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Bone-like Hydroxyapatite Formation in Human Blood

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ABSTRACT

The purpose of this study was to prove the mechanism of mineralization, when hydroxyapatite (HAP) is formed in blood plasma. These observations were substantiated by in vitro simulation of HAP crystallization in the plasma of healthy adults in a controllable quasi-physiological environment (T = 37°C, pH = 7.4) and at concentrations of dissolved Ca and P ions that resemble those in the blood of healthy adults. Another objective of the study was to investigate the role of Mg2+, Na+Cl - and serum albumin, the main blood protein, in the formation of calcium phosphate. Mineralized aortic and mitral valves of the heart were obtained in operations on patients with rheumatic or septic acquired valvular disease. Macroscopic and histological analyses of these samples were conducted. Primary nanocrystals of HAP formed in the blood may take part in the mineralization of both cardiac valves and vessels. The penetration of HAP nanocrystals may be caused by breaks in the endothelium of blood vessels. The lowering of pH may result from substantial degradation in the valve tissue. The experimental data make it possible to conclude that albumin and Fetuin-A are structural proteins that are responsible for subsequent incorporation of HAP nanocrystals in newly formed bone tissue.

KEYWORDS
Calcified tissue, hydroxyapatite, blood mineralization, scanning electron microscopy, heart valves

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Introduction

Calcium phosphate, existing in the mineral form of HAP can either build bones and teeth enamel and dentin or cause pathological calcifications in humans. The behavior of calcium phosphates in the organism depends on physicochemical conditions. In particular, the nucleation and growth of crystals is regulated thermodynamically by Ca and P super-saturation and by local concentrations of associate inorganic ions and organic macromolecules. Different elements in biological fluids either facilitate or inhibit biomineralization (Demer & Tintut, 2008). Spontaneous precipitation of HAP is often doubted (Yarbrough

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et al., 2010), though mammalian extracellular fluids are known to be metastable with respect to it. However, the present research showed that such considerations contradict experimental results (Giachelli, 1999; Titov, Larionov & Shchukin, 2000). The nature and parameters of the tissue fluid responsible for the physiological and pathological mineralization remain unexplained to date.

Mineralization in the cardiovascular system is known to exist solely as a phenomenon of pathogenesis. Mineral formations on natural heart valves, bioprostheses thereof, and blood vessels consist of calcium phosphate. Different calcium phosphates have different solubility and crystallization thermodynamics. The nature and parameters of the tissue fluid that is responsible for physiological and pathological mineralization remain undetermined. The mechanisms that control the calcification of heart valves and vessels are studied extensively nowadays.

Literature Review

Ectopic calcification is mostly a cellular and cell-induced process, similar to the processes that are observed in bones (London, 2013; Sage, 2011; Yiu, 2015; Egan et al., 2011; Miller, Weiss & Heistad, 2011; Thompson & Towler, 2012). Vascular calcification is an actively regulated process, in which cells could assume osteoblast-like functions. At present, one of the most popular theories is that calcium deposits form on the aorta and heart valves through a membranous mechanism of mineralization (Leopold, 2012; New & Aikawa, 2013; Reynolds, 2004; Golub, 2011; Thompson & Towler, 2012). The most commonly accepted hypothesis is the formation of primary calcium deposits on the aorta and heart valves by a membrane mechanism similar to bone mineralization (Titov, Larionov & Shchukin, 2000). Here, the role of the blood is limited to transporting Ca and P ions. In intercellular matrix vesicles with membrane properties, their concentration is high enough to form HAP Ca10 (PO4) 6 (OH) 2 or other calcium phosphates. Acid phosphates – octacalcium phosphate (OCP) - Ca8H2 (PO4) 6•5H2O or dicalcium phosphate digidrate (DCPD - brushite) - CaHPO4•2H2O – are assumed to be precursor phases that are transformed into HAP which is the most stable one (Titov, 2004; London, 2013; Sage, 2011).

The present research proves the existence of an alternative mechanism, which implies that HAP forms in the blood plasma without any intermediate phases. It is assumed that the mechanism participates in tissue mineralization, whereas the formation of more acid calcium phosphates results from secondary transformations.

This concept is based on: a) an investigation of the atomic structure and morphology of calcium deposits on heart valves; b) discovered HAP microcrystals in the human blood and lymph (Giachelli, 1999); c) experimental results from modeling the ionic blood composition in aqueous solutions.

