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The Effect of the Modified Coatings Titanium Implants on the Morphofunctional State of Tissues Adjacent to Area of the Bone Regeneration.

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ABSTRACT

The osteomuscle interaction is realized through vascular patterns, which state depends on the mechanism of injury, tissue microenvironment conditions, including the bone implant composition. The aim of this study was to study the effect of titanium and its coating on endothelium-dependent interactions and vascular component state of tissues surrounding the tubular bone fracture area. The muscle tissue, vascular component and expression of trophic factors (VEGF-A, TGFβ, endothelin-1 and wisfatin) of the area adjacent to the osteoreparation foci in conditions of experimental latent fracture of the femur without combining fragments and with used titanium implant without and with calcium-phosphate bioactive coating were studied. It was shown that the posttraumatic period in the tissues of animals with the use of a coated implant was characterized by adaptive histogenesis of connective tissue with no destruction of myofibrils. The dependence of the content of trophic factors and the number of vessels on the conditions of osteoregeneration is established. The smallest values of the average and the relative area of the positive reaction of VEGF-A, TGFβ, endothelin-1 and low indices of vascular remodeling of surrounding tissues were revealed in the tissues of animals with the using of coated titanium, which demonstrates its high biocompatible properties.

Keywords: bone, implants, muscles, vessels, vasoactive factors, VEGF-A, TGFβ, endothelin

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INTRODUCTION

The processes of bone regeneration are regulated by the complex of interstitial and cellular factors of the muscular and vascular components adjacent to the area of damage [1-3]. Muscular tissues adjacent to the tubular bone fracture are the source of mesenchymal stem cells involved in a regeneration process, and endothelium-dependent reactions in the vessels are variable with time and conditions of trauma [4-7]. The determinative value of the structural components of muscle tissue in the initiation of local inflammation of the fracture area and the subsequent regeneration of bone tissue was proved [8-10]. Thusosteomuscle interaction is realized through vascular patterns, which state depends on the mechanism of injury, tissue microenvironment conditions, including the bone implant composition. This state determines the vector and potency of the damaged tissue reparation. Activation of myoblasts, endotheliocytes, macrophages, and production of various paracrine factors by these cells determine the trend of development of the initial inflammatory process and the subsequent bone tissue restoration. These structural components of the intercellular matrix include transforming growth factor beta (TGFβ), which influences on cell proliferation, vascular endothelial growth factor (VEGF), which performs mitogenic functions related to endotheliocytes, endothelin with vasoconstriction effect, and others [11-13].It is shown that VEGF has a stimulating effect on innate immune cells and influences indirectly on the regulation of the osteoregeneration process [3, 14, 15]. On the other hand, it is indicated that number and activity decrease of myoblasts with the expression of immunity cells functions reduces the reparative osteogenesis activity [5, 16]. Therefore, it is obvious that the study of molecular-cellular mechanisms in tissues surrounding the bone regeneration area is an urgent problem.

The extensive use in recent years of titanium implants for the restoration of various bone injuries determines the necessity to study the chemical composition effect of the metal as well as its surface and bioactive coatingdesign on the osteoregeneration processes. It is shown that microelements in the implant composition affect the endothelium-dependent reactions in the area of bone tissue damage [12, 18, 19]. Hereby titanium oxidation products affect the factors of paracrine regulation of the vascular endothelium, promote the expression of endothelin-1, E-selectin, monocyte chemoattractant protein-1 and other humoral factors, leading to endothelial dysfunction [20, 21]. Whereas, the high content of hydroxyapatite in coatings on titanium reduces the tissue expression of endothelin-1 and increases osseointegration [22, 23]. At the same time, the implant composition influence on the state of the vascular component of the muscle tissue remains insufficiently studied. In accordance with the aforementioned data, the aim of this work was to study the effect of titanium and its bioactive coating on endothelium-dependent interactions and vascular component state of tissues surrounding the tubular bone fracture area.

MATERIALS AND METHODS

VT1-0 titanium implants (Ltd. Scientific Production Association "Deost", "Osteomed", Russia) (0.25 wt. % Fe; 0.12 wt. % Si; 0.07 wt. % C; 0.12 wt. % O; 0.04 wt. % N; 0.01 wt. % H; balance – Ti) with a surface roughness Ra = 0.12 µm were used as a samples. Plasma electrolytic oxidation (PEO) method was used to form coatings on the implant surface in the bipolar mode. The electrolyte composition for PEO includes 30 g/l of calcium glycerophosphate($C_3H_7O_6P$) Ca_2H_2O and 40 g/l of calcium acetate $Ca(CH_3COOO)_2 \cdot H_2O$.

