In Vitro Study of Matrix Surface Properties of Porous Granulated Calcium Phosphate Ceramic Materials Made in Russia

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We performed *in vitro* screening of monophasic (hydroxyapatite, β-tricalcium phosphate, carbonate-substituted hydroxyapatite with 0.59 and 5.9 wt% substitution with CO_3^{2-}) and biphasic (hydroxyapatite—tricalcium phosphate with various percentage of the components 80/20, 60/40, 20/80, silicon-substituted hydroxyapatite with 0.79 wt% SiO_2) porous granulated ceramics composed of calcium phosphate powders synthesized by methods of heterophasic interaction of reagents and precipitation from aqueous solutions using MTT test and cultured human fibroblasts. Acute toxicity of materials (24-h incubation with cell culture) and matrix properties (3, 5, 7, 14, 18, 21, 28 days in culture) were evaluated. We selected a batch of materials obtained by precipitation from aqueous solutions, which were non-toxic and were characterized by good matrix properties (for cells). Biphasic ceramics with hydroxyapatite—tricalcium phosphate ratio of 80/20 exhibited best characteristics, and ceramics on the basis of silicon-substituted hydroxyapatite showed moderate characteristics.

Key Words: *calcium phosphate bioceramics; human fibroblasts; viability*

Porous bioceramics of the basis of hydroxyapatite (HA) is one of the most widely used classes of synthetic osteoplastic materials applied for closing bone defects due to similarity of its chemical composition to the mineral component of the bone tissue. However, the rate of bioresorption for HA ceramics is low and therefore complete organitypic regeneration of the bone tissue cannot be achieved. Moreover, its clinical use is limited due to insufficient mechanical properties and low crack resistance [1,4,7,11]. Among the representatives of the new classes of calcium phosphate ceramic materials with increased biodegradation rate and improved mechanical properties, the most promising are biphasic and cation- and anion-substituted HA ceramic materials [2,3,6]. Different methods of the synthesis of calcium phosphate powders and technologies of preparing bioceramics of their basis provide possibilities for manufacturing a wide spectrum of materials with different microstructure, surface microrelief, and therefore, different matrix properties (for cells) [5,9,10,12,13].

The aim of the present study is *in vitro* evaluation of acute cytotoxicity and matrix properties

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of porous granulated calcium phosphate ceramic materials with nanostructured surface and selection of the most promising samples for further *in vivo* experiments.

MATERIALS AND METHODS

Two laboratory batches of porous granulated calcium phosphate bioceramic materials manufactured in Institute of Physicochemical Problems of Ceramic Materials, Russian Academy of Sciences were selected for *in vitro* screening studies. The initial substances were synthesized by methods of heterophasic interaction of reagents [1] and precipitation from aqueous solutions [11]. Each batch included 8 types of materials: 1) monophasic HA, β-tricalcium phosphate (β-TCP), and carbonate-substituted hydroxyapatite (CHA) with the content of carbonate groups of 0.59 and 5.9 wt%; 2) biphasic HA — β-TCP with different ratio of components (80/20, 60/40, 20/80) and silicon-substituted HA (HA-SiO_2) containing 0.79 wt% SiO_2. Porous granulated matrixes were prepared using an original technology developed in Institute of Physicochemical Problems of Ceramic Materials, Russian Academy of Sciences [2,3]: a suspension of calcium phosphate powders in aqueous gelatin solution (P-11, GOST 11293-89) was dispersed in vegetable oil (viscosity 0.585 Pa at 25°C) and mixed at a rate from 200 to 1000 rpm. Granules of near-spherical shape formed over 1 min were isolated by filtration on a Buchner funnel, washed, dried, and heated at 1000-1200°C for 1 h. A fraction with granule size of 300-600 μ isolated using a set of sieves was used in further experiments. Thus, all studied samples had similar granule size and differed by chemical composition, porosity, pore size, and specific surface of the granules (Table 1).

