Porous Materials from Titanium—Cobalt Alloys for Hybrid Implants

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We proposed a new method to increase the biocompatibility of porous materials that were synthesized from titanium and cobalt allows by the method of self-propagating high-temperature synthesis. This method suggested the introduction of calcium hydroxyapatite into the reaction mixture. Administration of calcium hydroxyapatite into the reaction mixture had a modifying effect on the structure and surface of the pore space and biocompatibility of composite materials. Administration of calcium hydroxyapatite crystals was followed by a significant decrease in the size of pores and appearance of water-soluble fractions, which inhibited the activity of cells. However, treatment with amorphous nanodispersed calcium hydroxyapatite increased the biocompatibility and adhesiveness of materials for mesenchymal stem cells. The pore space and mechanical characteristics of materials obtained with amorphous nanodispersed calcium hydroxyapatite were similar to the properties of natural bone. Moreover, these materials surpassed titanium—cobalt allows in biocompatibility. Our results indicate that the introduction of amorphous nanodispersed calcium hydroxyapatite into the reaction mixture during self-propagating high-temperature synthesis has a modifying effect on the pore space of composite materials and increases their biocompatibility and adhesiveness for cells. We conclude that these materials may be used as a carrier of stem cells and progenitor cells in hybrid implants.

Key Words: *titanium; cobalt; calcium hydroxyapatite; porous materials; hybrid implants*

Titanium and its alloys are used in the manufacture of orthopedic and dental implants for the reparation or equivalent substitution of bone cells. During the last decade, much attention is paid to open-pore materials that can integrate with bone tissue. Highly porous three-dimensional matrix structures (particularly ceramic and polymer materials) are the basic elements in modern engineering of bone and cartilage tissue. However, the elastic and strength properties of these materials differ from those of bone tissue. It results in the destruction of synthetic constructions. A unique biocompatibility, low specific density, and high strength of porous titanium contribute to the preparation of implants, whose struc-

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ture and mechanical properties are very close to those of the bone. The materials with these properties provide not only support function, but also biointegration (growth of cells and vessels into the structure of implant).

High biological inertness of titanium determines low adhesiveness, which prevents the migration of cells. Biologically porous titanium can stimulate cell migration and differentiation in an osteogenic direction. This material is obtained by various methods of surface treatment. Biomimetic coatings are applied by the method of chemical precipitation. However, the formation of these coating has several limitations in three-dimensional matrix structures [4,6,8]. Another method to obtain a bioactive and osteoinductive coating of the porous titanium implant is based on long-term cultivation of osteogenic precursor cells on its surface. Such cells cover this implant with the matrix, which contributes to migration and osteogenic differentiation of proper stem cells in a patient. The use of bioengineering constructions contributes to grafting of the implant (even under conditions of bone destruction due to the disease or implant revision) [3]. The development of bioactive porous materials for hybrid implants is an urgent problem of oral and maxillofacial surgery, orthopedics, and stomatology.

Porous biocomposite materials from titanium—cobalt alloys were obtained by the method of self-propagating high-temperature synthesis (SHS). This method is based on the exothermic interaction between two or several chemical elements or compounds, which occurs under conditions of direct combustion [2,7]. Published data show that SHS may be used to prepare the porous material from titanium—cobalt alloy with the specified structure and properties [5]. Calcium hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$, HAP) was introduced into the reaction mixture to provide osteointegration of materials. Biological activity of HAP-containing materials is determined by the phase state. Previous experiments were performed to evaluate the effect of HAP crystallinity on structural characteristics, mechanical properties, and interaction of materials with mesenchymal stem cells (MSC) and tissues. A method for the manufacture of HAP materials with the desired structure was developed and patented at the Institute of Biochemical Physics (Russian Academy of Sciences) and Research-and-Production Company BIOMED. The crystalline structure of such materials is very close to that of bone tissue. Moreover, these materials are characterized by high biocompatibility and bioactivity [1]. HAP samples were used for the synthesis of composites from titanium—cobalt alloys.

