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FORMATION OF EYEBALL ORBITAL STUMP USING TITANIUM NICKELIDE TISSUE-ENGINEERED CONSTRUCT AND AUTOLOGOUS BLOOD MONONUCLEAR LEUKOCYTES

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Objective: to study the morphological features of formation of the eyeball orbital stump using a titanium nickelide tissue-engineered construct and a suspension of autologous blood mononuclear leukocytes *in vivo*. **Materials and methods.** Experiments were performed on 54 sexually mature Wistar rats weighing 200–250 g. The animals were divided into 3 groups, depending on type of surgical intervention: group 1 (n = 18) consisted of animals in which eyeball orbital stump was formed after evisceroenucleation through implantation of a titanium nickelide tissue-engineered construct and a suspension of autologous blood mononuclear leukocytes in the scleral sac; group 2 (n = 18) – the eyeball orbital stump was formed through implantation of titanium nickelide tissue-engineered construct in the scleral sac; group 3 (n = 18) – orbital stump was formed using an Alloplant implant. **Results.** It was established that in group 1 rats, on day 7 following surgery, the specific volume of connective tissue was 7.9 times ($p_U = 0.048$) higher than in group 2 rats and 15.8 times ($p_U = 0.039$) higher than in group 3 rats. On day 14 after surgery, the volume of connective tissue in the eyeball orbital stump of group 1 rats reached the highest value compared to that in the other groups. The numerical density of newly formed vessels in the eyeball orbital stump of group 1 rats, starting from day 14 after surgery up to the end of experiment (day 21), was statistically significantly higher than that in the other groups. Moreover, on day 21, this indicator was 4.0 times ($p_U = 0.001$) higher in group 1 rats than in group 2 rats and 9.8 times ($p_U = 0,0003$) higher than in group 3 rats. **Conclusion.** Implantation of titanium nickelide tissue-engineered construct and a suspension of autologous blood mononuclear leukocytes into the scleral sac after evisceroenucleation in an *in vivo* experiment leads to accelerated maturation of the connective tissue and intensive vascularization in the eyeball orbital stump. This ensures strong fixation of the implant and reduces risk of rejection.

Keywords: blood mononuclear leukocytes, eyeball orbital stump, tissue-engineered construct, titanium nickelide, cell technology.

INTRODUCTION

Due to the achievements of up-to-date ophthalmology, the treatment of various serious diseases of the visual organ is becoming more effective. However, despite all the ongoing therapy measures, it is impossible to save the eyeball as an organ [1, 2]. It should be noted that about 75% of enucleations are performed without the formation of a musculoskeletal stump and implantation of an orbital insert [2, 3]. This, in turn, leads to the development of anophthalmic syndrome. The clinical pattern of this complication is featured with the sunken orbital-palpebral sulcus, eyelid deformity, ptosis, and an incomplete closure of the palpebral fissure [1, 4]. Treatment of anophthalmic syndrome is a time-consuming and multi-stage process aimed at restoring the volume of the conjunctival cavity and other anatomical structures, as well as restoring the orbital tissue volume deficiency through implanting an inert, biocompatible material [1, 3, 5]. Several materials are offered for use as an orbital implant. Some of them, cartilage, hydroxyapatite, and carbon composites are widely used in orbital surgery, others, e.g. tantalum, ceramics, injection hydrogel, monolithic silicone have limited use for their high cost and high adverse event rate [1, 3, 5].

At present, ophthalmic surgeons show increasing interest in porous materials with the structure providing a sufficiently rapid ingrowth of surrounding tissues, thus contributing to the strong fixation of the implant in orbit. Still, when using porous materials, especially in the long term, such complications as the implant exposure, its infection and rejection cannot be excluded. A possible solution to the problem is the use of cell technology during orbital implantation [6–12].

Purpose: to study the morphological features of formation of the eyeball orbital stump using a titanium nickelide tissue-engineered construct and a suspension of autologous blood mononuclear leukocytes *in vivo*.

MATERIALS AND METHODS

Experiments were performed on 54 sexually mature Wistar rats weighing 200–250 g (54 eyes).

