METHODS

In Vitro Regulation of Cell Behavior by Calcium Phosphates Synthesized by the Mechanochemical Method

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Translated from *Byulleten' Eksperimental'noi Biologii i Meditsiny*, Vol. 138, No. 9, pp. 356-360, September, 2004
Original article submitted February 10, 2004

Biological activity of eight hydroxyapatite powders synthesized by the mechanochemical method was studied *in vitro*. Introduction of copper and zinc atoms into synthetic hydroxyapatite structure promotes cell adhesion. Hydroxyapatites obtained by the mechanochemical method are promising base for a new class of materials and coatings with regulated bioactivity for orthopedics and traumatology.

Key Words: *mechanochemically synthesized apatites; extracts; myelokaryocytes; adhesion and viability in vitro*

Calcium phosphate materials and coatings for implants are now widely used in traumatology and orthopedics due to their high capacity to integration into bone tissue. Technologies for isolation, purification, and use of bioactive hydroxyapatite (HAP) were developed; this biomaterial is best of all compatible with the body, but is fraught with the risk of infection.

According to ASTM.F1185-89 standard, synthetic HAP, a complete chemical analog of mineral bone substance, is allowed for practical use. The structure of natural HAP crystals cannot be precisely reproduced under laboratory conditions [1]. One of the flaws of stechiometric HAP is its poor solubility at pH 7.2 [3]. It is believed that biological activity of the material is better, if it releases (as a result of partial dissolution) bioactive ions, primarily calcium and phosphorus ions, into the environment [10]. These ions are utilized by the organism for formation of microcrystals of biological apatite on the surface of implants [13], this leading to construction of the new implant—bone functional system.

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The main approaches at preparation of apatites with regulated biological activity are preparation of nonstechiometric HAP and HAP modification by ions other than the ions in its structure [3]. The method of hard body mechanometry helped solve both problems [8].

Biodegradation of the implant can be simulated *in vivo* and *in vitro* by physicochemical and biochemical dissolution of material in aggressive liquids and/or by bioresorption realized by cell systems of the body (macrophages, osteoclasts). The sensitivity of *in vitro* analyses is higher than that of *in vivo* ones. Functional methods for evaluation of cell activity (cell adhesion to plastic or glass) are highly sensitive to the nature of the test material [11].

Functional response of bone marrow cells to extracts of Ca-deficient stechiometric HAP $(Ca/P)_{at.}<1.67$) and HAP with substitutes in the cationic sublattice synthesized by the mechanochemical method attracted our attention.

MATERIALS AND METHODS

Mechanochemical analysis of apatites [8] was carried out in an EI-2×150 planetary mill in ceramic drums with ceramic balls for 10 or 30 min. Phase com-

0007-4888/04/1383-0316

position of the powders was evaluated by the data of x-ray phase analysis (RPA) and infrared spectroscopy (IRS).

Experiments were carried out in autumn-winter with biological material from 2 male Wistar rats (200 g) from vivarium of Institute of Cardiology, Tomsk Research Center. Before the study the animals received standard ration for 1 week. The rats were sacrificed under ether narcosis, bone marrow was isolated from the femoral bones, and myelokaryocytes were cultured in 24-well plastic plates in a concentration of 8.5×10^6/well for 1 h at 37°C in 1 ml culture medium of the following composition: 280 mg/liter L-glutamine (Sigma), 80 mg/liter gentamicin sulfate, 5% FCS (ICN), 95% Ca^{2+},Mg^{2+}-free Dulbecco balanced phosphate buffer (Vektor Firm).

The cytotoxicity of calcium phosphate powders (1 mg/ml cell culture) and their 7-day extracts was evaluated (Table 1). Sterile extracts of powders were prepared in accordance with ISO regulations under conditions of culturing in a concentration of 0.1 mg/ml in 0.9% NaCl solution at 37°C. This test system induced chemical degradation of materials.

The extracts in titers 1:8 and 1:4 of the final volume of culture medium (1 ml) were added to the wells. Cell cultures after addition of an equivalent volume (125 or 250 μl) of isotonic NaCl (solvent control) or extracts of biological non-toxic HAP (the absence of toxicity was confirmed in preliminary experiments) served as controls. After culturing, myelokaryocytes were divided into adhesive and nonadhesive fractions.

