NANOTECHNOLOGIES

Variability of the Biological Activity of Oxidized Titanium Implants

V. P. Shakhov, V. I. Vereschagin, T. S. Petrovskaya, V. P. Ignatov, and A. V. Krylatov*

Translated from *Byulleten' Eksperimental'noi Biologii i Meditsiny*, Vol. 152, No. 10, pp. 458-462, October, 2011
Original article submitted March 30, 2010

Oxidized titanium is a biologically inert material, but bioinertness reduces biomechanical characteristics of titanium implants. Modification of the structure of oxide surface layer of BT 5-1 titanium by increasing its thickness (by 1.7 times) and pore diameter (by 1.4 times) and by adding phosphorus, aluminum, and zinc oxides to its composition leads to radical modification of its biological characteristics. These implants acquire osteoinductive properties in *in vivo* systems not found in pure or oxidized BT 1-00 titanium and fairly well maintain *in vitro* growth of mesenchymal cells.

Key Words: *titanium; implant; bioinertness; bioactivity; mesenchymal stem cells*

Titanium implants are widely used in medical materials technology due to high biocompatibility, low thermal conductivity, low weight, and corrosion resistance [2,4,6,8]. Interaction of titanium with oxygen, water, other biological fluids and under specially created conditions leads to the formation of dioxide layer on its surface, due to which titanium implants become bioinert [5,6,8]. These implants are indispensable in medicine and dentistry in many cases. Calcium phosphate coating is formed on titanium implants in order to render them bioactive properties [1,3,4,5,7]. Calcium phosphate materials, including those in the form of coating, are characterized by osteoconductive and even osteoinductive properties providing high osteointegration of the implants and improving biomechanics of the entire construction. However, they are rather fragile and are rapidly destroyed in case of titanium deformation or under the effects of cyclic and dynamic loading of opposite directions [2,3,7]. In order to rule out the negative events, structural and functional characteristics of oxidized titanium implants should be transferred to a qualitatively new level by rendering them bioactive properties without application of a calcium phosphate coating.

We studied the possibility of rendering osteoinductive properties to bioinert titanium implants by modifying the structure and chemical characteristics of the surface oxide layer.

MATERIALS AND METHODS

Experiments were carried out on male BALB/c mice (n=83; 18-21 g) in winter-fall. The animals were kept on standard diet and handled in accordance with the regulations and ethical standards of the Helsinki Declaration [2]. BT 1-00 titanium implants shaped as disks 12 mm in diameter and 0.5 mm thick from BT 1-00 titanium (α-phase content up to 98.8 mas%) or from

Department of Silicate and Nanomaterial Technology, National University of Resource-Effective Technologies, Tomsk Polytechnic University; *Laboratory of Experimental Models, Institute of Cardiology, Siberian Division of the Russian Academy of Medical Sciences, Tomsk, Russia. ***Address for correspondence:*** vpshahov@mail.ru. V. P. Shakhov

0007-4888/12/1524-0475 © 2012 Springer Science+Business Media, Inc.

BT 5-1 titanium alloy (titanium α-phase content 90 mas%, the rest components Al, V, Fe, Zn) were used. Oxide coating was formed by anode spark oxidation in electric pulsed mode. Sulfuric and/or phosphoric acid solutions served as the electrolytes [2,8]. The coatings on the implants were studied by x-ray analysis, optical microscopy, and local micro x-ray analysis [2,3]. The microstructure and microhardness of the coating, thickness, size of pores, the coating porosity and qualitative composition were studied [2,3].

The implants were first degreased, washed in extra pure water, dried, and sterilized in dry air at 180°C for 40 min [2]. Bioinert and osteoinductive characteristics of the implants were studied by ectopic osteoformation by the standard method [2,8,9]. To this end, the bone marrow was washed out from the femoral bone with 1 ml RPMI-1640 (Sigma) into Petri dishes and transferred with forceps onto the surface of titanium implant, which was then implanted under the skin to recipient mice. After 1.5 months, the implants were removed and their surfaces were examined under a light microscope. Histological sections of some ectopic osteoformation foci were prepared and stained with hematoxylin or by van Gieson method [2].

