ISSN 1990-7931, Russian Journal of Physical Chemistry B, 2020, Vol. 14, No. 7, pp. 1158–1162. © Pleiades Publishing, Ltd., 2020.
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Supercritical Treatment of Xenogenic Bone Matrix in the Manufacture of Implants for Osteosynthesis

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Received June 10, 2019; revised June 25, 2019; accepted June 25, 2019

Abstract—A method for producing a new generation of high-purity xenogenic biomatrixes suitable for implantation in a living organism is proposed. Special biocompatibility can be achieved by additional treatment with supercritical carbon dioxide and the introduction of dosed amounts of polylactides with molecular weight of 20 and 40 kDa in the porous space of the matrix. In vivo testing of the obtained materials showed their high biocompatibility with varying degrees of integration with surrounding tissues.

Keywords: supercritical carbon dioxide, biological matrix, xenogenic bone tissue, resorbability, polylactide
DOI: 10.1134/S1990793120070064

INTRODUCTION

Implants made of xenogenic bone matrix obtained from farm animals intended to replace the affected areas of bone tissue are widely represented in the global market of osteoplastic materials. The different times of resorption (resorption in biological media) and formation of new bone tissue are common features to all implants of this type. This may result in either incomplete recovery of the recipient's bone tissue, if bone growth does not keep up with the rate of resorption material, or to slow recovery, if the material is resorbed more slower than bone tissue is regenerated [1–3]. With the instability of the implant–bone juncture and differences in biophysical and biomechanical characteristics of the material and bone tissue, interfacial destruction and clinical implant dysfunction may occur [4]. A disadvantage of the known variants of osteo-inductive biological matrices is reduced biocompatibility and uncontrolled accelerated resorption in the body [5, 6].

The implant material structure should replicate the open bimodal porous structure of bone tissue, which promotes angiogenesis, adsorption of proteins, and fixation of osteogenic cells on the contact surfaces of the implant [7, 8].

The xenogenic bone matrix (XBM) is a material similar in structure to natural bone tissue with analogous microstructure; it consists of type 1 collagen (organic component) and calcium phosphates (mineral component). An important technological step in creating XBM-based implants is the chemical treatment of interfibrillar spaces from cells of the fat and protein component of the original organism, which allows achieving biocompatibility and functioning of the implant after the operation. In addition, the osteogenic activity of the bone matrix increases after the removal of the mineral component of bone tissue due to the "opening" of an entire complex of protein regeneration factors [9, 10].

The existing methods of physico-chemical treatment of bone tissue aim to increase biocompatibility and osteoinductivity. They suggest the use of various chemicals: ethyl alcohol, diethyl ether, hydrochloric acid, urea, enzymes, antibiotics, etc. The disadvantage of using chemical reagents is the presence of residual impurities of solvents that reduces biocompatibility and preservation of the regenerative potential of the implant.

Application of supercritical (SC) media at the final stage of processing creates significant advantages in comparison with the traditional method of chemical cleaning of the initial matrix. This is due to the rapid mass transfer in SC-media due to low viscosity and high diffusion coefficient. As a result, acceptable cleaning of the most inaccessible areas of the matrix is achieved.

The present work is aimed at creating a new family of osteosubstituting matrices with increased biocompatibility and osteoinductivity using supercritical technologies.

In addition, we suggest that the introduction of complex antibiotic polylactide into the interfiber space is able to prolong the antibacterial effect of antibiotics after the implantation of the matrix into the recipient. For this reason, it seems important to study the effect of polylactide on the biocompatibility of bone material.

The purpose of this phase of the study is to develop a methodology for obtaining high-bone matrices with enhanced biocompatibility due to additional treatment of partially demineralized precursors in the SC-CO_2 medium and Introduction of lactide polymers of various molecular weights into the matrix structure.