Viability of nonadhesive karyocytes was evaluated in Goryaev chamber (by exclusion of 0.4% Trypan blue) before and 1 h after addition of synthetic calcium phosphate powders or their extracts. Trypan blue test shows severe necrotic impairment of the cell membrane caused by the toxic agent [6].

Cells adhering to the plastic were fixed in methanol for 3-5 min, stained with 0.2% Azur-II-eosin for 10 min, washed in tap water, and dried in air. The number of cells adhering to plastic was evaluated by computer-aided morphometry of digital images; two wells per sample were examined.

Quantitative parameters of cells adhesive to plastic were determined by measuring their optical characteristics [6].

The density of the object (arbitrary units of optical density) was examined using the Adobe PhotoShop 5.0 software according to the gray levels statistics; the

TABLE 1. Optical Density of Myelokaryocyte Culture after 1 h-Culturing with 7-Day Extracts of Synthetic and Biological HAP Extracts

HAP powder extract	Number of adhesive bone marrow cells, opt. dens. arb. Units	
	extract titer 1:8	extract titer 1:4
$Ca_9HPO_4(PO_4)_5(OH)+14H_2O$; $(Ca/P)_{at.}=1.5$	15.516±0.989 (n=10)$^{++++}$	9.986±0.756 (n=10)$^{*+}$
$Ca_{9.6}(PO_4)_6(OH)_{1.2}+15H_2O$; $(Ca/P)_{at.}=1.6$	16.190±0.574 (n=15)$^{*****++}$	14.445±0.425 (n=12)$^{*+}$
$Ca_{10}(PO_4)_6(OH)_2+15H_2O$; $(Ca/P)_{at.}=1.67$	18.525±0.529 (n=16)*	20.881±0.730 (n=12)
$Ca_{10.8}(PO_4)_6(OH)_{3.6}+15H_2O$; $(Ca/P)_{at.}=1.8$	18.042±0.501 (n=16)*	19.358±0.569 (n=12)
$Ca_{10}K_{0.04}(PO_4)_{6.04}(OH)_2+15H_2O$	18.021±0.546 (n=12)*	18.706±0.445 (n=10)
$Ca_{10}(Cu,Zn)_{0.08}(PO_4)_6(OH)_{2.16}$	14.628±1.288 (n=10)$^{+++}$	22.280±0.931 (n=7)$^{*****+++}$
$Ca_9Ba(PO_4)_6(OH)_2+23H_2O$	10.299±0.554 (n=12)$^{*****+}$	11.142±0.477 (n=12)$^{*+}$
$Ca_9Mg(PO_4)_6(OH)_2+14H_2O$	17.801±0.719 (n=13)**	17.096±0.742 (n=15)
Biological HAP	18.063±0.319 (n=18)*	18.824±0.783 (n=10)
Isotonic NaCl solution (solvent)	13.204±1.016 (n=10)	18.460±0.967 (n=12)

Note. n: number of measurement blocks in 2 wells. *p<0.001, **p<0.01, ***p<0.02, ****p<0.05 compared to the solvent; ^{+}p<0.001, ^{++}p<0.01, ^{+++}p<0.02, ^{++++}p<0.05 compared to biological HAP.