The number of viable cells after incubation of a suspension of myelokaryocytes from mouse femoral bone marrow in RPMI-1640 (concentration of viable myelokaryocytes was 10^6 cell/ml RPMI-1640) on disks from titanium BT 1-00, BT 5-1 alloy, or their oxidized forms was evaluated. The samples with cells applied onto them were incubated at 5% CO_2, 37°C, and 100% humidity. Viable karyocytes were counted after 2 h in a Goryaev chamber using 1% trypan blue (Merck) [2,3].

Colony growth from bone marrow multipotent stromal cells (MSC) was studied *in vitro* as follows. A suspension of viable karyocytes was prepared and diluted to a concentration of 10^6 cell/ml in complete nutrient medium of the following composition: 10% DMEM with low glucose content, 90% FCS, 200 mmol/liter L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (all reagents and media from Sigma). The suspension was then transferred into plastic flat-bottom flasks (Falcone) (25 ml/flask) and incubated in a CO_2 incubator at 5% CO_2, 37°C, and 100% humidity. After 3 days, nonadherent cells were removed, the supernatant was replaced with a fresh portion of complete medium, and culturing was continued for 14-15 days more. The

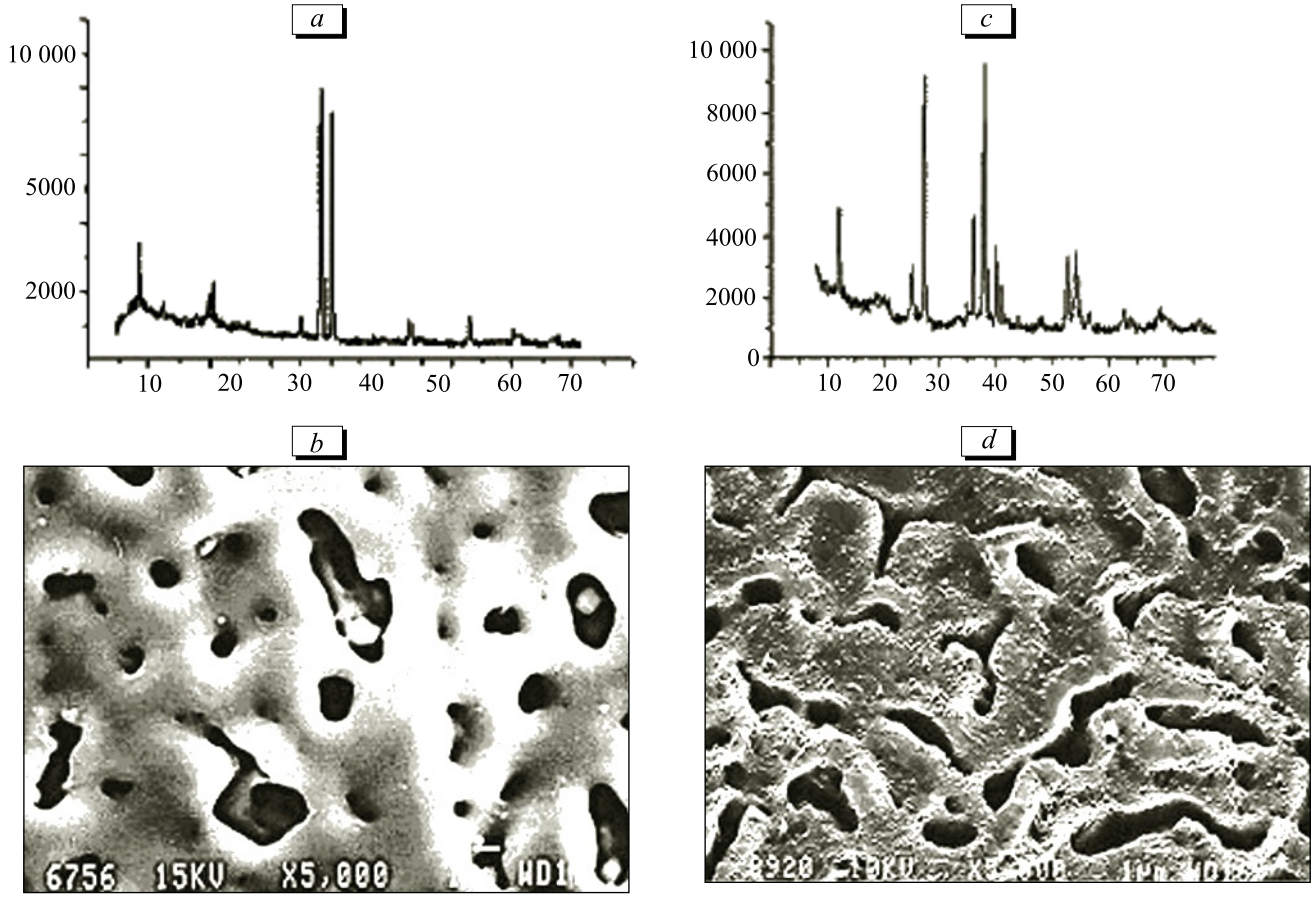

Fig. 1. Difractogram and electron microscopy of BT 1-00 titanium (*a*, *b*) and BT 5-1 alloy (*c*, *d*) after oxidation, ×5000.

TABLE 1. Micro X-Ray Analysis and Physicochemical Composition, Properties of BT 1-00 and BT 5-1 Titanium and Oxide Layer before and after Anode Spark Oxidation in Electrolyte (10% H_2SO_4+H_3PO_4) (*M*±*m*, *pt*<0.05)

Properties	BT 1-00, %	Oxide content in coating, mas% (BT 1-00)	BT 5-1, %	Oxide content in coating, mas% (BT 5-1)
Ti	99.5-99.9	79.1±2.7 (TiO_2)	89.6-93.7	69.9±3.7 (TiO_2)
Fe	≤0.12	-	≤0.3*	-
C	≤0.05	-	≤0.1	-
Si	≤0.08	-	≤0.15*	-
N	≤0.04	-	≤0.05	-
O	≤0.1	-	≤0.15	-
P_2O_5	-	19.3±3.1	-	25.1±4.3*
H	≤0.008	-	≤0.02*	-
Al	-	-	4.3-6.0	1.0±0.3 (Al_2O_3)
Zn	-	-	2-3	0.6±0.2 (ZnO_2)
Zr	-	-	≤0.3	-