This work was designed to study the effect of HAP crystallinity on structural characteristics, mechanical properties, and biocompatibility of porous materials from titanium—cobalt alloy synthesized by the method of SHS. We evaluated whether these materials can be used in hybrid implants.

MATERIALS AND METHODS

Three types of materials from titanium and cobalt were synthesized by the method of SHS. They differed in an initial composition of the reaction mixture. For the reaction of SHS, metal powders were mixed at a stoichiometric ratio of titanium and cobalt. HAP (10 wt %) was added to the reaction mixture. Titanium hydride served as a gasifying additive to produce the porous structure of materials. The mixture was pressed to obtain cylindrical samples (diameter 12 mm, height 15-16 mm). The samples were put vertically in an argon-filled reaction chamber. The initial temperature varied from 300 to 600°C. The local reaction was initiated by a hot wolfram spiral. After the induction of this reaction, a steady-state combustion wave spread over the sample (continuation of the process under conditions of SHS). The synthesized materials were sawed into plates (thickness 2-3 mm) and used in further experiments.

HAP samples were prepared by the method of self-precipitation [5]. Amorphous HAP was obtained by drying at 130°C. As differentiated from amorphous HAP, crystalline HAP was subjected to annealing at 900°C. An elemental analysis showed that these samples have the stoichiometric composition (Ca/P ratio 1.67). A roentgen phase analysis of the dried sample (130°C) revealed a typical diffraction pattern of HAP. All peaks were significantly widened, which reflects the amorphous phase state. The annealed sample (900°C) was characterized by a well-developed crystalline structure of HAP. An infrared spectroscopic study showed that the amorphous sample is hydrated (as differentiated from the crystalline sample).

The cytotoxic properties and adhesive characteristics of materials were estimated. The cells were isolated from human embryonic musculocutaneous tissue (6-8 weeks gestation). The cells were cultured in DMEM/199 medium (1:1) containing 10 fetal bovine serum (FBS), 100 U/ml penicillin, and 100 U/ml streptomycin at 5% CO_2. Experiments were performed with the culture of passage 11 cells ($CD133^-$, $Cd117^-$, $CD45^-$, $CD90^+$, $CD54^-$, $CD62L^-$, $CD62P^-$, $CD9^+$, $CD34^-$, $CD31^-$, $CD71^-$, $CD20^-$, $CD157^-$, $CD106^+$, and $CD62E^+$). These cells were inoculated to the surface of test samples

(35,000 cells/cm^2) and cultured for 72 h. Morphological characteristics and viability of cells were evaluated by the method of staining with 0.0002% acridine orange in phosphate buffer using an Axiovert 200 microscope (Carl Zeiss).

Cytotoxicity of materials was estimated in the MTT test. This test is based on the reduction of a colorless tetrazolium salt by mitochondrial and cytoplasmic dehydrogenases of metabolically active living cells. This reaction results in the formation of blue formazan crystals that are soluble in dimethylsulfoxide (DMSO). The samples of materials were maintained in physiological saline at 37°C for 15 and 244 days. The volume of solution was 1 ml per 0.2 g material. The cells were put in wells of a 24-well plate (up to 85,000 cells/cm^2) in DMEM/199 medium with 5% FBS. After 1 day, the medium was replaced by 400 ml serum-free DMEM/199 medium. The extract of materials (100 m) was added. Incubation was performed for 1 day. After incubation, 50 ml solution of MTT were added to each well (5 mg/ml in PBS solution (150 mM sodium phosphate buffer, pH 7.2; 150 mM NaCl) filtered through filters with a pore size of 0.2 m). The mixture was maintained in humid atmosphere at 37°C and 5% CO_2 for 3 h. The liquid was removed. DMSO (500 ml) was added. The plates were shaken at room temperature for 5 min to dissolve formazan salts. The development of staining was recorded by measuring the optical density in