EXPERIMENTAL

Xenogenic Bone Block Demineralization Technique

Demineralization of XBM blocks measuring 10 × 10 × 10 mm (with a deviation of ±1.2 mm on one side, but not more than ±1.5 mm on three sides) and weighing 0.25–1.05 g was carried out in a solution of hydrochloric acid of various normality at low nominal pressure (200 torr) and vigorous stirring (rotation speed of mixers 100 rpm) for 3–90 min.

After a specified time, the hydrochloric acid solution was drained, the blocks washed three times with distilled water and dried by lyophilization. For 1 g of a XBM sample 50 mL of HCl solution was taken.

Determination of Residual Calcium in Demineralized Blocks

The determination of residual calcium was carried out using the weight method by the mass of calcium phosphates present in the samples before and after demineralization. The mass of the calcium phosphates remaining in the sample after demineralization was determined by weighing the samples after heat treatment at 850°C for 3 h to remove the organic component. The amount of calcium phosphate removed during demineralization was determined by the difference in the mass of the samples before and after processing. In the calculations, the following ratios were used:

$$1)\ N = M_{\text{init.}} - M_{\text{dem.}},$$

where N is the mass of removed calcium phosphates, g; $M_{\text{init.}}$ is the mass of the original sample, g; $M_{\text{dem.}}$ is the mass of the demineralized sample, g;

$$2)\ E = N + P,$$

where E is the mass of calcium phosphates in the sample before demineralization, g; and P is the mass of calcium phosphates remaining after demineralization of, g;

$$3)\ M_{\text{Ca(init.)}} = 0.3785E,$$
$$M_{\text{Ca(dem.)}} = 0.3785P,$$

where $M_{\text{Ca(init.)}}$ and $M_{\text{Ca(dem.)}}$ is the mass of calcium in the initial and demineralized samples, respectively, g; 0.3785 is the average mass fraction of calcium in the biological hydroxyapatite gross formula $Ca_{8.3}(PO_4)_{4.3}(CO_3)_x(HPO_4)_y(OH)_{0.3}$, $x + y = 1.7$;

$$4)\ Ca_{\text{rem.}} = M_{\text{Ca(dem.)}}/M_{\text{Ca(init.)}} \times 100\%,$$

where $Ca_{\text{rem.}}$ is the percentage of residual calcium.

Bone Matrix Cleaning Using Supercritical CO_2

For preliminary purification of XBM samples from fat and protein components by extraction in a Soxhlet apparatus, a number of organic solvents—diethyl ether, acetone, pentane, methanol, methylene chloride, pentane, as well as their various combinations in successive extractions—were tested. Optimal extraction conditions were determined: acetone (8 h), then pentane (8 h).

After extraction, the bone blocks were dried in air at room temperature for 12 h. The residual solvent was removed in vacuo (40 min at 10 mm Hg). The final cleaning of the samples was carried out in a flow reactor (Fig. 1), stirring at 46 or 53°C and at a pressure of SC-CO_2 10 MPa, to feed which a high-pressure pump was used (TharSFC). In all cases, the necessary extraction time was determined by adjusting the samples to constant weight according to the results of control weighing with an accuracy of ±1 mg.

The Introduction of Resorbable Polylactides Into the Porous Structure of the Bone Matrix

PARASORB PDL 02 and 04 polylactides (PLA) (Corbion) with molecular weights (M) of 20 and 40 kDa, respectively, were used to introduce purified samples into the pores. The absorption of the PLA solution by the volume of samples occurred due to their natural porosity in one go, which provided more uniform polymer adsorption within the implant. Repeated procedure increases the polylactide concentration gradient along the height of the sample due to solvent evaporation. The bone blocks preliminarily purified in SC-CO_2 were placed in a polymer solution in methylene chloride (0.7 mL). After complete absorption of the solution, the residual solvent was removed in vacuo (40 min at 10 mm Hg). For further biological experiments, 4 series of samples were prepared, containing PLA with M = 20 and 40 kDa in the amount of 5 and 10% of the initial mass of the bone block.