Before the start of the study, all bioceramics specimens were thoroughly washed in 5-6 portions of distilled water, dried (60°C, 4 h), and sterilized (160°C, 2 h). Then, sterile granules were placed in 24-well Costar plastic plates (100 mg per well, 3 wells per sample), sterile DMEM (M. P. Chumakov Institute of Poliomyelitis and Viral Encephalites, Russian Academy of Medical Sciences) was than added, and the plates were placed into a thermostat for 2-3 h for complete saturation of granules with the medium. Immediately before addition of cells, the medium was removed from the plates with bioceramic materials.

Acute cytotoxicity of biomaterials and matrix properties of their surface (for cells) were evaluated using immortalized human fibroblasts (HF, clone 1608) obtained from cell collection (Medico-Genetic Research Center, Russian Academy of Medical Sciences). The cells were maintained in complete growth medium (CGM: DMEM, 10% FCS (FURO), 600 mg/liter glutamine, 50 μg/ml gentamicin) and were used in log phase of the growth. For preparing the suspension of dissociated cells, the monolayer of HF was treated with 0.25% trypsin (Sigma). The cell suspension was thoroughly washed by centrifugation in a large volume of CGM (2 times), the cells were counted, and their viability was determined by staining with 0.04% trypan blue. Than HF in 1.5 ml CGM were added to plates (190,000 cells/cm^2, 380,000 cells per well) with and without ceramic specimens (experiment and control, respectively) and incubated for the evaluation of acute cytotoxicity for 24 h and for evaluation of matrix properties for 3, 5, 7, 14, 18, 21, 28 days with regular replacement of CGM (2 times a week). All procedures were performed under sterile conditions, culturing was carried out in wet air containing 5% CO_2 at 37°C.

The number of viable HF throughout the experiment was determined by MTT test based on reduction of 3-(4,5-dimethylthiazolyl-2-yl)-2,5-diphenyl tetrazolium bromide (MTT, Sigma) to blue water-insoluble formazan crystals [8]. Previous experiments showed that the amount of formed formazan can characterize proliferative activity (viability/quantity) of various types of human and animal cells. After incubation with HF, 1000 μl medium was taken from each well and 125 μl MTT (5 mg/ml) was added. After 3-h incubation (5% CO_2, 37°C), 250 ml medium was discarded from each well. The formed formazan was dissolved in isopropyl alcohol (750 μl per well). The sediment formed after precipitation of proteins in isopropanol was removed by centrifugation of plates at 3000 rpm for 10 min. Then, 100 μl supernatant from each well was transferred to a 96-well flat-bottom plate (Costar) and optical density of formazan solution was measured at λ=540 nm on a MSS-340 spectrophotometer. Samples with pure CGM and samples containing the test material without cells were used as spectrophotometric controls (blanks).

The pool of viable cells (PVC) and changes in the pool of HF were calculated by formulas:

$$PVC = \frac{OD_{exp}}{OD_{contr}} \times 100\%$$

and

$$D = \frac{OD_{current} - OD_{previous}}{OD_{previous}} \times 100\%,$$

TABLE 1. Physicochemical Characteristics of Granulated Porous Calcium Phosphate Bioceramics

Samples	Granule size, μ	Porosity, %	Pore size, μ	Specific surface, m^2/g
HA	300-600	57	1-10	0.55
β-TCP	300-600	54	1-5	0.51
HA-TCP (80/20)	300-600	55	5-30	0.32
HA-TCP (60/40)	300-600	49	5-30	0.19
HA-TCP (20/80)	300-600	51	5-30	0.23
CHA (0.59 wt% CO_3^{2-})	300-600	45	1-10	0.58
CHA (5.9 wt% CO_3^{2-})	300-600	52	1-15	0.33
HA-SiO_2	300-600	54	5-20	0.47

where OD is optical density of formazan solution (arb. units).

The data were processed statistically using Student test. The difference between the means was significant at $p<0.05$.

RESULTS

During the first stage of the experiments, acute toxicity of all 8 samples synthesized by the method of heterophasic interaction was revealed (Table 2): After 24-h culturing of HF with these samples, PVC for different biomaterials varied from 6.2 to 40.5% from the control. Bioceramic materials synthesized by precipitation from aqueous solutions produced no appreciable toxic effect on HF pool: after 1-day culturing with these samples PVC was 69.0-89.0% (Table 2).