The experimental reproduction of the latent fracture was performed on eugamic male rats of the Wistar line(36 rats) with the weight of about 200-250 g in accordance with Helsinki Declaration provisions and European Community Directive (86/609 G)recommendations. The design of this study was approved by the interdisciplinary ethics committee of PSMU of Russia Public Health Ministry (protocol No. 6, 18.10.2014).The surgical procedure was made at aseptic conditions under ether anesthesia. Animals were withdrawn from the experiment 14 days after the trauma by intraperitoneal injection of 3% sodium thiopental. The animals were divided into three groups. The first one was the control group (12 rats), in which a femur fracture was reproduced without combining the debris. The second onewas the Group 1 (12 rats), in which a titanium implant was implanted in the fracture area. The third one was the Group 2 (12 rats), in which a PEO-coated implant was inserted. The bone fracture was performed by manual flexion effort, which allowed simulating a closed, minimally invasive osteosynthesis that provides an indirect increment. Implants with and without PEO-coating was inserted into the femur intramedullary canal through the inlet formed by a thin trocar in the intercondylar fossa. To immerse the screw cap into the subchondral layer, the hole in the bone was widened by the countersink, and through it the screw was inserted up to the middle third of the thigh diaphysis. The

wound was closed using one or two sutures, after which a bone fracture was made. For implant fixing the standard VT-1 titanium screws with and without a bioactive calcium-phosphate PEO-coating were used.

The connective and muscular tissues were obtained from the area adjacent to the site of osteosynthesis(14 and 28 days) and fixed in 10% neutral buffered formalin (24 h).According to the standard method tissues were embedded in paraffin and cross-sections with the thickness of about 5-7 μm were made. These cross-sections were stained with hematoxylin eosin. Mouse monoclonal antibodies (IgG) raisedagainst VEGF-A, TGFβ, endothelin-1 (End-1) and visfatin (Vis) (Abcam, USA) at 1:300 dilution were used for immunohistochemical detection of localization in growth factor tissues.Polyclonal antibodies to mouse IgG, markedby peroxidase were used as secondary antibodies. Preparations were evaluated using a CX41 microscope (Olympus, Japan) equipped with a digital camera.

Morphometric analysis of the obtained images of sections stained with hematoxylin eosin was made using the program ImageJ 4.1. As indicators, the values in pixels of the capillaryspecific volume (CSV), the vascular index (VI, the ratio of the vessel wall thickness to its diameter) and the trophic index (TI, the ratio of the capillary specific volume to the parenchyma specific volume) were used [24]. The tissue immunohistochemical reaction was assessed in terms of the absolute (arbitrary unit) and the relative area of the positive reaction (%), its average size (arbitrary unit), the average content of the component according to the color intensity, which was estimated by the optical density in pixels (relative unit).

Statistical analysis of the results was made using Statistica 6.0 (StatSoft, USA). The normality of the indicators distribution was estimated according to the value of the median (Me) [25th; 75th percentile]. Since the distribution of values in the studied samples did not correspond to normal (in accordance with Kolmogorov), the Mann-Whitney U-test and the Kruskal-Wallis nonparametric H-test (K-W) was used to evaluate the reliability of the differences in comparing the two and three groups of variables, respectively. Connection between variables was identified using the Spearman's rank correlation coefficients. Differences were considered reliable for $\rho<0.05$.