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Experiments were performed on 54 sexually mature Wistar rats weighing 200–250 g (54 eyes) from the vivarium of the FGBOU VO SibGMU (Siberian State Medical University) of the Ministry of Health of Russia. Before the experiment, all animals were quarantined for a week under vivarium conditions in the usual food regime. The experimental studies protocol was approved by the local ethics committee of the GOU VPO SibGMU of Roszdrav (Russian Federal Service on Surveillance in Healthcare) of November 29, 2010, Reg.No.1715.

The animals were divided into 3 groups, depending on type of surgical intervention: group 1 (n = 18) consisted of animals in which eyeball orbital stump was formed after evisceroenucleation through implantation of a titanium nickelide tissue-engineered construct and a suspension of autologous blood mononuclear leukocytes in the scleral sac; group 2 (n = 18) included rats with the eyeball orbital stump formed through implantation of titanium nickelide tissue-engineered construct in the scleral sac; group 3 (n = 18) consisted of animals with the orbital stump formed using an Alloplant implant.

The titanium nickelide implant is made of porous titanium nickelide filament TN-10 of 100 μm thickness (Certificate of conformity No. РОСС RU ЛЯ79НО93 37 of 15.04.2011) [13], rounded, 4–5 mm in diameter. The implant is produced in the facilities of the Scientific Research Institute of Medical Materials (supervised by Professor V.E. Gyunter, Doctor of Technical Sciences).

The Alloplant biomaterial implant is manufactured by the FGU VTsGiPKh (Russian Center for Ophthalmic and Plastic Surgery of the Ministry of Health of Russia, Ufa, Russian Federation) of subcutaneous fat of human sole, rounded, 5 mm in diameter.

Mononuclear leukocytes from the experimental animals' blood were isolated immediately prior to surgery by fractionation on a ficoll-verographin separation solution (1.067–1.077 g/ml density). The cells purity was 96–98%, the percentage of stained (dead) cells was 1.5–2%, not exceeding the allowed (no more than 3%) limit.

Under operating conditions with etherization, all animals of 3 groups (54 rats) underwent evisceroenucleation of an eye, followed by placement of the corresponding implant in the scleral sac of the eyeball. Group 1 animals (18 rats) were injected with 0.1 ml of a freshly isolated autologous mononuclear blood leukocytes suspension (cell density: 200 thousand cells / 1 ml) in the structure of the titanium nickelide implant. All animals of 3 groups were postoperatively instilled with Tobramycin solution (6 times a day) in the conjunctival cavity of the operated eye.

The experiment lasted 21 days. During the experiment, on the 1st, 3rd, 7th, 14th, 21st days after the operation, an external examination was made, biomicroscopy of the operated eyes to assess the condition of the palpebral conjunctiva and the eyeball stump, of the edges of the surgical wound and sutures, as well as the presence and nature of the discharge in the conjunctival cavities.

On days 7, 14, and 21 after surgery, six rats from each group were removed from the experiment with the operated eyeball stumps removed, the obtained material fixed and hematoxylin-eosin stained by van Gieson for 200-, 300- and 400x light microscopy. At all stages of the experiment, the experimental animals were euthanized in compliance with the EU rules and norms (86/609EEC) and Helsinki Declaration.

Data processing

In slices morphometry with the ImageJ 1.50i software, mono- and polynuclear leukocytes, plasmocytes were counted, the volume of the stroma and the numerical percentage density of the newly formed vessels were determined. The results were statistically analyzed with the IBM SPSS Statistics 20 statistical package. The normality of distribution of indicators was checked by Kolmogorov–Smirnov test. The variables with a normal distribution were analyzed with Student's t-test. The results are presented in the M ± m form, where M is the arithmetic mean value, m is SEM. If the data distribution did not correspond to the normal distribution law, a nonparametric criterion was used, Mann–Whitney test (p_U). Differences were considered statistically significant at $p < 0.05$.

RESULTS

One day after the operation, the external examination of the animals of all 3 groups (54 rats) showed moderate edema and hyperemia of the eyelid's conjunctiva and the operated eyeball stump, which gradually decreased by the 3rd to the 4th days. The formed eyeball stump in all experimental rats (100%) was rounded and moderately mobile. Biomicroscopy showed adapted edges of the surgical wound in the suture area, and a small amount of mucous discharge was found in the conjunctival cavity of the operated eyes in rats. Throughout the experiment (21 days), no cases of exposure or rejection of an implant placed in the scleral sac of the operated eyeball were revealed in animals of all 3 groups.