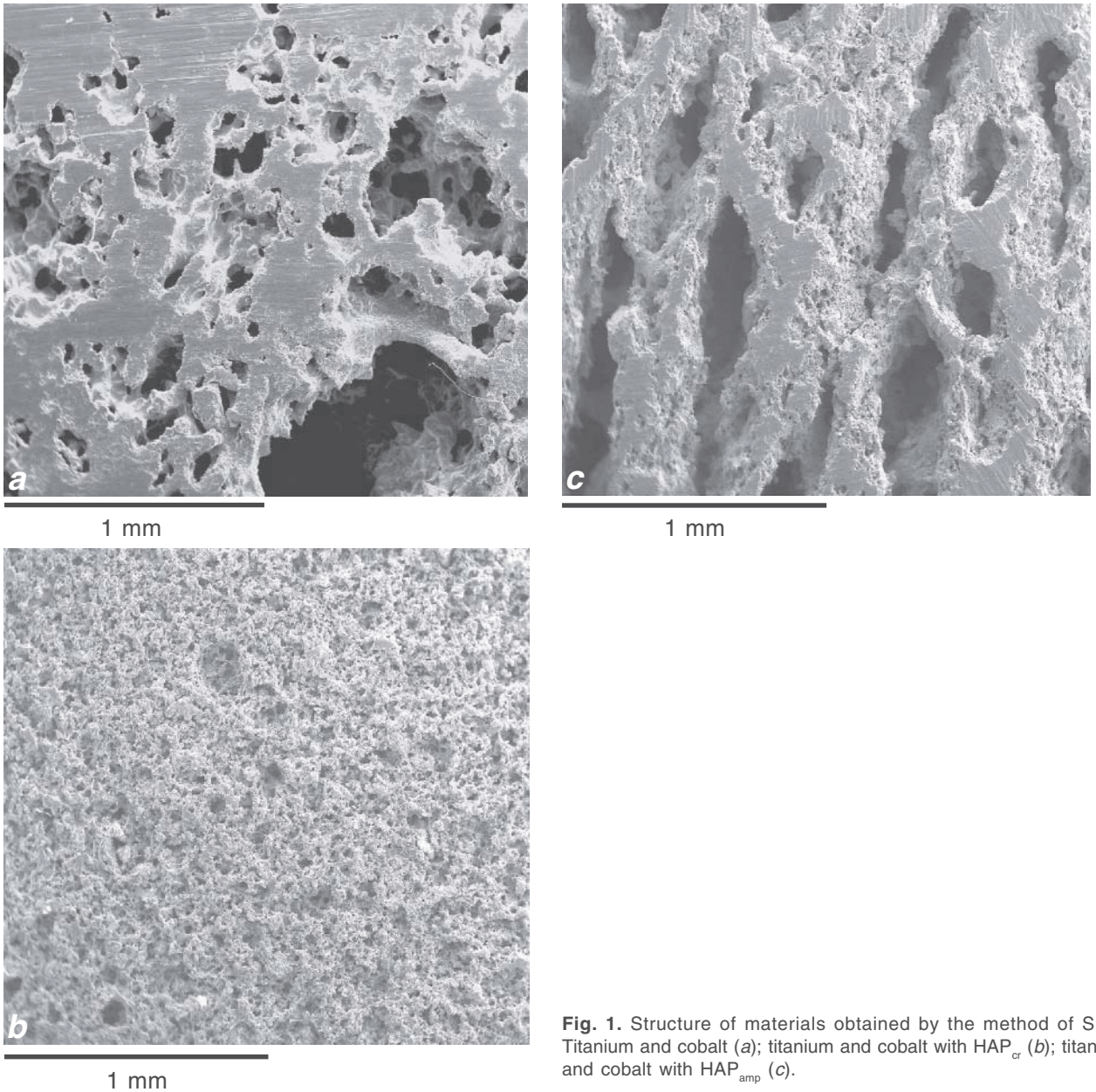

Fig. 1. Structure of materials obtained by the method of SHS. Titanium and cobalt (*a*); titanium and cobalt with HAP_{cr} (*b*); titanium and cobalt with HAP_{amp} (*c*).

wells of a 96-well plate at 540 nm using a BIO-RAD 680 photometer. The measurements were performed in at least three parallel experiments. The results were analyzed by Student's *t* test (Origin software). The differences between mean values were significant at $p<0.05$.