Aim of the Study

The aim of this study was to prove the mechanism of mineralization, when HAP is formed in blood plasma. Investigate the role of Mg2+, Na+Cl - and serum albumin, the main blood protein, in the formation of calcium phosphate.

Research questions

The overarching research question of this study was as follows:

What is mechanism of mineralization, when HAP formed in blood plasma? What is the role of Mg2+, Na+Cl - and serum albumin, the main blood protein, in the formation of calcium phosphate?

Methods

The research was approved by the biomedical ethics committee of the J.L. Tsivyan Novosibirsk Research Institute of Traumatology and Orthopedics of the Ministry of Health of the Russian Federation by Minutes No. 065/15 dated September 25, 2015. The research followed ethical principles for medical research involving human subjects (Anon, 2013).

Mineralization Subjects in Vivo

The present study used intraoperative material – mineralized aortic and heart valves obtained from patients with acquired valvular diseases of rheumatic and septic genesis. A macroscopic and histological analysis of the material was performed. In addition, blood was taken from patients with ultrasound-confirmed heart valve mineralization. The dry residue of the blood was studied. A similar analysis was performed for comparison purposes on donor blood and the pulmonary lymph of a patient with disseminated sclerosis.

Extraction of HAP from Plasma

Collected sterile venous blood (50 mL) was held in glass vials for 3 hours until it coagulated; the transparent liquid fraction was then sampled with a pipette. Prior to extraction, the plasma samples were diluted in distilled water (1:4) to make them less viscous. In order to avoid nucleation of apatite during the preconditioning of the sample, the obtained precipitate was diluted with distilled water to achieve a magnitude lower than the concentrations of ions. During each step, the supernatant was removed, and the liquid was centrifuged at g=45,000 for 2 hours.

After the final centrifuging, the precipitate was annealed in a muffle furnace in air at 670°C for 1 hour. The annealing temperature was estimated empirically against the standard samples of valve calcification HAP and inorganic bone tissue. At this temperature, HAP did not transition to the anhydrate state (whitlockite) and preserved its original morphology and size of particles almost perfectly. The precipitate was diluted in distilled water in a 1 mL glass vial, for examination under a transmission electron microscope. The suspension was then spread on carbon-coated copper grids with a diameter of 3 mm and dried for TEM examination.

Conditions of Calcium Phosphate Synthesis

Calcium phosphate mineralization in human blood was modeled in aqueous solutions in controllable conditions (pH=7.4□0.05 and 37□0.2°C), using analytical grade chemicals and twice-distilled water. The starting K2HPO4 (+NaCl) and CaCl2 (+MgCl2) solutions were mixed at 6.4-6.6 mL/min, the total volume of the final solution being 800 mL. The reagents were mixed slowly to resemble the conditions of a human organism as closely as possible. Bovine serum albumin (BSA - fraction V, USA) was used as a model protein. The albumin powder was added after the inorganic part of the solution had been mixed. In all experiments, the solution was mixed continuously at the set

temperature for 7 hours. The solid fraction was extracted by precipitation from the aqueous solution, while the residue was diluted four times with twice-distilled water (1:10). The calcium phosphate particles obtained with albumin were annealed in a muffle furnace in air at 600ºC for 1 hour to remove the inorganic component prior to electron microscopy.

Visualizations of Results

The techniques for structural and elemental analyses included high-resolution transmission electron microscopy (HR TEM), scanning electron microscopy (SEM), electron diffraction, and energy-dispersive X-ray spectroscopy (EDX). Electron microscopy was performed on a JEM2010 transmission electron microscope (acceleration voltage 200 kV, resolution 1.4 Å) equipped with an EDAX EDS detector (spectral resolution 130 eV) and a TESCAN MIRA3 scanning electron microscope with an Oxford EDS detector (resolution 128eV) and built-in INKA ENERGY software. The electron microscopic images were processed in Gatan Digital Micrograph. In addition, the D8 GADDS X-ray diffractometer made by Bruker Corporation was used.

HAP stoichiometry in the heart valves was determined by X-ray microanalysis (WDX). Synthetic apatite was used as a standard. The Ca/P molar ratios in the deposits were in the range of 1.72-1.80. The total concentration of Na, Mg, Cl, S, Si, Fe, and Mn in each sample did not exceed 1%.

According to IR spectroscopy data, the valve HAP contained about 4 – 5 wt % of CO32- ions. The presence of carbonate is typical for human apatite's, where CO32- ions substitute PO43- groups in the structure (Yiu, 2015).

