Materials and Methods

The purpose of this research was to study biointegrative characteristics of implants coated by polyazolidine ammonium modified by the hydrate flavonoids' halogen ions and propolis at different concentrations. Materials for the study included implants, which were thermally treated by high-frequency induction currents, as well as human dermal fibroblasts, isolated from healthy donor skin, which was provided by plastic-surgery clinics of Saratov on approved schedule. The study involved clinical and cytological methods as well as light and electron microscopy. Cell culture of dermal fibroblasts was obtained using the tissue explants method [22]. Biopsy samples of the skin, taken in the surgery suite, were immersed into special vials with a sterile proliferating mixture. Further work on the source material and the cell cultures was performed under sterile conditions in a laboratory, specially equipped with set of "pure" laminar flow units (2nd protection class ["Nuaire", USA]).

Several titanium workpieces coated with polymer film (polyazolidine ammonium modified by the hydrate flavonoids' halogen ions) and propolis at different concentrations were used as a substrate for the experiment: sample #1—control; sample #2—biologically active substance (BAS), 10 mg/ml; sample #3—BAS, 5 mg/ml; sample #4—BAS, 2.5 mg/ml; sample #5—BAS, 1.25 mg/ml; sample #6—functional substance (FS), 1%; sample #7—FS, 0.1%; sample #8—FS, 0.01%; sample #9—FS, 0.001%; and sample #10—FS, 0.0001%. Before the experiments, titanium blank samples were sterilized in a hot-air oven ("Ekros", Russia), placed further in 24-well plates ("Costar", USA), and then subjected to cell culture seeding (at the concentration of 1×10^5 cells per sample in 2 ml of mixture). Nutrient mixture DMEM ("Biolot", Russia) with the addition of 10% of fetal bovine serum (FBS) ("HyClone", UK) and antibiotic-antimycotic blend was used for cultivation. Plates were placed in a CO_2 incubator Sanyo MCO-18M ("Sanyo", Japan) at a temperature of 37°C and carbon dioxide content of 5%. Assessment of the cells, cultured on the experimental materials, was performed using light-microscopy techniques. The change in the shape and number of cells during cultivation was observed using an inverted microscope ("MIB-R", Russia).

Adhesion and proliferation factors of cell culture on the implants were examined using an electron microscope (MIRA\LMU, "Tescan"). Biointegration of experimental implants was investigated in rabbits of "Grey Giant" breed in the course of a clinical experiment conducted at the clinical hospital of the Department of Veterinary Medicine, Food, and Biotechnology at Saratov State Agrarian University. The experiment used 9-month-old animals having a live weight of 4.0-4.2 kg. Based on the principle of analogues, they were divided into 2 groups, 5 animals in each group. Due to the weak strength properties of rabbit jaws and complexity in viewing the oral cavity of the animal, the clinical test of biointegrity was performed by placing implants into the astragalus of rabbits.

Implants installed in the rabbits had a cylindrical shape, were 3.5 mm in diameter and 10 mm in length, and had a spiral groove. Their surface was coated by titanium dioxide TiO_2, employing induction heat treatment carried out by a special unit driven by high-frequency currents. Induction heat treatment was conducted at a temperature of 800 ± 10°C for 2 min followed by cooling.

The implants with titanium dioxide coating were mounted in the animals of the first (control) group, while implants coated with titanium dioxide and polymer film (polyazolidine ammonium) and modified by nanoaggregates of flavonoids were mounted in the animals of the second (experimental) group.

The coating on the test samples was formed as follows: BAS, 1.25 mg/ml and FS, 0.0001%, using an immersion and drying technique at a temperature of 5°C until complete dehydration. Postsurgical therapy consisted of standard procedures in veterinary practice. All the experiments were performed in accordance with GOST RISO 10993.

Neuroleptanalgesia with xylazine and zoletil was applied to the experimental animals. Further, canals were drilled in the rabbit's femur, in the area between the metaphysis and middle third of the diaphysis, for mounting the implants. Implants were screwed into the prepared beds to a state of sufficient mechanical resistance.