RESULTS

Muscle tissue reparation in animals with a fracture without implant insert occurred by the formation of a granulation tissue with transformation into a muscle callus. The formation of these structures was related with incomplete restoration of the muscle fibers continuity. Around the vascular endothelium, the collagen fibers destructuring was realized with a total area increase of the connective tissue with immune cells and adipocytes accumulation along the periphery of endomysia, which demonstrated a disruption of the complete recovery process. Foranimals with implantation of the metal nail without coating into the injured femur, the numerous collagen fibers in tissues and adipocytes in the granulation tissue near the capillaries were found as well asdestructuredfuchsinophilicbundles. Clusters of fibroblastic cells in different degrees of differentiation, lymphocytes, and macrophages were observed between the muscle fibers and near the vascular endothelium.The stroma formation was accompanied by the development of an abundant network of capillaries, by the appearance of numerous free myocytes with the formation of growth buds and strands of newly synthesized thin muscle fibers.The formation in tissues of a granulation connective tissue with a predominance of fibroblastic diferon cells was determined after the implantation of the metal nail with a calcium-phosphate coating in the damaged femur. The connective tissue was significantly developed and was distinguished by compacting the intercellular space and presence of numerous collagen fibers with different morphology. It was revealed in endomysium the proliferating myoblast accumulations with single, thickened, undulate collagen fibers around them.These fibers can be determined as ones of mature differentiated connective tissue. Moreover, the similar proliferation of connective tissue was detected in the presence of fibroblasts atvarious degrees of differentiation. The development of the capillary network of myonumvessels with the presence of highly differentiated cell components of endothelium anddeveloped connective-tissue capsule was determined. Therefore, the post-traumatic period in the tissues of this animals group was characterized by adaptive histogenesis of connective tissue in the absence of myofibrils destruction.

Morphometric analysis of the muscle tissue state showed an increase in the specific volume of the parenchyma against the background of anindex decrease for capillaries ($H = 9.9$, $\rho = 0.007$; $H = 9.3$, $\rho = 0.0095$, **table 1**) for animals with insertion of implantwith a calcium-phosphate coating, as compared to animals tissues of other groups. The parenchymal-stromal ratio was also significantly higher in tissues of animals with PEO-

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coated implant (H = 9.7, ρ = 0.0052), whereas the lowest one was fixed in the muscles of animals without an implant (U = -2.47, ρ = 0.01). TI was significantly higher in the muscles of animals with uncoated implants (H = 9.3, ρ = 0.009). Therefore, the morphometric evaluation of the vascular component state of muscle tissue showed significantly low values of CSV, SI, and TI for animals with PEO-coated implantand high ones for animals with bare titanium implants (**Table 1**).

Table 1: The indexes of the morphometric analysis vascular component in the area muscle tissues adjacent to the fracture site (Me [Q25; Q75])

Indexes,Me	Control	Group 1	Group 2	H K-W criteria, p
Capillary specific volume (CSV)	0.0034 [0.0031; 0.0039]	0.0044 [0.0040; 0.0049]*	0.0014 [0.0013; 0.0014] *ǂ	H=9.3, ρ=0.0095
Vascular index (VI)	0.29 [0.28; 0.31]	0.34 [0.31; 0.36] *	0.17 [0.16; 0.20] *ǂ*	H=18,9, ρ=0,0001
Trophic index (TI)	0.0042 [0.0040; 0.0046]	0.0056 [0.0048; 0.0074] *	0.0015 [0.015;0.0016] *ǂ	H=9.3, ρ=0.0095
Parenchymaspecific volume (PSV)	0.77 [0.76; 0.80]	0.83 [0.75; 0.86] ǂ	0.89 [0.88; 0.89] *	H=9.9, ρ=0.0072

Footnote: in Table 1 and Table 2

* differences are reliable when compared with the control group, ρ<0.05;

ǂ differences are reliable when compared with Group 1, ρ<0.05.

Immunohistochemical study of the content of biologically active components, which influence on the repair process of damaged tissues showed the expression values dependence on osteoregeneration conditions (**Fig. 1**). Thus, intensity indices of the average and relative area of the positive reaction, reflecting the specific content of the vascular endothelial growth factor (VEGF-A), transformation growth factor (TGFβ) and endothelin-1 (End-1), were significantly higher in the tissues of the control animals as compared to samples of animal experimental groups (**Figure 1a**, **Table 2**). The criterion of Kraskel-Wallis H-test was H = 7.1, (ρ = 0.03) and H = 11.3 (ρ = 0.0034) for VEGF-A, H = 9.5 (ρ = 0.008) for TGFβand H = 20.7 (ρ = 0.000001) for End-1. The lowest expression indices of VEGF-A, TGFβ and End-1 were detected in the tissues of animals withPEO-coated titanium implant with the indices of Mann-Whitney U-test: U = -2.37 (ρ = 0.02), U = -2.74, (ρ = 0.006); U = -2.88 (ρ = 0.004), U = -2.10 (ρ = 0.04); U = -3.87 (ρ = 0.0001), U = -3.81, (ρ = 0.0001), respectively (**Figure 1c**). The average size of the positive reactiondepositof the muscular tissue to endothelin-1 was the smallest in the control group (H = 12.3, ρ = 0.002), with insignificant differences for the experimental groups (z = -0.53, ρ = 0.6).