Implantation of Modified Matrices in the Subcutaneous Tissue of Laboratory Animals

Animal studies were carried out in compliance with the international rules of bioethics in accordance with

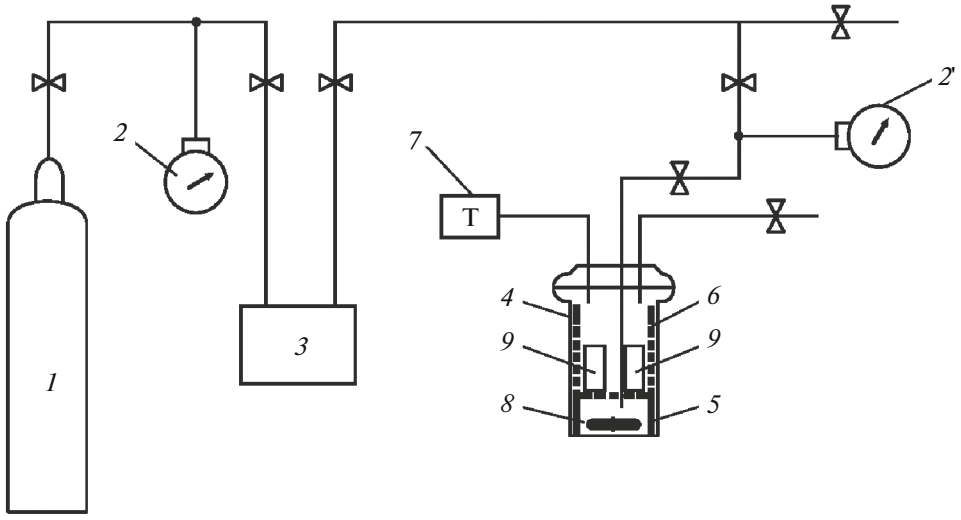

Fig. 1. Schematic diagram of the installation with a flow reactor: (*1*) cylinder with CO_2; (*2*, *2*') pressure gauges; (*3*) high pressure pump; (*4*) flow reactor; (*5*) teflon ring; (*6*) perforated teflon glass; (*7*) thermocouple; (*8*) magnetic stir bar; (*9*) matrix samples.

the requirements of the Helsinki Declaration of the World Medical Association and guidelines on the humane treatment of laboratory animals, as well as the standard *GOST P ISO 10993-2* "Medical devices. Assessment of the biological effects of medical devices. Part 2. Requirements for animal welfare conditions."

The implantation was performed on 3-month-old Wistar rats weighing 250–300 g. Under general anesthesia in aseptic conditions, linear access was performed in the projection of the spine, through which pockets for implanting the material were formed in the subcutaneous tissue. The observation period was 30 days, after which the studied samples of the matrix were dissected and transferred to histological examination.

Production of Histological Sections

For the study, the samples were prepared according to the standard Merkulov's histological scheme with demineralization [11]. The sections were dyed with hematoxylin and eosin. The microphotographs obtained with a digital microscope with 200-fold magnification were studied using ImageView and HistoMorph v.2.2. programs.

The obtained histological data served to evaluate the biological compatibility of samples according to the following parameters: inflammation, integration with the surrounding tissues and angiogenesis (formation of new blood vessels). For the convenience of processing the results, a scoring system has been adopted.

For Inflammation:

3 points – absence of inflammation;

2 points – no signs of purulent inflammation, a small leukocyte infiltration

1 point – pronounced leukocyte infiltration, without foci of purulent inflammatio;

0 points – severe purulent inflammation.

For Integration with Surrounding Tissues:

3 points – the sample is surrounded by a transparent thin connective tissue capsule, partially or fully resorbed;

2 points – connective tissue densities of the capsule are noted on the sample capsules, resorption is not pronounced;

1 point – the sample is partially surrounded by a dense unformed connective tissue capsule;

0 points – the sample is completely surrounded by a dense unformed connective tissue, delimited from its own tissues.

