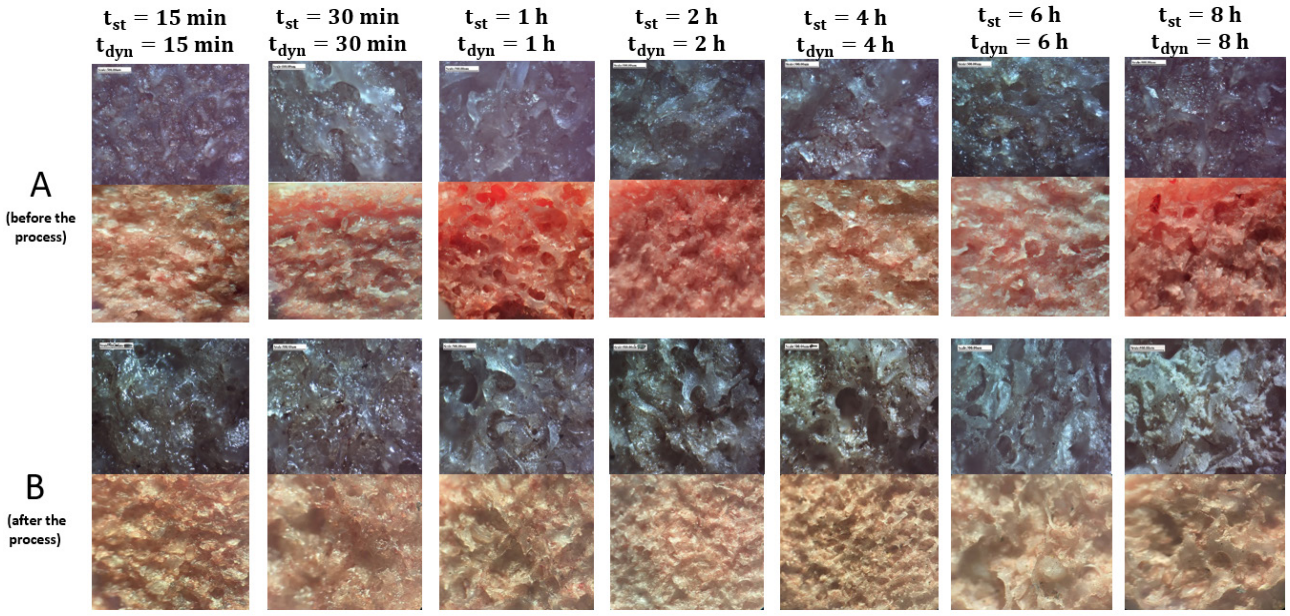

Figure 6 – Microscopic images of bone tissue surface morphology

The results of microscopic analysis of samples obtained by decellularization in SC-CO_2 in static and dynamic modes with preliminary exposure in 96% ethyl alcohol are shown in Figure 7.

Usage of 96% ethyl alcohol as a co-solvent and an increase in the time of the process has a positive effect on the morphology of bone tissue surface and the removal of ICC takes place most completely.

With the help of spectrophotometric analysis, the DNA concentration of the last samples was determined in each method (Figure 8).

The concentration of DNA in bone tissue after the decellularization process in SC-CO_2 in dynamic mode with constant flow is 69.4 ng/µl, after decellularization in SC-CO_2 in static and dynamic modes is 61.5 ng/µl, after decellularization in static and dynamic modes with preliminary exposure in 96% ethyl alcohol is 33.4 ng/µl.

The concentration of DNA extracted from bone tissue using the decellularization method in static and dynamic modes with preliminary exposure in ethyl alcohol is almost 2 times greater than in samples obtained by other methods.

Table 5 shows the optimal conditions for obtaining ECM using the decellularization method.