Note. **Pu*<0.05 between oxidized BT 1-00 and BT 5-1 titanium implants.

medium was replaced every 5 days. Colonies of at least 50 cells were counted, after which the material was dried, fixed in methanol, and stained with azur II-eosin [2]. The data were processed using parametric (Student *t* test) and nonparametric (Mann—Whitney—Wilcoxon *U* test) tests (Statistica 6.0 software).

RESULTS

Variations of the anode-spark oxidation modes led to the formation of coating of up to 25.7±0.5 μ thick for BT 1-00 titanium and up to 44.1±0.9 μ for BT 5.1 alloy (*pt*<0.05). Qualitative composition of the coating, its structure, and size of pores depended on the electrolyte type and chemical composition of the metal implant. After processing of pure titanium (BT 1-00), the mean size of micropores in the electrolyte (mixture of sulfuric and phosphoric acid solutions) was 14.3±0.1 μ and nanopores 10-25 nm. X-ray structural and local micro X-ray analysis showed that titanium oxide (TiO_2) and phosphorus oxide (P_2O_5) predominated in the coating (Fig. 1, Table 1). Processing of BT 5-1 alloy led to the formation of pores of 20.6±0.2 μ. The content of TiO_2 in the coating was significantly lower, while the levels of P_2O_5, Al_2O_3, and ZhO_2 increased (Table 1). Oxidized BT 1-00 titanium or oxidized BT 5-1 alloy exhibited no effects of any kind on cell survival during co-incubation (Table 2), which was in line with the previous data [3,6,9].

According to the common mesomechanic laws, the surface structure determines the bioinformation properties of the implant. If the micropores in the oxide layer are <14.3 μ, the implants are bioinert and do not support the growth of bone and stem mesenchymal cells *in vivo* and *in vitro* (Table 2, Figs, 1, 2). If the diameters of micropores on the oxide layer surface are brought (by the anode spark oxidation) to the size of subunits forming the microstructure of the bone osteon (≈20.6 μ), these implants will exhibit bioactivity. The area of oxide layer increases significantly from