Data, Analysis, and Results

The examination of the mineralized parts of the heart valves with optical and SEM demonstrated that calcificates existed as an aggregate of fine particles, which formed larger particles of varying density up to several centimeters in size.

According to TEM data, the calcificate aggregates of the heart valves consist of primary particles of different size, morphology, and structural ordering. The largest crystals comprising a small part of the volume in the samples of different mineralization degree have a plate-like shape and are about 5 nm thick and 1-5 μm in cross size. They have a well-ordered structure and sometimes show hexagonal habitus typical for apatite crystals. The most developed planes in these crystals are basal planes (0001). The major part of the valve HAP consists of 10-100 nm nanocrystals (Giachelli, 1999) similar to bone apatite (c-axis in the crystal plane). Large grains normally consist of disoriented blocks 5-20 nm in size. It is remarkable that the samples that are poorly crystallized according to XRD data are observed as block microcrystals in the electron microscope.

The other type of mineralization was observed in some heart valves with HAP mineralization. Plate-like crystals and aggregates of sharp-cornered crystals growing from a common center were found (Figure 1). They are 2-10 μm in size and are distributed throughout the entire valve tissue. These deposits are also calcium phosphate, and only P, Ca, and O signals are registered by EDX. The Ca/P ratios obtained from the comparison with the apatite standard are between 0.95 and 1 for various measurements, which corresponds better to

brushite. Brushite crystals do not adhere to the surrounding fibers in an oriented fashion, but rather tear the interfiber connections.

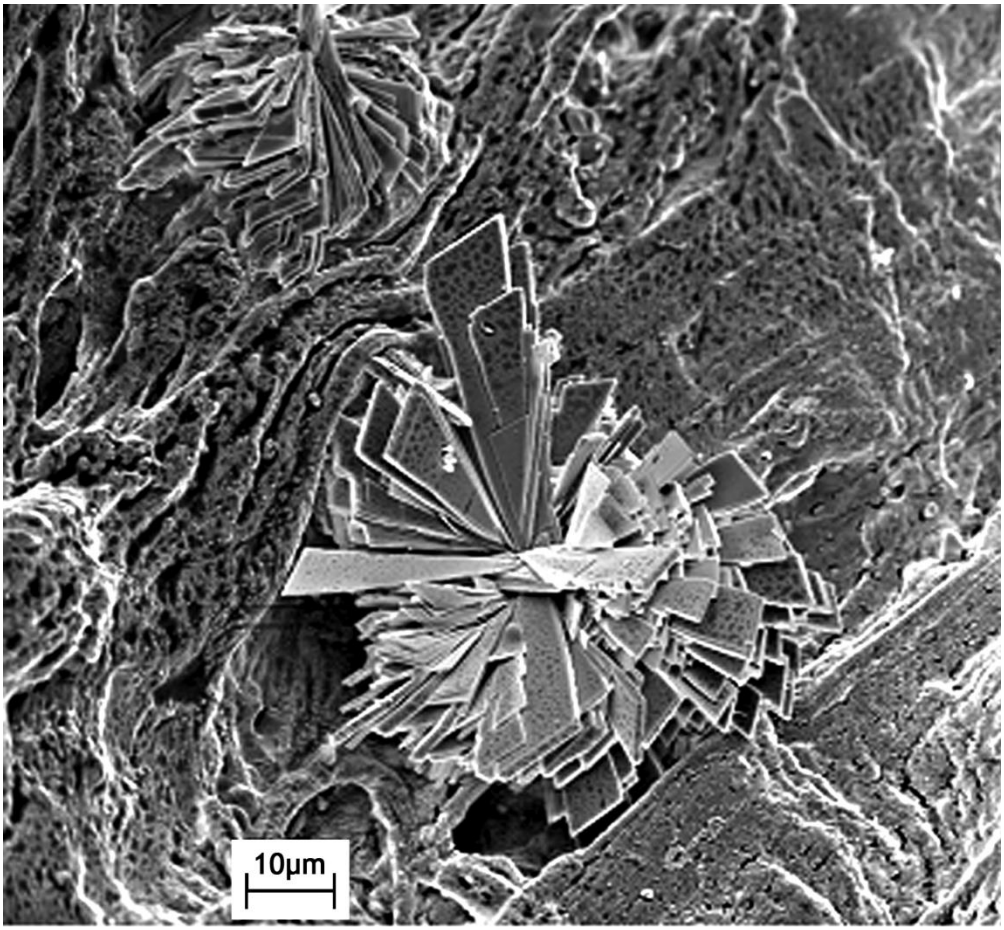

Figure 1. Electron microscopic image of calcium phosphate of natural heart valve – SEM morphology of DCPD microcrystals in the valve tissue