For scanning electron microscopy (SEM), the cells were fixed on the surface of materials. Seventy-two hours after inoculation of cells, the samples were washed in 0.1 M PBS (pH 7.4) and fixed with 2.5% solution of glutaraldehyde in PBS for 2 h. After removal of the fixating solution, all samples were washed with PBS and dehydrated. Ethanol was removed. The samples were maintained in hexamethyldisilazane for 30 min and dried. The final drying of samples was performed by means of transition through a critical point on a Hitachi CPD-1 device (Critical Point Dryer). They were put on an objective table and sprayed with the mixture of gold and palladium (Eiko-IB3 device, Ion coater). The spraying conditions (ion current 6 mA, interelectrode voltage 1.5 kV) allowed us to obtain a 25-nm spay layer. The samples were examined on a CamScan S-2 device (Cambridge Scanning) under recording of secondary electrons (accelerating potential 20 kV). The images were captured and analyzed on a personal computer (Microcapture 2.2 hardware-software system for microscopy and analysis).

The biocompatibility of materials was assessed in accordance with GOST R ISO 10993.6-99. The dynamics of capsule formation was studied during subcutaneous administration of metallic implants to adult male Wistar rats weighing 200-250 g (10 animals for each type of materials). The surgery was performed under sterile conditions. The operative field was treated. The animals were anesthetized with calipsol (16.9±1.1 mg calipsol per 100 g body weight). The interscapular skin and subcutaneous fatty tissue of the back were cut. Sterile implants (discs; 10 mm in diameter, 0.5 mm in height) were introduced into a soft tissue defect and fixed with soft tissues. Interrupted sutures (polyglycolide 4/0) were applied to the wound. The implant was completely covered. Hemostasis was performed during the surgery. The animals were killed by ether in a lethal dose on day 10. The local tissue response was evaluated. Implants and intact surrounding tissue were fixed in 10% formalin to study the local tissue response. Metallic samples were removed from the tissue before histological treatment. The samples were dehydrated with alcohols and embedded in paraffin. Serial sections were stained by hematoxylin and eosin. Tissue samples were studied by histological methods.

RESULTS

The samples had a microporous structure with the prevalence of open pores (Fig. 1).

The samples of materials from titanium—cobalt alloys were characterized by irregular porosity (pore size 100-800 m) and alternating regions of a monolithic (pore-free) material. The samples of titanium—cobalt alloys with 10% amorphous HAP (HAP_{amp}) had a regular porous structure (pore size 200-500 m). The structure of partition walls was also porous (pore size 50-80 m). The structure of this material is optimal for cell migration and growth of small vessels. The samples of materials from titanium—cobalt alloys with 10% crystalline HAP (HAP_{cr}) were of regular porosity. The size of pores was 50-80 m, which could not provide cell migration.

The behavior of cells was studied during cocultivation with samples of materials. The samples of materials from titanium—cobalt alloys, as well as those from titanium—cobalt alloys and 10% HAP_{amp}, had no adverse effect on cells. By contrast, the materials from titanium—cobalt alloys and 10% HAP_{cr} inhibited the activity of cells. The introduction of HAP_{cr} is probably followed by the formation of soluble phases that have an adverse effect on cell function. This hypothesis was confirmed by the MTT test. This test was conducted to evaluate metabolic activity of cultured cells in the presence of extracts from study materials (Fig. 2).