On the basis of these results we chose a batch of nontoxic for cells biomaterials synthesized from calcium phosphate powders and isolated by precipitation from aqueous solutions.

TABLE 2. Pool of Viable HF after 24-h Culturing on Bioceramic Samples

Material	PVC, % of control	
	I*	II**
HA	27.6	76.0
β-TCP	26.1	77.0
HA-TCP (80/20)	18.2	88.0
HA-TCP (60/40)	20.6	89.0
HA-TCP (20/80)	23.2	70.0
CHA (0.59 wt% CO_3^{2-})	40.5	69.0
CHA (5.9 wt% CO_3^{2-})	30.5	85.0
HA-SiO_2	6.2	73.0

Note. I*: synthesis of calcium phosphate powders by heterophasic interaction; II**: synthesis of calcium phosphate powders by precipitation from aqueous solutions.

During the next stage, adhesion properties of biomaterials and their capacity to maintain HF proliferation in culture were evaluated. We found that the pool of HF progressively increased with increasing the time of culturing in both control and experimental cultures. The cells more actively populate the biomaterial surface and more intensively proliferate than in the control. For instance, optical density of formazan solution in the control increased from 0.634 to 3.173 arb. units over 28 days, while in experimental wells containing samples of biphasic ceramics HA-TCP with component ratios of 80/20 and 60/40 this parameter increased from 0.559 to 4.295 and from 0.563 to 4.268 arb. units, respectively. On the whole, optical density of formazan by the end of the experiment (28 days) significantly surpassed the control in 6 of 8 bioceramic materials (Tables 3 and 4).

Counting of the pool of viable HF during culturing on these materials (compared to the control at each stage of the experiment) confirmed this result.

We found that biphasic materials HA-TCP (80/20 and 60/40) demonstrated the best adhesion properties of the surface (1-day culturing) and the highest capacity to maintain HF proliferation (culturing for 3-28 days), while HA-SiO_2 was least suitable for culturing.

Then we analyzed HF growth rate of bioceramic matrixes (Table 5).

The most active colonization of all tested materials was observed during the first week in culture: the growth of HF population at this term varied from 130% (CHA, HA-SiO_2) to 212% (HA-TCP, 20/80), whereas in the control this parameter was ≤85% (Table 5). During the second week in culture and later, the rate of cell growth decreased; the higher this parameter was during the first week, the

TABLE 3. Dynamics of Optical Density of Formazan Solution (MTT Test) and PVC (%) during HF Culturing on Polystyrene and Bioceramic Samples

Material	Day of observation							
	1		3		7		10	
	OD	PVC	OD	PVC	OD	PVC	OD	PVC
Polystyrene (control I)	0.634±0.031		0.810±0.004		1.072±0.052		1.301±0.033	
HA	0.484±0.012*	76.3	0.752±0.028	92.8	1.282±0.046	120.0	1.615±0.019*	124.1
HA-TCP (80/20)	0.559±0.002	88.2	0.872±0.007*	107.7	1.396±0.036*	30.2	1.900±0.035*	146.0
HA-TCP (60/40)	0.563±0.011	88.9	0.851±0.015	105.1	1.402±0.082	130.8	1.949±0.102*	150.0
CHA (0.59 wt% CO_3^{2-})	0.436±0.008*	68.8	0.658±0.060*	81.2	1.255±0.100	117.1	1.588±0.013*	122.1
CHA (5.9 wt% CO_3^{2-})	0.538±0.004	84.9	0.557±0.073	68.8	1.235±0.074	115.2	1.637±0.018*	125.8
Polystyrene (control II)	0.455±0.001		0.700±0.016		0.878±0.089		1.669±0.089	
β-TCP	0.348±0.023*	76.5	0.740±0.006	105.7	0.978±0.013	111.4	1.942±0.118	116.4
HA-TCP (20/80)	0.313±0.016*	68.8	0.755±0.039	107.9	0.975±0.051	111.1	1.842±0.052	110.4
HA-SiO_2	0.333±0.037	73.2	0.702±0.060	100	0.783±0.043	89.2	1.772±0.096	106.2

Note. Here and in Table 4: control I was used for samples HA, HA-TCP (80/20), HA-TCP (60/40), CHA (0.59 wt% CO^{2-}_3), and CHA (5.9 wt% CO^{2-}_3); control II was used for samples b-TCP, HA-TCP (20/80), and HA-SiO_2. *p<0.05 compared to the control.