HAP crystals (10-70 nm) were discovered, which are structurally and morphologically similar to bone apatite and the fine fraction of the heart valve calcification. They were found both in the solid residue of the blood plasma of patients with calcinosis, and in the blood of healthy donors (Giachelli 1999). Figure 2a shows a TEM microphotograph of particles extracted from the donor blood, which are similar in size, morphology, and structure to bone apatite and to big HAP deposits in native heart valves. The respective diffraction image of the aggregate shows several rings with spacing that corresponds to the d/n of HAP (3.4, 3.1, 2.8, 2.3, and 1.8 Å). Furthermore, plate-like crystals up to 2.5 µm in size (Figure 2b) were found in the lymph of the disseminated sclerosis patient; the crystals were similar to the microcrystals with planes (0001) in the heart valves. The blood also contained needle-like nanocrystals, but these differed from the nanocrystals of the fine fraction of valve HAP. HAP of the solid residue of blood and lymph was identified by electron diffraction and X-ray spectra (EDX).

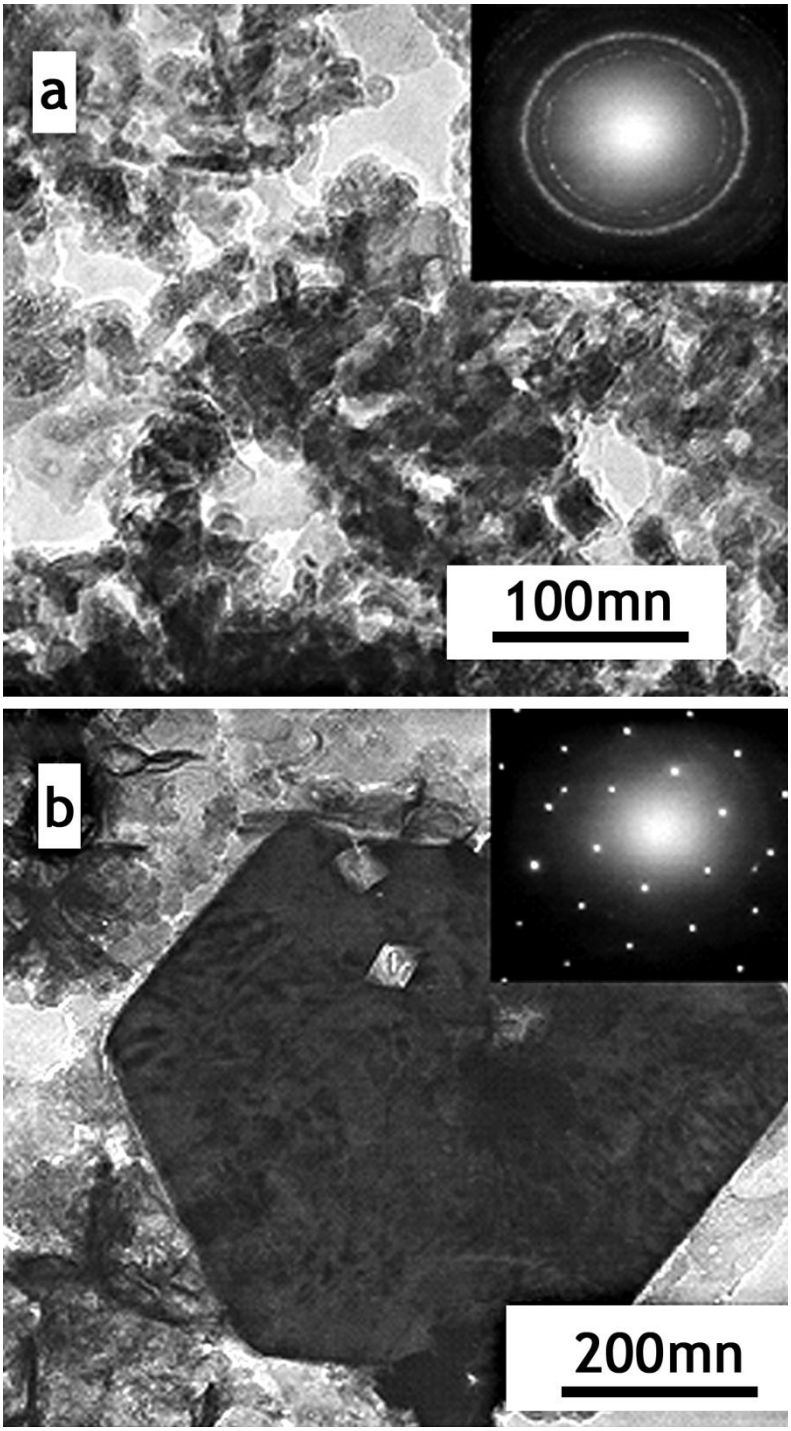

Figure 2. TEM images of HAP: (a) of nanocrystalline HAP extracted from healthy donor blood; (b) in pulmonary lymph of a patient with disseminated sclerosis. Inset shows the respective diffraction pattern

To prove further the hypothesis regarding the participation of the blood HAP in tissue calcification and use it in the diagnostics of mineral metabolism disorders in humans, model experiments were conducted under controlled conditions. These observations were substantiated by in vitro simulation of HAP crystallization in the plasma of healthy adults in a controllable quasi-physiological environment (T = 37°C, pH = 7.4) and at concentrations of dissolved Ca and P ions that resemble those in the blood of healthy adults (Table 1). Another objective of the study was to investigate the role of Mg2+, Na+Cl - and serum albumin, the main blood protein, in the formation of calcium phosphate.

Table 1. The concentration of ions measured in plasma of a healthy adult and the respective concentrations in the final aqueous solution after mixing during in vitro experiments

	Concentration, mM				
№	Ca^{2+}	P^{5+}	Mg^{2+}	Na^{+} Cl^{-}	Albumine g/L
In blood	1.05 – 1.3	1.00 – 1.5	0.75 – 1.25	130-156	35 - 55
1	1.33	1.50			
2	1.33	1.50	0.40		
3	1.33	1.50	0.80		
4*	1.33	1.50	1.00		
5	1.33	1.50		50	
6*	1.33	1.50		150	
7*	1.33	1.50	1.00	150	
8	1.33	1.50			38.6
9	1.33	1.50	0.40	150	38.6