According to light microscopy, in animals of the group 1 (6 rats), on the 7th day after evisceroenucleation and implantation of a tissue-engineering titanium nickelide construct and a suspension of autologous mononuclear blood leukocytes in the eyeball stump, extensive accumulations were found in the scleral sac of the mononuclear leukocytes (6259.0 ± 1646.0 cells per 1 mm^2 section) (Fig. 1), a small number of plasmocytes (443.6 ± 200.5 cells per 1 mm^2 section) and single polymorphonuclear leukocytes (PML) (344.9 ± 165.1 cells per 1 mm^2 section). Around the titanium nickelide implant, multiple focal accumulations of immature fibroblasts were detected. Thin collagen fibers were moderately edematous,

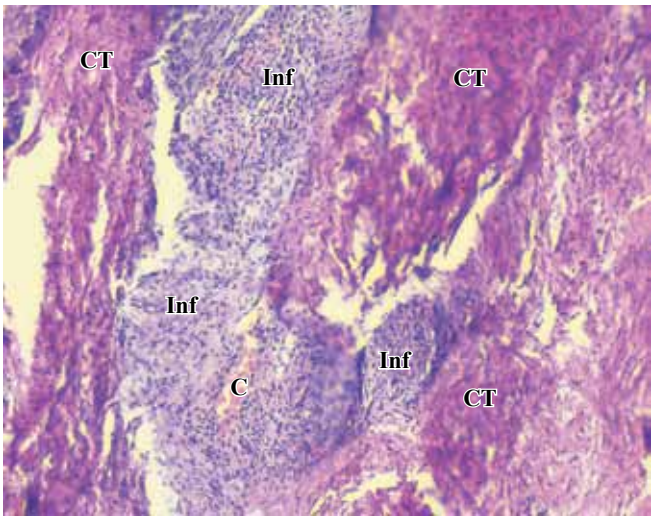

Fig. 1. Growth of loose fibrous connective tissue, focal mononuclear infiltration and newly formed vessels in the orbital stump of the 1st group of animals on the 7th day after evisceroenucleation with implantation of a tissue-engineering construction from titanium nickelide and suspension of autologous blood mononuclear cells. CT – connective tissue, Inf – cell infiltration, C – vessels. Stained with hematoxylin and eosin. ×400

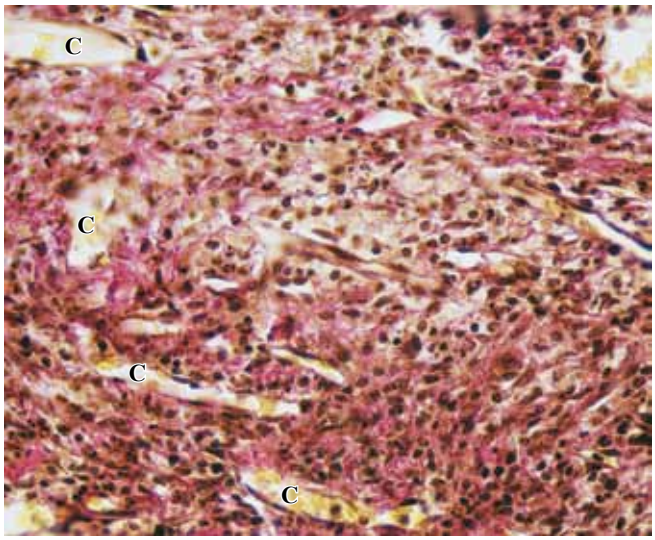

Fig. 2. Diffuse lymphocytic leukocyte infiltration and single newly formed vessels in the orbital stump of the 2nd group of animals on the 7th day after evisceroenucleation with implantation of titanium nickelide. C – vessels. Stained with hematoxylin and pikrofuksin by the method of Van-Giezon. ×300

Table

Quantitative ratio of stroma and newly formed vessels in 1 mm² section of the eyeball stump in animals, depending on the type of implant, M ± m, p_U