TABLE 2. Bone Marrow Cell Viability after Contact with the Test Material, Osteoconduction (OC; Ectopic Bone Formation Focus on Titanium Disk) *In Vivo* and Number of MSC Grown *In Vitro* from Bone Marrow Cells of BALB/c Mice on Oxidized BT 1-00 and BT 5-1 Titanium Implants (*M*±*m*, *pt*<0.05)

Titanium	Electrolyte	Thickness of coating, μ	Pore diameter/depth, μ	Cell viability, %	OC, $μ^2$	Number of MSC, ×10^6
BT 1-00	10% H_2SO_4+H_3PO_4	25.7±0.5	14.3±0.1/7.1±0.2	97.5±2.5	3.9±0.5	4.5±2.6
BT 5-1	10% H_2SO_4+H_3PO_4	44.1±0.9*	20.6±0.2*/12.3±0.1*	98.4±2.1	20.3±0.7*	16.3±2.7*

Note. **pt*<0.05 between oxidized BT 1-00 and BT 5-1 titanium implants.

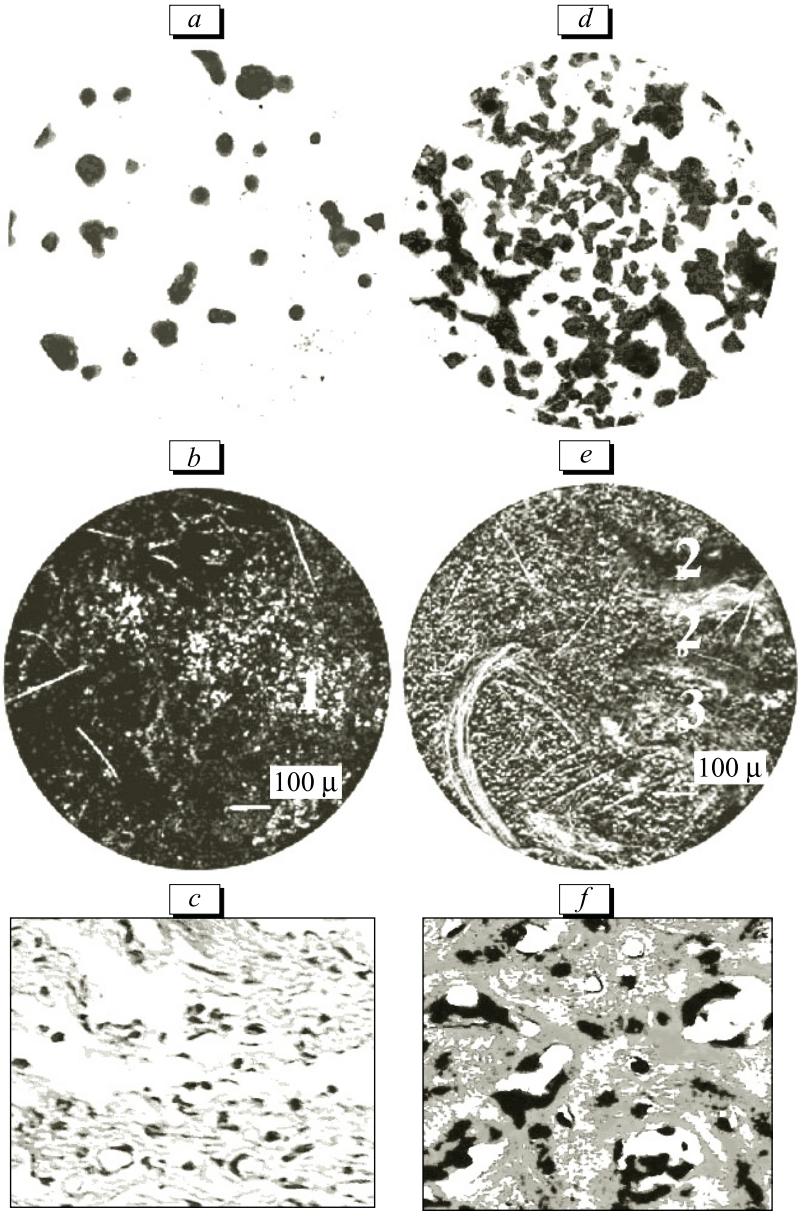

Fig. 2. Bone marrow cell growth on BT 1-00 (*a*-*c*) and BT 1-5 (*d*-*f*) titanium disks cultured during 14 days *in vitro*. Solitary cells on BT 1-00 titanium (*a*); MSC colonies on BT 5-1 titanium (*d*); azur II-eosin staining, ×900 (*a*), ×400 (*d*). Ectopic bone formation (*b*, *c*, *e*, *f*), oxidized BT 1-00 (*b*) and BT 5-1 (*e*) titanium surface after application of the bone marrow and 1.5-month subcutaneous implantation *in vivo*; native preparation, ×10. Histological section of tissue from the implant surface (*b*): *1*) connective tissue cells (*b*); *2*) ectopic bone formation (*e*, *f*); *3*) stromal tissue (*e*). Connective tissue (*c*, *f*); hematoxylin (*c*) and van Gieson staining (*f*), ×900.

113.0±0.3 mm^2 (BT 1-00 titanium) to 180.4±4.5 mm^2 (Pu<0.05; BT 5-1 titanium). Active *in vitro* growth of mesenchymal colonies is seen on their surface in tissue culture (Table 2, Fig. 2). These materials acquire osteoconductive properties *in vivo* (Table 2, Figs. 1, 2), which is characteristic of composite calcium phosphate implants and some types of biological glass and plastic [1-3]. Migration and growth of immature coarse fiber bone tissue with myelopoiesis foci were seen on their surface (Fig. 2). On the other hand, no colonies of bone marrow MSC were formed on pure titanium, just solitary mononuclear cells were seen on its surface (Fig. 2). Testing of biological characteristics of BT 1-00 titanium by ectopic bone formation showed the formation of stromal tissue from the bone marrow on its surface. No