Study materials had different adhesiveness for cells (Fig. 3). Staining with acridine orange produced a green color of cell nuclei. These data illustrate viability of cells and absence of destructive processes. A SEM study showed that the introduc-

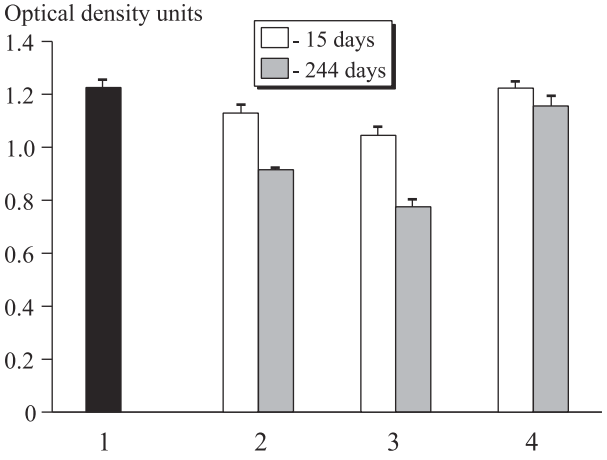

Fig. 2. Dependence of optical density (MTT test) on the chemical composition of samples and time of their maintenance in physiological saline. Control (*1*); titanium and cobalt (*2*); titanium and cobalt with HAP_{cr} (*3*); titanium and cobalt with HAP_{amp} (*4*).

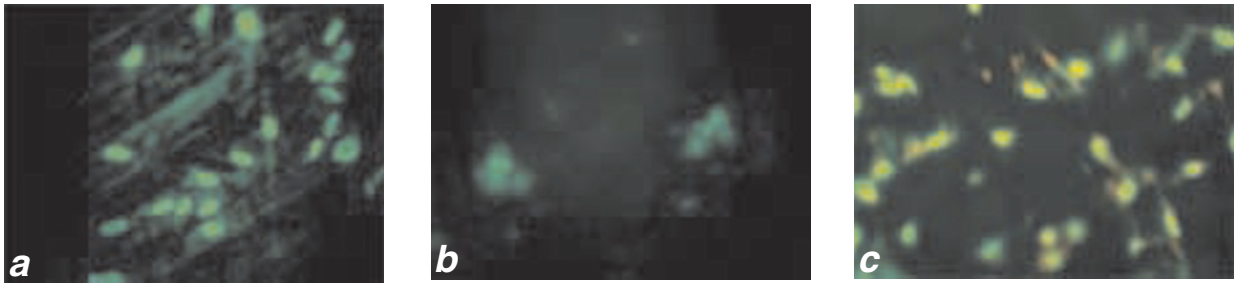

Fig. 3. Cultured cells on the surface of materials obtained by the method of SHS. Titanium and cobalt (*a*); titanium and cobalt with HAP_{cr} (*b*); titanium and cobalt with HAP_{amp} (*c*). Fluorescence microscopy, acridine orange staining (×200).

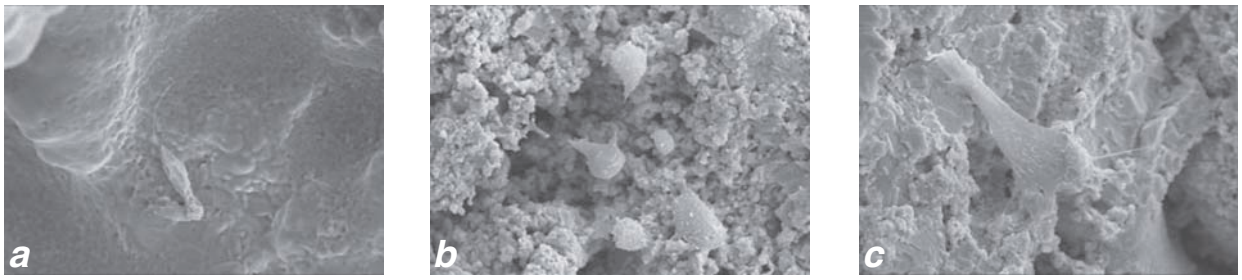

Fig. 4. Cultured cells on the surface of materials obtained by the method of SHS. Titanium and cobalt (*a*); titanium and cobalt with HAP_{cr} (*b*); titanium and cobalt with HAP_{amp} (*c*). Electron microscopy (×500).