For Angiogenesis:

3 points – the area of the newly formed vessels is more than 5%;

2 points – the area of the newly formed vessels is in the range of 3–5%;

1 point – the area of the newly formed vessels is in the range of 2–4%;

0 point – the area of the newly formed vessels is in the range of 1.5–3%.

RESULTS AND DISCUSSION

In order to obtain XBM samples with different calcium contents, they were treated with hydrochloric

Table 1. Successive change in bone block mass during extraction under optimized conditions

No.	Initial mass, g	Mass after extraction, g		Decrease in mass after extraction, %	Extraction conditions in CO_2		Mass after extraction with SC-CO_2	Total decrease in mass, %
		with acetone	with pentane		time, h	temperature, °C		
1	0.929	0.349	–*	62.43	14	46	0.342	63.19
2	1.105	0.470	0.464	58.01	8	46	0.463	58.10
3	1.092	0.448	0.443	59.43	6	53	0.441	59.62
4	0.806	0.284	0.282	65.01	6	53	0.28	65.26
5	1.474	0.699	0.695	52.85	6	53	0.694	52.92

* Without using pentane.

Table 2. The effect of molecular weight and concentration of PLA on biocompatibility

Sample no.	PLA content, %	PLA molecular weight, kDa	Parameters in scores**			
			inflammation	integration with surrounding tissues	angiogenesis	total
1*	0	–	3.00	1.25	0.50	4.75
2	0	–	3.00	1.75	0.75	5.50
3	5	40	3.00	2.25	2.75	8.00
4	5	20	3.00	2.25	2.25	7.50
5	10	20	3.00	2.00	1.75	6.75

* The sample was treated with organic solvents but not treated with SC-CO_2.
** Averaged data for 10 samples.

acid at a concentration of 0.15, 0.3, and 0.6 N for various times, as a result of which blocks of varying degrees of demineralization were obtained.

It was established that when using blocks with a mass of 0.6–1 g to obtain partially demineralized samples with the residual calcium content of 70–90%, treatment with a solution of HCl with a concentration of 0.15 N should be performed for 10 min or 0.3 N for 3 min. To obtain partially demineralized blocks with the residual calcium content of 40–60 and 10–30%, treatment can be conducted with 0.3 and 0.6 N HCl solutions for 10–60 and 20–90 min, respectively, depending on the mass of the block.

Data on the change in mass of the XBM samples during sequential processing with organic solvents and SC-CO_2 are given in Table 1. Additional use of pentane allowed to reduce the processing time in the SC-CO_2 medium until achieving constant mass from 14 to 8 h at 46°C and to 6 h at 53°C.

In order to establish the dependence of the matrix biocompatibility parameters on the treatment conditions, we used XBM samples with a low degree of demineralization (with the residual calcium content of 70–80%), which, in shorter in vivo experiments, usually allows evaluating the rate of angiogenesis and level of biocompatibility [12].

The results of studies on the biocompatibility of partially demineralized XBM samples treated with organic solvents purified in SC-CO_2 and additionally processed by applying various amounts of PLA with different molecular weights are given in Table 2.

The data obtained show that additional purification in the SC-CO_2 increases the biocompatibility of the matrix. In the investigated series, the sample obtained by the introduction of 5% PLA (molecular weight 40 kDa) in the bone matrix, additionally purified in SC-CO_2, had the best biocompatibility parameters.

Thus, the presented methodology for cleaning biological matrices with SC-CO_2 may allow us to obtain an implant with increased potency to angiogenesis, a greater tendency to integrate with the surrounding tissues, and reduced ability to cause an inflammatory reaction. Introduction of bioresorbable polymers (polylactides) into the porous structure of the bone matrix leads to a further increase in its biological compatibility.

FUNDING

The study was financially supported by the Russian Foundation for Basic Research (project 18-29-06010) and technically supported by the IPK LIK.

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Translated by N. Smolina