Results and Discussion

Research conducted on the condition of dermal fibroblasts has shown that the cells were well adhered to the samples No. 1, 2, 3, 4, and 5. It was clearly seen that there was high proliferative activity (accumulation) in the vicinity of these samples. The sample 7 (Figure 1)

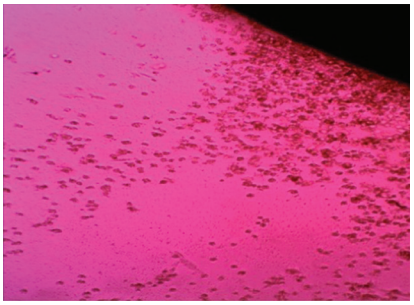

Figure 1: Sample #7

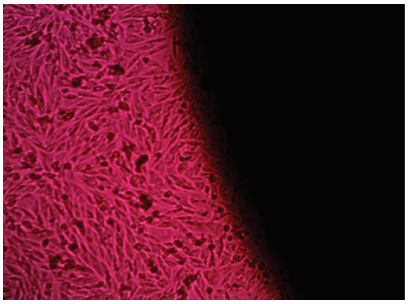

Figure 2: Sample #9

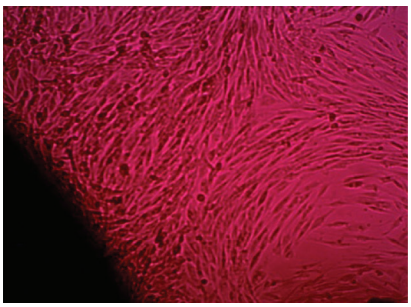

Figure 3: Sample #10

has shown growth inhibition of cells and then their subsequent death. This is evidenced by change (rounding) in shape and the lack of cell growth.

Samples 8, 9, and 10, where the polymer concentration was consistently reduced (Table 1), have demonstrated improved adhesive and proliferative abilities of the cell culture as evidenced by the presence of distinctive spindle-shaped configuration nearby the cell samples (Figures 2 and 3).

The best results were obtained from sample 10. During the control microscopy after 24 h, it was noted that the culture was in a good condition, the shape of the cells was predominantly spindle-like, outgrowths were well expressed, and the nucleus was clearly defined. Table 1 presents the research results on the effect of different concentrations of functionally and biologically active substances on the functional characteristics of the fibroblast culture on the first and second days of the experiment.

The clinical evaluation of implants' biointegration involved the measurement of the animal's body temperature, behavior; the evaluation of the animal's ability to support its injured extremity; the evaluation of the response of the animal to the compression in the vicinity of the implant as well as the availability of inflammation that could be detected by the degree of swelling and exudation. During the first week after surgery, no significant differences in the clinical condition of animals were revealed in the animals of experimental and control groups. During the first day of the surgery, 5 control animals and 3 animals of the experimental group refused food and water. The increase in body temperature was not recorded. The ability of animals to lean on the operated limb was observed as early as on the next day and did not violate in subsequent days.

Local inspection of all animals revealed weakly expressed inflammation pattern in the area of "implant bone" the day after surgery. At this time, swelling and redness of soft tissues, as well as slight tenderness to palpation were observed. After the first week of clinical presentation and characterization, the condition of the animals in the control group did not change significantly, while the inflammation symptoms of the soft tissues in the animals of the experimental group almost disappeared, and palpation did not cause any concern to animals. The skin in the area of operating stitches was not infiltrated; that testified to the normalization of hemodynamics in the "implant-bone" contact zone during a relatively short time.

First day			Second day		
No.	**Sample**	**Assessment**	**No.**	**Sample**	**Assessment**
1	Control	+	1	Control	+
2	BAS 10 mg/ml	+	2	BAS 10 mg/ml	±
3	BAS 5 mg/ml	+	3	BAS 10 mg/ml	±
4	BAS 2.5 mg/ml	+	4	BAS 2.5 mg/ml	±
5	BAS 1.25 mg/ml	+	5	BAS 1.25 mg/ml	+
6	FS 1.0%	---	6	FS 1%	--
7	FS 0.1%	---	7	FS 0.1%	--
8	FS 0.01%	--	8	FS 0.01%	±
9	FS 0.001%	±	9	FS 0.001%	±
10	FS 0.0001%	+	10	FS 0.0001%	±

Note: + normal, ++ good, - partial loss, -- dominating loss, --- total loss, ± availability of the living and the dead fibroblasts.

Table 1: Impact assessment of the different concentrations of functionally and biologically active substances on the functional characteristics of the fibroblast culture

Changes in the condition of the tissues around the implants, persisting during the experiment, were related to the surface properties of implants formed by various treatment methods—thermal oxidation and modification by nanoaggregates of flavonoids. After the end of the experiment, when removing the implants from the bones of animals in the experimental group, exertion to extract the implant required greater effort than that of the animals in control group. The surface of the implants extracted from animals' bones of the experimental group had quite a considerable number of bone tissue fragments (Figures 4 and 5).