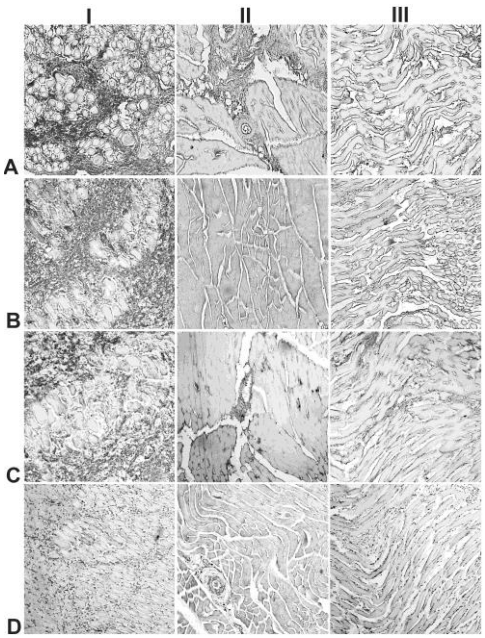

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Fig 1: Localization of paracrine regulation factors and visfatin in the tissues: I – the evident positive reaction in connective tissue and endomisis of animals with femur fracture without fragments combining; II – ill-defined reaction to antigens in the tissues of animals with implanted into the bone the uncoated titanium nail and III – the nail with a calcium-phosphate coating. A - reaction to TGFβ; B - to VEGF-A; C - on End-1; D - Visfatin. Immunohistochemical method.Increase X100.

Table 2: The indexes of the paracrine regulation factors content and visfatin in tissues adjacent to the osteoregeneration site (Me [Q25; Q75])

Indexes,Me	Control	Group 1	Group 2	H K-W criteria, p
TGFβ				
Average reaction area, arbitrary unit	108502.8 [75173.0; 167315.7]	42149.4 [33348.5; 59131.6] *	32053.7 [26028.3; 42847.4] *	H=9.5, ρ=0.0085
Average deposit size, arbitrary unit	37.48 [19.00; 59.11]	11.58 [9.19; 17.55] *ǂ	8.86 [8.24; 9.18] *	H=11.6, ρ=0.0030
Relative area, %	11.77 [8.16; 18.15]	4.70 [3.66; 6.43] *	3.48 [2.82; 4.81]*	H=9.5, ρ=0.0085
Average content, relative unit	102.79 [99.91; 104.33]	108.32 [105.64; 108.95] *ǂ	106.03 [104.47; 107.41]	H=6.9, ρ=0.0327
VEGF-A				
Average reaction area, arbitrary unit	315559.8 [130305.7; 352995.6]	124246.8 [100116.9; 152614.3]	81644.3 [63184.3; 122183.5]*	H=7.1, ρ=0.0286
Average deposit size, arbitrary unit	86.39 [57.99; 106.24]	35.44 [33.59; 54.55] *ǂ	28.60 [18.53; 31.99] *	H=8.6, ρ=0.0138
Relative area, %	34.24 [14.14; 38.30]	7.79 [7.28; 10.86]*ǂ	5.29 [2.37; 7.15] *	H=11.3, p=0.0034
Average content, relative unit	142.26 [141.82; 142.54]	114.63 [111.05; 151.07]	125.77 [104.76; 148.67]	H=0.8, ρ=0.6609
End-1				
Average reaction area, arbitrary unit	211445,0 [127049,5; 325969,9]	120248,1 [76246,3; 215882,5]	52285,8 [50056,7; 53623,3] * ǂ	H=20,7, ρ=0,000001
Average deposit size, arbitrary unit	188,79 [180,55; 192,30]	200,47 [189,72; 204,42] *	199,77 [196,94; 200,05] *	H=12,3, ρ=0,002
Relative area, %	20,20 [6,13; 15,72]	5,79 [3,68; 10,41]	2,52 [2,41; 2,59] * ǂ	H=20,7, ρ=0.000001
Average content, relative unit	148,00 [146,00; 152,00]	166,50 [162,00; 174,00] *	159,00 [151,00; 161,00] * ǂ	H=18,9, ρ=0,0001
Visfatin				
Average reaction area, arbitrary unit	27648,0 [25067,5; 27915,3]	85121,3 [80455,7; 115997,2] *	89185,5 [86780,2; 122964,5] *	H=21,0, ρ=0,00001
Average deposit size, arbitrary unit	184,79 [183,37; 189,06]	207,97 [206,39; 208,63] *	224,48 [212,48; 225,45] * ǂ	H=22,5, ρ=0,000001
Relative area, %	3,00 [2,72; 3,03]	4,10 [3,88; 5,59]*	4,30 [4,18; 5,93]*	H=10,2, ρ=0.006
Average content, relative unit	128,00 [126,00; 131,00]	177,00 [175,00; 180,00] *	191,00 [187,00; 194,00] * ǂ	H=25,00, ρ=0,00001