10	1.33	1.50	0.75	150	38.6

X* - calcium phosphate is not formed

Magnesium inhibits the growth of calcium phosphate (Yiu, 2015); the effect of Na+Cl- is apparently caused by the high concentration and ionizing strength of its solutions, including plasma; albumin can adsorb onto HAP mineral surfaces (Leopold, 2012; Egan et al., 2011) and either accelerate or delay calcification in different conditions (Miller, Weiss & Heistad, 2011). About 45% of the 2.5 mmol of calcium circulating in the body is free, 45% is bound to serum proteins, chiefly albumin, and the remaining 10% is complexed with small molecules such as citrate and phosphate (Thompson & Towler, 2012); only free Ca ions are biologically active in extracellular fluids.

In all experiments of in vitro calcium phosphate synthesis HAP was obtained, which formed without precursor phases at low Ca and P concentrations and physiological pH = 7.4. The first experiment resulted in precipitation of nanometer acicular crystals of HAP (Figure 3a) with the crystallographic c direction along the crystal plane (see HR TEM micrograph and respective Fourier image in Figure 3b). There were found needlelike crystals up to 200 nm in length and 5 nm in width with a typical contrast ratio on TEM photos – a light central line and darker edges (Figure 3a). The needlelike crystals with a similar contrast ratio on TEM-photos were found in the patient's

lymph. The remaining part consisted of thin plates with different structural ordering, varying from an aggregate of nanocrystals consisting of several elementary cells to large crystals with a long-range structural order. As expected, the total mass of the synthesized product reduced significantly when the concentrations of dissolved Mg (experiments 2-4) or NaCl (experiments 5 and 6) increased, while the precipitated HAP nanocrystals were 1.5-2 times smaller than those in experiment 1. Precipitation from an aqueous solution laden with bovine serum albumin (BSA), but free from Mg and NaCl (experiment 8) likewise produced acicular HAP crystals, almost twice shorter on average (~100 nm) than with an albumin-free solution (experiment 1). The final product looked like a mineral-organic coagulate or gel while albumin, which is highly soluble in water, became insoluble and resisted even multiple rinsing in bi-distilled water, i.e., BSA and HAP formed a complex. In experiment 3, when dissolved Mg increased to 0.75 mM, the HAPs changed in both size and morphology and turned into 40-70 nm platy crystals.