formation of bone structures was seen (Fig. 2, Table 2). It is logical to suppose the universal biological nature of the common regularities of physical mesomechanics, because similar results were previously obtained for hydroxylapatites and calcium phosphate biological glass. It is possible that phosphorus, aluminum, tin, and other admixtures

(iron, sulfur, zirconium, hydrogen) oxides, present in the metal and electrolyte and forming in the course of electrochemical reactions, are involved in these processes (Table 1) [1-3,5,8].

Thus, bioinert/bioactive characteristics of titanium implants are largely determined by many factors: chemical composition of the metal, electrolyte, oxide film and microstructure of its surface. If the surface of oxidized BT 1-00 titanium has pores less than 14.3 μ, the implant is bioinert. Creation of pores larger than 20.6 μ on the surface of BT 5-1 titanium implants leads to enlargement of the area and volume of working surface, this stimulating the growth of mesenchymal stem cells and bone tissue *in vivo* and *in vitro*.

REFERENCES

1. T. S. Petrovskaya, V. P. Shakhov, V. I. Vereschagin, and V. P. Ignatov, *Medical Materials Technology and Creation of Biomaterials for Traumatology and Orthopedics* [in Russian], Tomsk (2010).
2. A. V. Karlov and V. P. Shakhov, *External Fixation Systems and Regulatory Mechanisms of Optimal Biomechanics* [in Russian], Tomsk (2001).
3. L. Hanch and D. Jones, *Biomaterials, Artificial Organs, and Tissue Engineering* [in Russian], Moscow (2007).
4. A. Dawidowicz, S. Pielea, D. Paluch, *et al.*, *Polym. Med.*, **35**, No. 1, 3-14 (2005).
5. L. Le Guehennec, E. Goyenvalle, M. A. Lopez-Heredia, *et al.*, *Clin. Oral Implants Res.*, **19**, No. 11, 1103-1110 (2008).
6. P. J. Manders, J. C. Wolke, and J. A. Jansen, *Clin. Oral Implants Res.*, **17**, No. 5, 548-553 (2006).
7. F. Nakahira, T. Konishi, K. Yokota, *et al.*, *J. Ceram. Soc. Jpn.*, **114**, 46-50 (2006).
8. H. Nakayama, T. Kawase, H. Kogami, *et al.*, *J. Biomater. Appl.*, **24**, No. 8, 751-768 (2010).
9. V. Yao and T. J. Webster, *J. Nanosci. Nanotech.*, **6**, Nos. 9-10, 2680-2692 (2006).
10. J. Yao, X. Li, C. Bao, *et al.*, *J. Biomater. Appl.*, **24**, No. 7, 607-624 (2010).