TABLE 4. Dynamics of Optical Density of Formazan Solution (MTT Test) and PVC (%) during HF Culturing on Polystyrene and Bioceramic Samples

Material	Day of observation							
	14		17		21		28	
	OD	PVC	OD	PVC	OD	PVC	OD	PVC
Polystyrene (control I)	1.362±0.070		2.010±0.000		1.993±0.055		3.173±0.007	
HA	1.737±0.016*	127.5	2.178±0.157	108.4	2.522±0.131	126.6	3.722±0.090*	117.3
HA-TCP (80/20)	2.003±0.082*	147.1	3.118±0.270*	155.1	3.089±0.023*	155.0	4.295±0.150*	135.4
HA-TCP (60/40)	1.958±0.082*	143.8	2.981±0.180*	148.3	3.156±0.058*	158.4	4.268±0.089*	134.5
CHA (0.59 wt% CO_3^{2-})	1.606±0.043	117.9	2.615±0.062*	130.1	2.524±0.422	126.6	3.926±0.013*	123.7
CHA (5.9 wt% CO_3^{2-})	1.687±0.102	123.9	2.820±0.069*	140.3	2.468±0.140	123.8	3.765±0.042*	118.7
Polystyrene (control II)	2.061±0.029		2.509±0.077		3.186±0.106		3.412±0.00	
β-TCP	2.346±0.024*	113.8	2.973±0.055*	118.5	3.586±0.122	112.6	3.931±0.061*	115.2
HA-TCP (20/80)	2.059±0.089	100	2.586±0.018	103.1	3.262±0.016	102.4	3.500±0.00	102.6
HA-SiO_2	1.750±0.046*	84.9	2.425±0.065	96.7	3.134±0.073	98.4	3.277±0.038	96.0

more drastically it decreased (Table 5), which indirectly attested to gradual saturation of the material with cells and contact inhibition of their growth.

Thus, we performed *in vitro* screening of 8 types of granulated porous bioceramic materials and tested two series of samples of the same chemical composition, but obtained by different methods (heterophasic interaction and precipitation from aqueous solutions). It was found that materials obtained by the first method are cytotoxic, while materials precipitated from aqueous solutions exhibit no cytotosicity under the same experimental conditions *in vitro*. Therefore, in further experiments we used granulated bioceramics manufactured from calcium phosphate powders obtained by precipitation from aqueous solutions.

All bioceramic materials had satisfactory adhesion properties and maintained proliferation of HF on their surface throughout culturing (28 days). The cell growth rate on granulated bioceramics was maximum during the first week, but then decreased (with increasing cell density). The best matrix properties (for HF) were found in HA-TCP (80/20 and 60/40), whereas HA-SiO_2 was least suitable for culturing. On the whole, all porous bioceramic materials obtained by precipitation from aqueous so-

TABLE 5. Dynamics of HF Pool Growth (%) during Culturing

Material	Weeks			
	1	2	3	4
Polystyrene (control I)	81.0	81.0	50.0	33.0
HA	165.0	36.0	45.0	48.0
HA-TCP (80/20)	150.0	44.0	54.0	39.0
HA-TCP (60/40)	149.0	40.0	61.2	35.2
CHA (0.59 wt% CO_3^{2-})	189.0	28.0	57.0	52.0
CHA (5.9 wt% CO_3^{2-})	130.0	37.0	46.0	53.0
Polystyrene (control II)	93.0	135.0	55.0	36.0
HA-TCP (20/80)	212.0	111.0	58.0	7.0
β-TCP	181.0	140.0	53.0	9.6
HA-SiO_2	135.0	124.0	79.0	5.0

lutions can be recommended for evaluation of biocompatibility and further preclinical biomedical studies (replacement of bone defects in isolated application and as components of bioengineering constructions, in particular, with multipotent mesenchymal stromal cells).

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