tion of HAP had a modifying effect on surface roughness of the pore space in materials (Fig. 4). It was probably associated with a change in the adhesive properties of materials.

The cells were slightly spread on the smooth surface of materials from titanium—cobalt alloys, as well as on the rough surface of titanium—cobalt alloys and HAP_{cr}. The surface of materials formed in the presence of HAP_{amp} was rougher than the surface of titanium—cobalt alloys. These features provided the better conditions for cell adhesion. The cells were spread over the surface of these materials and migrated to the inner zone of the pore space. These processes probably contribute to the integration of materials and bone tissue.

Studying the cytotoxic and adhesive properties of composite materials allowed us to select the samples for *in vivo* experiments. They included the materials from titanium—cobalt alloys, as well as from titanium and cobalt with HAP_{amp}. Titanium—cobalt alloy with HAP_{cr} had the non-optimal structure, potential cytotoxicity, and poor adhesive properties and, therefore, was excluded from further experiments.

The biocompatibility of materials was evaluated from the dynamics of capsule formation after

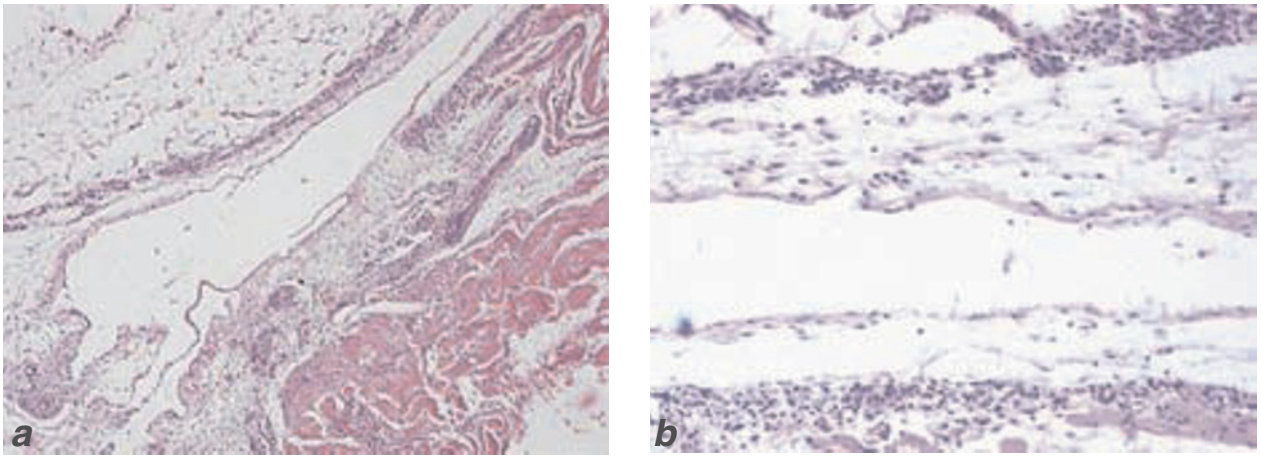

Fig. 5. Histological preparations of tissues around the samples of titanium—cobalt materials. Hematoxylin and eosin staining. Wide layer of connective tissue with dense focal infiltrates of lymphoid and macrophage cells (×50, *a*); loose connective tissue capsule with a considerable number of inflammatory cells in the bottom layer (×100, *b*).