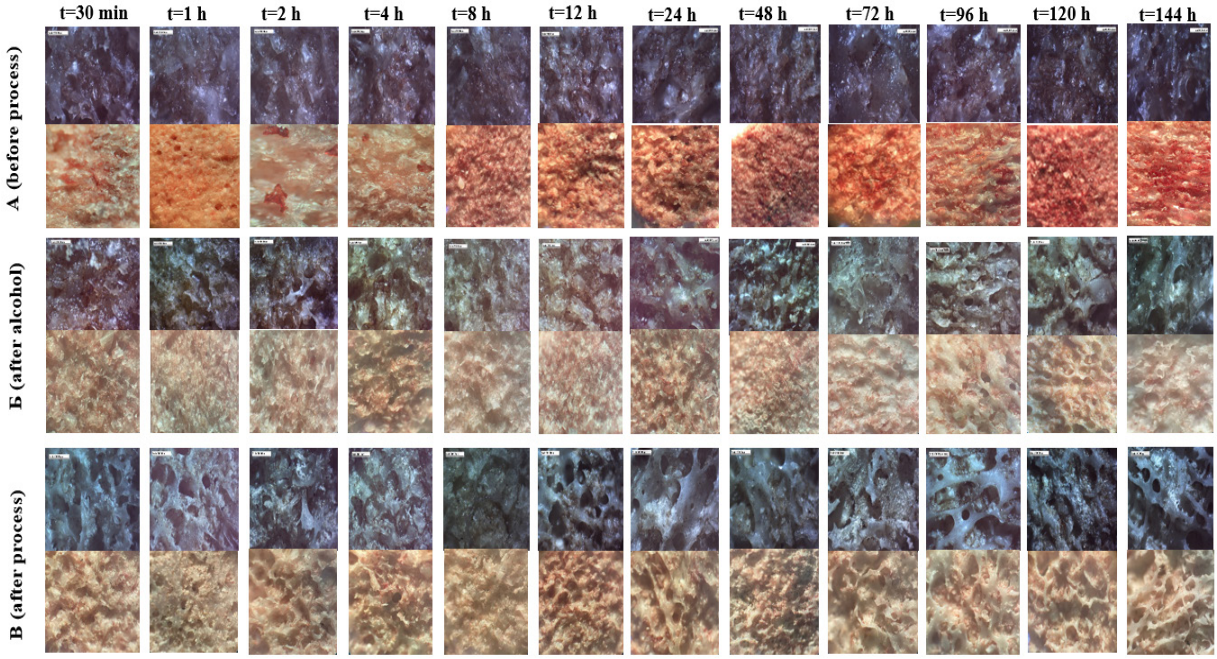

Figure 7 – Microscopic images of bone tissue surface morphology

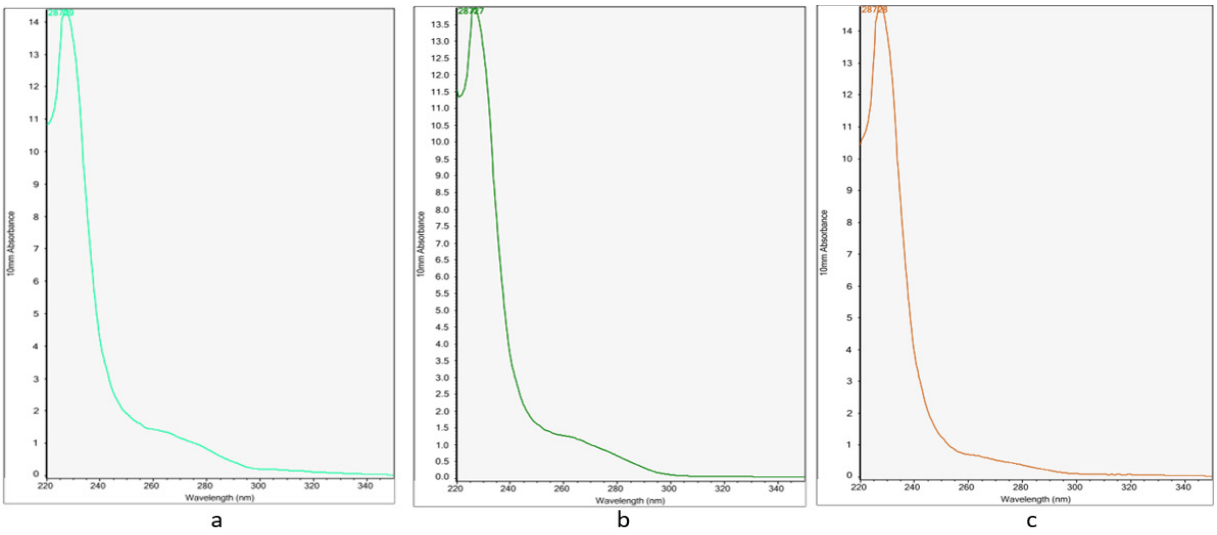

Figure 8 – Dependence of absorption coefficient on wavelength in the spectrophotometric determination of DNA (a) the process of decellularization in dynamic mode; (b) the process of decellularization in static and dynamic modes; (c) the process of decellularization in static and dynamic modes with preliminary exposure in 96% ethyl alcohol

Table 5 – Optimal conditions for obtaining ECM

#	Exposure time in ethyl alcohol, hours	Decellularization time in static mode, hours	Decellularization time in dynamic mode, hours
1	144	6	6

Also, according to [23], if the concentration of DNA in the decellularized material is less than 50 ng/μl, the use of this transplant is possible, since the appearance of the recipient's immune response is minimal.

Conclusion

According to the obtained microscopic images after histological examination, it can be concluded that the removal of ICC during the process of decellularization in SC-CO_2 in a dynamic mode with a constant flow occurs by only 70%, and the completeness of the decellularization process is 55% (visual calculation based on microscopic images of the bone surface morphology), whereas with decellularization in static and dynamic modes with preliminary exposure in ethyl alcohol, the removal of ICC is 98%, and the completeness of the decellularization process is 97%. In overall, use of the obtained ECM by decellularization in static and dynamic modes with preliminary exposure in ethyl alcohol is promising since its effect on the human body will be minimal. From an economic point of view, the use of this method is also promising, since the use of the static method leads to the possibility of increasing the process time, reducing energy consumption and wear of the pump.

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