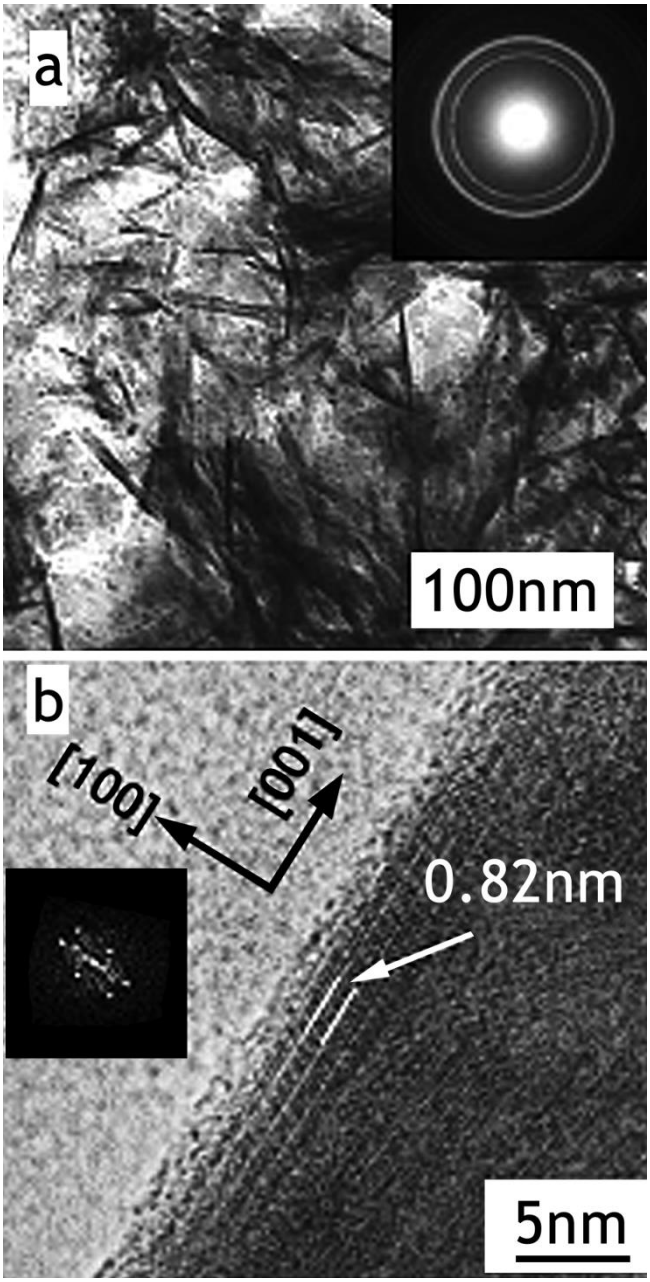

Figure 3. a: A TEM micrograph of nanocrystalline HAP synthesized in controlled in vitro experiments at T = 37°C and pH=7.4 (experiment 1, Table 1); b: An HR TEM image of an HAP crystal and its Fourier pattern

Experiments 6 and 7 gave no calcium phosphate precipitation. At a NaCl concentration of 0.15 M and a concentration of ions in the aqueous solution of Ca -1.33 mM and P - 1.5 mM (experiment 5), the synthesized HAP had nanocrystals with a structure that was similar to experiment 1, but significantly smaller in size (100-150 nm). Under the conditions of experiment 6 with the NaCl concentration of 0.15 M, and Mg concentration of 0.75 mM in the final aqueous

solution containing 1.33 mM calcium ions, and 1.5 mM concentration of phosphorous ions, calcium phosphate was not produced even after the solution was aged for one year at room temperature. Thus, dissolved Mg and NaCl can fully inhibit the nucleation of HAP within the Ca and P levels in the blood of healthy human adults, but albumin induces HAP nucleation in similar solutions (experiments 9 and 10).

It should be noted that the duration of the experiment (7 hours) turned out to be insufficient to produce the amount of calcium phosphate required to prepare samples and diagnose reliably the phase composition of the obtained product. However, the precipitation in the solution after 7-days incubation at room temperature was correctly identified as HAP by electron diffraction and X-ray analysis (EDX). The TEM images (Figure 4) show HAP as thin elongated plates, 50-150 nm in length.