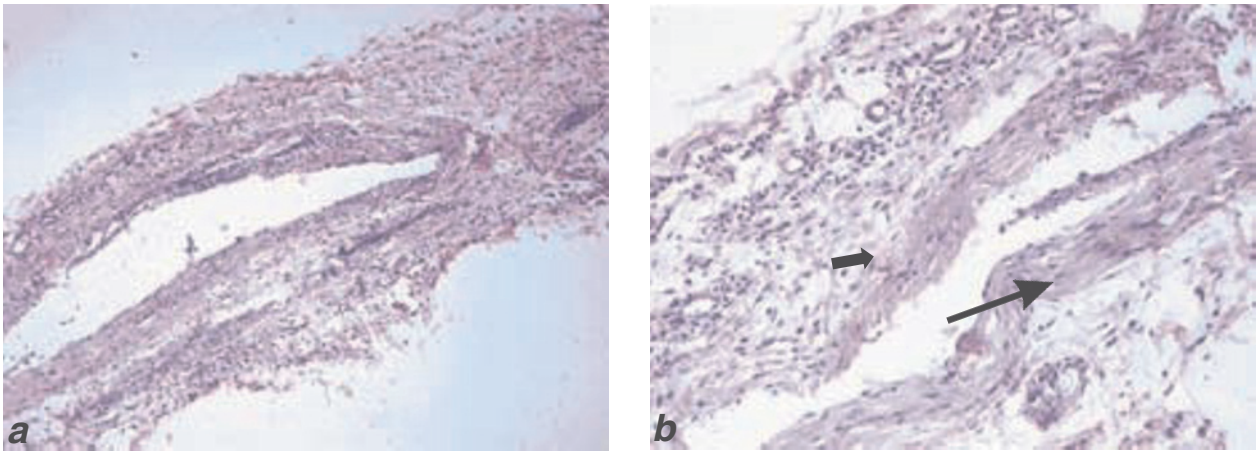

Fig. 6. Histological preparations of tissues around the samples of titanium—cobalt materials with 10% HAP_{amp}. Hematoxylin and eosin staining. Thick capsule (25 mm) with infiltrates of lymphoid and macrophage cells in the bottom layer of loose connective tissue (*50, *a*); connective tissue capsule in the form of circular collagen fibers (arrow) and adjacent loose connective tissue (thick arrow; *100, *b*).

subcutaneous administration of implants to male rats. Study materials had no hepatotoxic properties. A well-developed capsule was found on day 10 after subcutaneous administration of implants. Macroscopic signs of inflammation were not observed.

A histological study of implants from titanium—cobalt alloys revealed the signs of inflammatory cell infiltration in the connective tissue layer of a broad capsule and focuses of lymphoid and macrophage cells (Fig. 5).

A histological study was also performed with implants from titanium—cobalt alloys and 10% HAP_{amp}. A loose connective tissue capsule was formed below the implant on day 10. The capsule was rich in fibroblast cells and included a small number of lymphoid and macrophage cells (Fig. 6).

A histological study showed that these materials have no toxic effect on the surrounding tissue. The biocompatibility of titanium—cobalt alloy with HAP_{amp} was higher than that of titanium—cobalt alloy.

The introduction of HAP into an initial mixture for the manufacture of materials by SHS had a modifying effect on the structure of composite materials. Administration of HAP_{cr} resulted in the appearance of a fine-pore structure and cytotoxicity of materials. It is associated with the formation of soluble phases that inhibit the activity of cells.

HAP_{amp} contributed to the formation of pores and produced a modifying effect on the surface properties of the pore space. The pore space of these materials was characterized by high roughness, which provided the conditions for cell adhesion and growth.

The introduction of HAP_{amp} into an initial mixture under SHS conditions may be considered as a new approach for osteointegration of implants from titanium—cobalt alloys. These materials hold much promise as carriers of stem cells and precursor cells in hybrid implants.

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