The lowest values of the average and relative area of the immunohistochemical reaction for the visfatin (Vis) presence in muscle tissue adjacent to the fracture zone were established in animals of the control group (H = 21.0, ρ = 0.00001, H = 10.2, ρ = 0.006, **Table 2**). Whereas, the average and relative area of the positive reaction, indicating the visfatinpresence in tissues, was significantly higher in the tissues of animals with implants relative to control ones (z = 3.87, ρ = 0.0001, z = 3.87, p = 0.0001), and did not differ in the

experimental groups (z = 0.91, ρ = 0.4).The average size and the content of visfatinwas significantly higher in the group of animals, whosebone fragments were fixed with an implant with both bioactive calcium-phosphate coating (respectively: z = 3.87, ρ = 0.0001; z = 3.87; ρ = 0.0001) and without it (respectively: z = 2.12, ρ = 0.03, z = 3.17, ρ = 0.001) in comparison with the control group.

DISCUSSION

Thereby, it was established in this work that the structural organization of tissues surrounding the osteointegration area depends on the properties of the implant used. Thus, with the implantation of titanium into the bone fracture area, the vascular remodeling in muscles is revealed by a significant increase in CSV, SI, TI, total number of capillaries and thickening of their endothelium, which can be characterized as nonadaptiveangioproliferation. Our results are corroborated with data of other researchers who demonstrate the titanium oxide (TiO_2) ability to influence the endothelium and smooth muscle cells proliferation, as well as the expression of proinflammatory cytokines - tumor necrosis factor and interleukin-8 [21, 25]. Inconditions of experimental metalosteosynthesisof a tubular bone using a bioactive calcium-phosphate coating, vascular remodeling of surrounding tissues was observed to a lesser extent, since it was determined by the effect of a higher implant biocompatibility.The ratio of calcium to phosphorus in the composition of PEO-coating formed in this work is close to the one in bone tissue composition, which determines its ability to initiate insignificant stimulation of innate immunity cells [26]. In available literary sources, we have not found studies that confirm or disprove the negative effect of calcium phosphate coatings on the remodeling of tissues surrounding the osteosynthesisarea. Whereas, data of this study show that metalosteosynthesis with a bioactive coating shows a little vascular remodeling with adaptive changes in muscle tissue, which as a whole has a positive effect on the osteosynthesisprocess. This conclusion is confirmed by indices of paracrine regulation of the vascular endothelium, which demonstrate the concentrated and local distribution of these components in tissues. It is known that increased expression of endothelin-1, VEGF-A, and TGFβ initiates macrophage chemotaxis, increases vascular wall permeability and activates angiogenesis in the hypoxia vector, which contributes to low bone tissue regeneration [3, 4, 10].It was also established that use of bone implants with a highhydroxyapatite content showed a low tissue concentration of endothelin-1 and increased osseointegration in the fracture area [11, 6, 8]. At the same time, being a multifunctional adipokine, visfatin has protective properties towards to endothelium.A direct correlation of visfatincontent with vascular remodeling in tissues was determined [9].

CONCLUSION

In this study, it was shown for the first time that after implantation of metal structures with a bioactive calcium-phosphate coating, the normal geometry of the vascular wall in muscles was observed against the background of a low tissue level of endothelin-1 and high expression of visfatin, which demonstrates the conjugacy of osteosynthesis and remodeling processes of tissues surrounding the implant. Thus, the evaluation of the influence of the bone implants composition on the state of the vascular tissue component promotes an understanding of bone regeneration processes taking into account the implant biocompatibility and provides an opportunity for a rehabilitation prognosis.

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