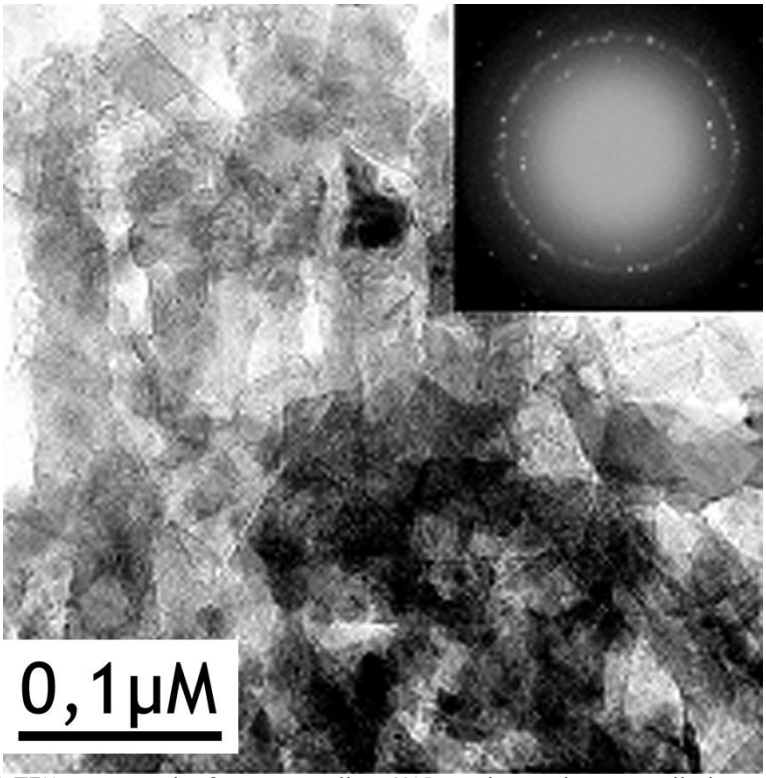

Figure 4. A TEM micrograph of nanocrystalline HAP synthesized in controlled in vitro experiments at T = 37°C and pH=7.4 (experiment 10, Table 1). Inset shows their diffraction patterns

Discussion and Conclusion

Calcification of the forming bone tissue implies deposition of calcium phosphates into a neogenic matrix. Pathological calcification causes calcium phosphate deposition in the tissues of various organs. It is known that bone apatite consists of thin-plate microcrystals elongated along the c-axis with an average size of ~ 45×20×3nm (New & Aikawa, 2013). Based on the similarity of the microstructure and the chemical composition between the inorganic bone

tissue and calcinosis masses on heart valves, it was assumed that their mineral elements are formed in the human blood. The HAP microcrystals found in the solid blood residues of healthy donors are structurally and morphologically similar to bone apatite (Titov, Larionov & Shchukin, 2000). Needle-like HAP crystals found in the lymph of ill patients are similar to those obtained in experiments 1 and 4, where there was no Mg or its concentration was much lower (experiment 2) than the normal concentration in the human blood. Magnesium not only inhibits HAP formation, but also makes the microcrystals more isometric (Reynolds, 2004) and similar in shape to bone apatite (experiment 3). Identical needle-like microcrystals were also found during in vitro mineralization of tendon (Golub, 2011) and were formed in an aqueous solution with polyacrylic acid (Le Geros, 2001). It is significant that these experiments were performed without Mg.

The effect of NaCl as an inhibitor of HAP formation is evidently caused by an increase of the ionic stress in the solution. However, sodium salt can completely inhibit HAP formation in an aqueous solution (experiments 4 and 5).

The joint action of Mg and NaCl in an aqueous solution in concentrations close to those of the blood plasma (experiment 6) completely inhibits HAP formation. The blood proteins facilitate HAP formation. The presence of HAP nanocrystals in the blood of healthy donors is indicative of their physiological origin. The experimental findings indicate that the formation of HAP microcrystals in the blood is possible only in the presence of albumin and, presumably, other proteins and enzymes, which may accelerate or inhibit the formation of HAP.

The formation of HAP in the plasma is similar to precipitation from a colloid solution. Serum albumin, a major blood component and an important agent in biomineralization, reduces the interfacial energy of HAP nuclei and thus stabilizes those that are smaller than the HAP nuclei in an albumin-free solution (Thompson & Towler, 2012). Albumin adsorbed on the surfaces of HAP crystals blocks their active growth sites and impedes both growth and aggregation. Thus, albumin regulates HAP nucleation, which is why changes of its concentrations in the blood may interfere with calcification patterns.