When conducting hematologic and biochemical studies, we have revealed that one day after surgery, the level of erythrocytes decreased from 5.8 ± 0.3 to $3.3 \pm 0.4 \times 10^{12}$/l in the first group and from 5.7 ± 0.2 to $3.9 \pm 0.1 \times 10^{12}$/l in the second group, respectively. Noted fluctuations were insignificant and testified to minor injury rate of the selected experiment and, as a consequence, the minor effect of bone injury on erythropoiesis. By the 30th day, the amount of erythrocytes in the experimental group reached baseline equal to $5.5 \pm 0.1 \times 10^{12}$/l, while in the control group, the number of red blood cells by the end of the experiment was $4.3 \pm 0.2 \times 10^{12}$/l that was below the presurgical indices. During the first 3 days, the animals of control group showed a significant leukocytosis, and this trend continued up to 14 days. The number of leukocytes in the first-group animals increased from 7.4 ± 1.0 to $11.2 \pm 1.3 \times 10^{9}$/l a day after surgery and to $10.3 \pm 0.3 \times 10^{9}$/l 3 days after; in the animals of the second group, the number of leukocytes increased from 8.5 ± 0.9 to 8.6 ± 1.9 in the first day and to $10.1 \pm 1.0 \times 10^{9}$/l in the third day, respectively. This

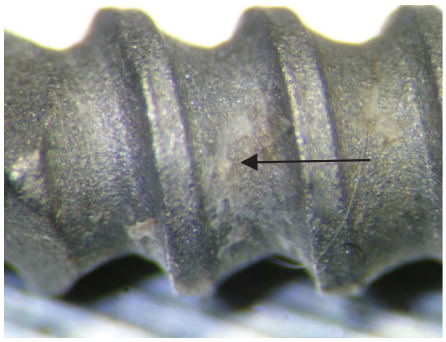

Figure 4: Control implant under a magnifying glass (×5)

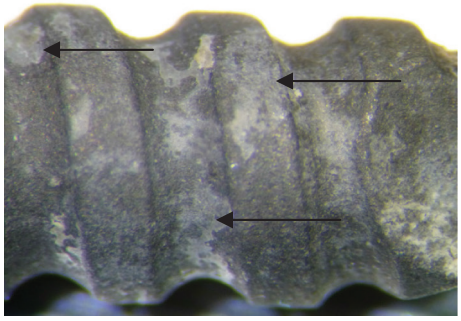

Figure 5: Experimental implant under a magnifying glass (×5). Fragments of newly formed bone tissue

condition was associated with expression of protective-compensatory reaction of the animal's organism aimed at the relief of the inflammatory process, initiated by the decay products of blood resulting from trauma. By 30th day, in the blood of rabbits in control and experimental groups, there was a slight decrease in the number of leukocytes to 9.0 ± 0.4 and 8.6 ± 0.9 × 10^9/l, respectively.

The level of hemoglobin in the animals of control group was characterized by an insignificant yet negative dynamics. Before the experiment, it amounted to 110.0 ± 2.0 g/l; a day after the surgery, to 75.4 ± 18.4 g/l; 3 days after, to 84.4 ± 6.6 g/l; and 30 days after, to 90.7 ± 5.7 g/l. Positive dynamics in the level of hemoglobin was observed in animals of the experimental group; before the experiment, it amounted to 109.0 ± 2.4 g/l; a day after, to 104.8 ± 3.8 g/l; 3 days after, to 86.0 ± 0.3 g/l; and 30 days after, to 111.3 ± 1.9 g/l. These data suggest an activation of the erythrocytes' saturation with oxygen because bone trauma inevitably leads to hypoxia of the tissues.

Parameters of total protein and albumin in animals of both groups showed small growth. In the first group presurgical values of total protein amounted to 75.0 ± 3.8 g/l; a day after the surgery, 98.7 ± 9.5 g/l; 3 days after, 111.0 ± 10.4 g/l; and by the 30th day, 101.5 ± 9.7 g/l; pre-surgical values of albumins amounted to 22.2 ± 1.3 g/l; a day after surgery, 36.8 ± 1.5 g/l; 3 days after, 41.7 ± 1.9 g/l; and by the 30th day, 29.3 ± 1.9 g/l, respectively. In the second group presurgical values of total protein amounted to 81.7 ± 4.9 g/l; a day after the surgery, 93.4 ± 13.4 g/l; 3 days after, 104.4 ± 5.5 g/l; and by the 30th day, 83.0 ± 4.6 g/l; pre-surgical values of albumins amounted to 25.3 ± 2.0 g/l; a day after the surgery, 34.7 ± 1.2 g/l, 3 days after, 40.0 ± 0.6 g/l; and by the 30th day, 26.0 ± 2.2 g/l, respectively that was associated with the availability of slight inflammation in the area of the osseous defect.