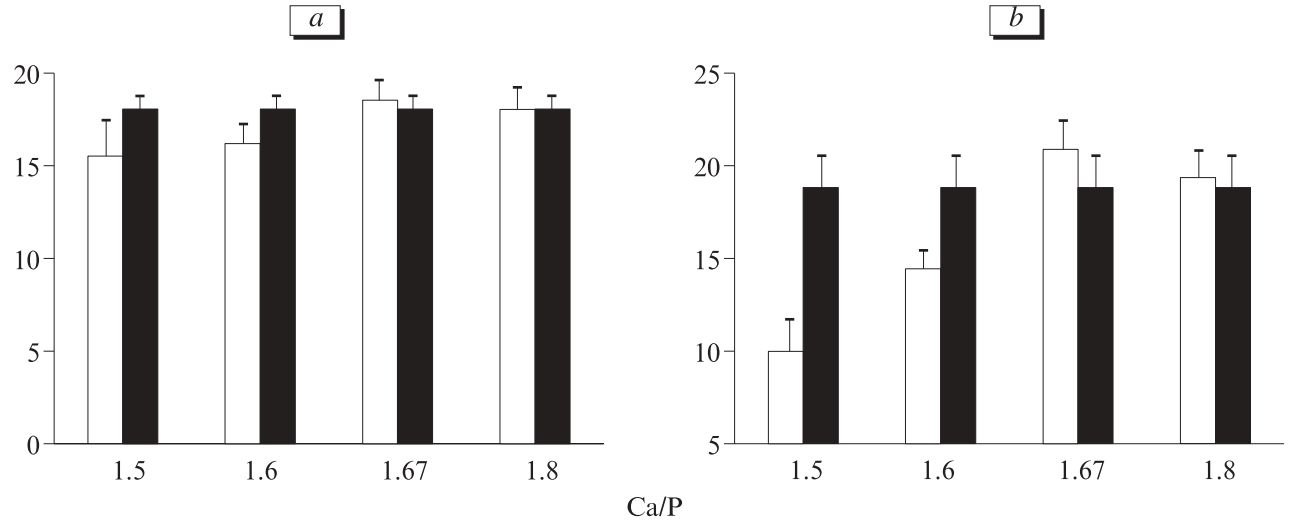

Fig. 1. Effects of 7-day extracts of synthetic calcium phosphates with different Ca/P ratio on bone marrow cell adhesion to plastic. *a*) extract titer 1:8; *b*) extract titer 1:4. Abscissa: Ca/P atomic ratio in apatite molecule. Ordinate: optical density of adhesive cell culture. Light bars: synthetic hydroxyapatite; dark bars: biological hydroxyapatite.

regions of interest (ROI) consisting of blocks with fixed area were distinguised [12].

The integral reflection coefficient:

$$R_{R,G,B}=S_{R,G,B}/W_{R,G,B},$$

where S is the brightness of tested region and W brightness of white reference (255).

Optical density of the object [5]:

$$D=100\lg(1/R_{R,G,B}).$$

The values should be standardized for different background:

$$X_R=R_T/R_B,$$

where R_T is tissue reflection coefficient and R_B background reflection coefficient (plastic near the well). Then

$$D=100\lg(1/X_R)=100\lg(S_B/S_T).$$

The results were statistically processed using Student's *t* test and Wilcoxon—Mann—Whitney's nonparametrical *U* test.

RESULTS

RPA and IRS demonstrated the possibility of HAP synthesis with replacement of Ca atoms in the molecule with other cations. Ca-deficient (non-stechiomet-

TABLE 2. Myelokaryocyte Survival in Liquid Culture after 1-h Culturing with 7-Day Extracts of Synthetic and Biological HAP Powders

Powder extract, *n*=3	Percentage of viable bone marrow cells	
	extract titer 1:8	extract titer 1:4
$Ca_9HPO_4(PO_4)_5(OH)+14H_2O$; $(Ca/P)_{at.}=1.5$	93.06	90.27
$Ca_{9.6}(PO_4)_6(OH)_{1.2}+15H_2O$; $(Ca/P)_{at.}=1.6$	93.37	96.83
$Ca_{10}(PO_4)_6(OH)_2+15H_2O$; $(Ca/P)_{at.}=1.67$	90.18	96.64
$Ca_{10.8}(PO_4)_6(OH)_{3.6}+15H_2O$; $(Ca/P)_{at.}=1.8$	98.00*	95.29
$Ca_{10}K_{0.04}(PO_4)_{6.04}(OH)_2+15H_2O$	93.46	93.58
$Ca_{10}(Cu,Zn)_{0.08}(PO_4)_6(OH)_{2.16}$	87.87	96.53
$Ca_9Ba(PO_4)_6(OH)_2+23H_2O$	91.26	94.11
$Ca_9Mg(PO_4)_6(OH)_2+14H_2O$	93.98*	94.30
Biological HAP	86.26	97.13
Isotonic NaCl solution (solvent)	88.87	86.00

Note. *$p<0.05$ compared to biological HAP.

ric) apatites are characterized by high resorption [3] promoting ion release into the solvent.

Calcium ions play an important role in cell adhesion. Other cations also possess pronounced biological activity [2].

Adhesion of bone marrow cells to plastic was studied in Ca^{2+},Mg^{2+}-free phosphate saline buffer in the presence of little volume of FCS. This model helps to record biological activity of soluble (extracted) fractions of synthetic HAP.