HAP microcrystals formed in the blood may also participate in the mineralization of heart valves and cardiovascular calcification. An endothelium injury can facilitate the penetration of HAP microcrystals formed in the blood into collagen fibers, which is followed by their deposition. At the late stage of mineralization, the transformation of HAP to brushite in the valve tissue may be accompanied by pH decrease. The latter may result from deep degrading changes in the valve tissue related to functional abnormalities due to calcification or septic processes.

The formation of HAP in the blood plasma is similar to precipitation from a colloid solution. At that, albumin reduces the interfacial energy of HAP nuclei and thus stabilizes those that are smaller than the HAP nuclei in an albumin-free solution (Combes & Rey, 2002). Albumin adsorbed on the surfaces of HAP crystals blocks their active growth sites and impedes both growth and aggregation. Thus, albumin regulates HAP nucleation, which is why changes of its concentrations in the blood may interfere with calcification patterns.

During HAP formation in the blood, the regulatory function apparently is performed by another serum protein – Fetuin-A, which many researchers

consider a strong inhibitor of mineralization (Smith, 2013; Dautova, 2014; Cai, Smith & Holt, 2015; Jahnen-Dechent, 2011; Sage, 2011). Fetuin-A is known to bind strongly calcium and forming apatite crystals. In particular, Fetuin-A molecules can form mineral colloids with calcium and phosphorus, thus preventing uncontrollable mineralization in pathological conditions. Paper (Wu, 2009) demonstrated mineral-protein complexes in the form of layered granules 50-500 nm in size, formed in a fetal bovine serum and an adult human serum. In terms of chemistry and morphology, these protein-mineral particles resemble granules discovered in the blood serum and so-called nanobacteria (NB) that were previously found in geological samples, soil, water, and other media. Similar 20-25 nm nanocrystals were found in vascular calcification and mineralized heart valves (Neven, 2011; Schlieper, 2011; Miller, 2004). Their nature is associated with biological liquids. NB were first characterized as pathogenic agents that produce diseases, including calcification. In fact, NB are a devitalized HAP complex with organic compounds (Raoult et al., 2008) and are bound strongly in human tissues with plasma proteins albumin and Fetuin-A. The presence of proteins and other inhibiting factors generally blocks the formation of apatite nuclei and stabilizes forming nuclei in the form of amorphous or partially crystalline spherical nanoparticles. However, such complexes, which are formed during the interaction between calcium phosphate complexes and albumin and Fetuin-A, become centers of origination and growth of HAP nanocrystals when saturated with calcium and phosphorus. The dual role of Fetuin-A in the blood plasma allows concluding that it is a systemic regulator of mineralization (Jahnen-Dechent, 2011).

The results of this research and abovementioned data from literary sources imply that the discovered HAP nanocrystals in the blood of patients and donor blood (Titov & Larionov, 2007; Titov, Larionov & Shchukin, 2000; Titov, 2004) and the protein-mineral complexes that were formed in the blood plasma have a common nature. However, as a result of the applied technique of HAP nanocrystal extraction from blood plasma to identify them with TEM (annealing at T=670°C), the organic part of these complexes was destroyed.

Protein-mineral complexes play an important role in physiological and pathological mineralization. The common property of albumin and Fetuin-A is their participation in the formation of bone tissue. Papers show that the matrix of a growing bone intensively consumes both albumin and Fetuin-A from the blood. Albumin and Fetuin-A are two most common non-collagen proteins in bone. It is also known that albumin is easily produced during bone demineralization (Combes & Rey, 2002).

The initial stage of bone tissue formation is associated with the formation of an extracellular matrix that mostly includes collagen fibers. The above experimental facts allow assuming that albumin and Fetuin-A are structural proteins responsible for subsequent integration of HAP nanocrystals into the newly formed bone tissue.

Primary HAP nanocrystals that were formed in the blood can also participate in the mineralization of heart valves and vessels. An endothelium injury can facilitate the penetration of HAP microcrystals formed in the blood into collagen fibers, which is followed by their deposition. At the late stage of mineralization, the transformation of HAP to brushite in the valve tissue may be accompanied by pH decrease. The latter may result from deep degrading

changes in the valve tissue related to functional abnormalities due to calcification or septic processes.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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