The values of aminotransferases (AST and ALT) in control and experimental groups varied within physiological norms that corresponded to the type of mechanical trauma. In the animals of control group, presurgical indicators of AST equaled 38.7 ± 3.5 u/l, increased during a day up to 58.0 ± 3.3 u/l, 3 days after, up to 67.0 ± 2.9 u/l but by the 30th day decreased to 53.7 ± 3.8 u/l. The level of ALT before the surgery was 22.2 ± 4.6 u/l; a day after, 29.8 ± 2.0 u/l; 3 days after, 35.1 ± 1.7 u/l; and by the 30th day, 26.0 ± 2.6 u/l. In animals of the experimental group, the AST concentration before surgery was 24.5 ± 4.1 u/l, a day after, 51.0 ± 3.9 u/l; 3 days after, 66.3 ± 0.4 u/l; and by the 30th day, 26.3 ± 4.1 u/l. The level of ALT prior to the surgery was 15.5 ± 2.2 u/l; a day after, 19.2 ± 4.0 u/l; 3 days after, 18.5 ± 1.9 u/l; and by the 30th day, 16.3 ± 1.7 u/l.

Dynamics of total bilirubin remained without significant fluctuations, indicating the absence of hemolytic and hepatotoxic processes in the organism. Perhaps, a slight increase in the amount of creatinine in the blood within the physiological norm during the period from the first to the third day could be caused due to damaged muscle fibers and toxic effects of the decay of anesthesia components. Subsequently, the level of creatinine decreased to normal.

The rates of calcium and phosphorus ranged within the limits of the presurgical values that were associated with a low degree of bone trauma and slight lysis of bone fragments. In the first group, the level of calcium prior to the surgery was 2.1 ± 0.1 mmol/l; a day after the surgery, 2.2 ± 0.1 mmol/l; 3 days after, 2.2 ± 0.1 mmol/l; and 30 days after, 1.9 ± 0.1 mmol/l; the phosphorus level was 1.0 ± 0.1 mmol/l, 1.2 ± 0.1 mmol/l, 1.2 ± 0.1 mmol/l, and 0.9 ± 0.1 mmol/l, respectively. In the second group, the level of calcium prior to the surgery was 2.2 ± 0.1 mmol/l; a day after the surgery, 2.4 ± 0.1 mmol/l; 3 days after, 2.3 ± 0.1 mmol/l; and 30 days after, 2.2 ± 0.1 mmol/l; the level of phosphorus was 1.0 ± 0.1, 1.1 ± 0.1, 1.1 ± 0.1, and 1.0 ± 0.1 mmol/l, respectively.

Given that surgical approach was minimally invasive, fluctuations in the level of urea were insignificant and ranged from 5.3 ± 0.4 to 6.0 ± 0.2 mmol/l in the control group, while in the experimental group, from 5.6 ± 0.9 to 7.6 ± 0.2 mmol/l.

According to the literature, normally, C-reactive protein is not detected in animal serum or is detected in small amounts. In our study, C-reactive protein detected in the animals of the control group a day after the surgery amounted to 30.6 ± 9.2 mg/l; 3 days after, 33.6 ± 1.8 mg/l; and by the end of the experiment, 35.6 ± 5.2 mg/l, whereas in the animals of the experimental group proper figures corresponded to 19.9 ± 4.3, 23.5 ± 2.6, and 3.4 ± 0.7 mg/l, respectively.

Conclusions

1. The analysis of the functional condition of dermal fibroblasts, cultivated on titanium workpieces with the coated polymer film and propolis at different concentrations, has shown that for the formation of nontoxic biofunctional coating on the surface of implant material, it is necessary to use the following agents at the respective concentrations: propolis at the concentration of 1.25 mg/ml on the active substance and polymer at the concentration of not more than 0.0001%. The neutrality of the samples is ensured by the lack of damaged cells in culture and by the capability of cells to adhere to the experimental substance, namely, polyazolidine ammonium modified by the hydrate flavonoids halogen ions.
2. The absence of inflammatory complications in animals of the experimental group in the early postsurgical period (3 and 7 days after surgery) and the need for considerable efforts to extract the implants at the end of the experiment can serve as evidence of the biointegration of the studied implant coatings that have passed thermal oxidation and have been modified by nanoaggregates of flavonoids.
3. Experimental implants do not exert inhibitory action on both erythropoiesis and leukopoiesis, since fluctuations in such indicators as the total number of erythrocytes, hemoglobin, and total leukocytes were minor and consistent with the staging of osteoreparative process. At the same time, it should be noted that in the experimental group, these figures reached presurgical values earlier (14 days after the surgery).
4. The lack of sharp fluctuations in the level of calcium and phosphorus; the dynamics of bilirubin, creatinine, and urea within the physiological limits; as well as the recovery of the initial level of AST, ALT, and C-reactive protei in animals of the experimental group at the early stages testify to the lack of toxic influence of implants with thermally oxidized surface treated by induction thermal method and modified by nanoaggregates of flavonoids.

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