The extracts of "acid" HAPs (Ca/P<1.67) in the studied concentrations clearly suppressed cell capacity to adhere to plastic in comparison with biological HAP (titers 1:8 and 1:4) and the solvent (titer 1:4). Suppression of the parameter was statistically significant (p<0.05) and dose-dependent (Fig. 1, Table 1). This is in line with the data on the cytotoxicity of amorphous (soluble) calcium phosphates [14]. Increase of velocity of ceramic resorption in the body stimulates osteoinductive activity, but deteriorates biocompatibility of the material.

The increase of the Ca/P ratio in the synthetic HAP molecule appreciably increased adhesive activity of bone marrow cells, particularly at high concentrations of the extracts (Fig. 1). By their effects on cell adhesion, extracts of stechiometric (Ca/P=1.67) and "alkaline" HAP corresponded to biological HAP extracts. Moreover, extracts of the alkaline HAP powder in 1:8 titer even improved cell survival in culture in comparison with the biological analog (Table 2).

Biological activity of HAP synthesized by the mechanochemical method can be regulated by inserting trace- and macroelements in the apatite lattice. For example, soluble fraction of apatite powders with one Ca ion replaced by Ba had an unambiguously negative effect on the myelokaryocyte adhesion in culture (Table 2). On the other hand, insertion of K or Mg atom in HAP structure seemed to stimulate cell adhesion (1:8 titer; Table 2). HAP solutions with Ca partially replaced by Mg somewhat improved the viability of bone marrow cells (Table 2).

HAP extracts containing 0.04% Cu and Zn ions in the structure used in high concentrations produced the maximum stimulatory effect on cell adhesion (Table 1).

As the body trace elements, copper and zinc modulate cell and tissue functions through dependent metalloenzymes [7]. They possess bactericidal effects. An effective cheap low-toxic antibacterial material, releasing antibacterial agent at a constant therapeutically active level, which can be used in many devices and materials, and with a reasonable shelf life, remains in need.

Synthetic HAPs with Cu and Zn atoms in the molecule can be useful for the formation of large-volume articles and for metal coating with regulatory and antibacterial activities. It will be a new class of articles, which will help to solve complex problem at the bone-implant interface [9] and hence, be biocompatible.

TABLE 3. Myelokaryocyte Survival in Liquid Medium after 1-h Culturing with Synthetic and Biological HAP Powders

Powder (n=3)	Percentage of viable bone marrow cells
$Ca_9HPO_4(PO_4)_5(OH)+14H_2O$; $(Ca/P)_{at.}$=1.5	96.87
$Ca_{9.6}(PO_4)_6(OH)_{1.2}+15H_2O$; $(Ca/P)_{at.}$=1.6	96.25
$Ca_{10}(PO_4)_6(OH)_2+15H_2O$; $(Ca/P)_{at.}$=1.67	97.13
$Ca_{10.8}(PO_4)_6(OH)_{3.6}+15H_2O$; $(Ca/P)_{at.}$=1.8	93.89
$Ca_{10}K_{0.04}(PO_4)_{6.04}(OH)_2+15H_2O$	92.79
$Ca_{10}(Cu,Zn)_{0.08}(PO_4)_6(OH)_{2.16}$	96.65
$Ca_9Ba(PO_4)_6(OH)_2+23H_2O$	98.67
$Ca_9Mg(PO_4)_6(OH)_2+14H_2O$	95.67
Biological HAP	96.46

Extracts of all specimens had satisfactory toxicity parameters in cell culture *in vitro* (Table 2). Testing of powders also indicates the possibility of their use *in vivo* (Table 3).

Hence, powders of HAP synthesized by the mechanochemical method are bioactive. Depending on their composition, they exert oppositely directed regulatory effects on functional activity of bone marrow cells by dissolving in biological liquids. All the tested synthetic calcium phosphates exhibited satisfactory cytotoxicity. Creation of Ca deficiency in apatite molecule (Ca/P<1.67) and replacement of one Ca ion in the elementary cell with Ba suppressed adhesive activity of myelokaryocytes *in vitro*. By contrast, introduction of Cu and Zn atoms into the structure of synthetic HAP promoted cell adhesion (it is known that copper, zinc, and magnesium prevent bone loss [4]). These metals possess an antibacterial effect, which is significant for the prevention of postimplantation infection.

Synthetic HAP obtained by the mechanochemical method are a promising basis for making a new class of articles and coatings with regulated bioactivity for orthopedics and traumatology.

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