Experiment days	Stroma volume, % (n = 10)			Blood vessels numerical density, % (n = 10)		
	Group 1	Group 2	Group 3	Group 1	Group 2	Group 3
Day 7	0.158 ± 0.1	0.02 ± 0.01	0.01 ± 0.005	0.04 ± 0.027	0.02 ± 0.014	0.01 ± 0.006
Day 14	95.0 ± 2.1 p_1 = 0.0002; p_2 = 0.0001	0.3 ± 0.14	0.2 ± 0.11	5.0 ± 2.1 p_1 = 0.04; p_2 = 0.03	0.04 ± 0.03	0.02 ± 0.01
Day 21	79.1 ± 3.4 p_2 = 0.0005	94.7 ± 1.9	5.3 ± 1.9	21.6 ± 3.1 p_1 = 0.001; p_2 = 0.0003	5.3 ± 1.9	2.2 ± 1.2

Note. p_1 – significance level of differences compared with the data of group 2; p_2 – significance level of differences compared with the data of group 3; M – sampling mean, m – error of mean.

loosely located. Newly formed vessels were found between the fibers (Table, Fig. 1).

In animals of the group 2 (6 rats) on the 7th day after evisceroenucleation and titanium nickelide construct implantation in the scleral sac in the cavity of the eyeball stump, diffuse, uniform lymphocytic leukocyte infiltration (1916.6 ± 495.3 cells per 1 mm² section), a small number of immature fibroblasts, and edematous collagen fibers loosely located around the implant were detected. Among the fibers, single newly formed vessels were revealed (Table, Fig. 2).

In animals of the group 3 (6 rats), on the 7th day after evisceroenucleation and Alloplant biomaterial construct implantation in the scleral sac, the cavity of the eyeball stump was filled with adipose tissue interrupted by single mononuclear leukocytes (111, 5 ± 41.8 cells per 1 mm² section), a small PML amount (9.6 ± 4.8 cells in 1 mm² section), thin collagen fibers (Fig. 3) and single newly formed vessels were found (Table).

On the 14th day after the operation, in animals of group 1 (6 rats), there was an extensive proliferation of fibrous connective tissue in the cavity of the eyeball stump. Collagen fibers were arranged more orderly than on the 7th day. Accumulations of mononuclear leukocytes were detected between the fibers (9093.8 ± 891.0 cells per 1 mm² section). Around the titanium nickelide implant, newly formed vessels were found (Table), most of which began to differentiate into arterioles and venules.

In animals of group 2 (6 rats), on the 14th day after the operation, in the cavity of the eyeball stump, there was an overgrowth of loose fibrous connective tissue with significant edema and moderate lymphocytic macropha-

ge infiltration (4744.0 ± 928.0 cells per 1 mm^2 section). Thin-walled capillaries were found between collagen fibers, single arterioles and venules appeared (Table).

In animals of group 3 (6 rats), on the 14th day after the operation, in the cavity of the eyeball stump there was a slight lymphocytic macrophage infiltration (103.6 ± 49.4 cells per 1 mm^2 section) and the proliferation of thin collagen fibers between the fat segments of the Alloplant biomaterial implant. Around the implant, single newly formed vessels were found (Table).

On the 21st day after the operation, mature connective tissue was found in the animals of group 1 (6 rats) in the cavity of the eyeball stump. Thick bundles of collagen fibers were arranged compactly and orderly (Fig. 4). Small focal accumulations of mononuclear leukocytes

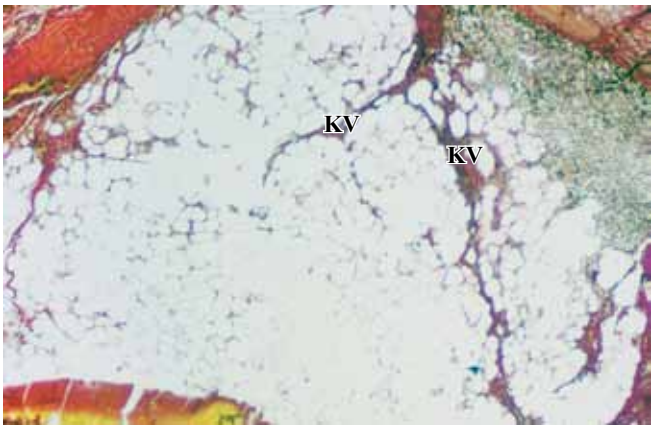

Fig. 3. Thin, single collagen fibers between the segments of adipose tissue in the orbital stump of the 3rd group of animals on the 7th day after evisceroenucleation with implantation of biomaterial "Alloplant". KV – collagen fibers. Stained with hematoxylin and eosin. ×200

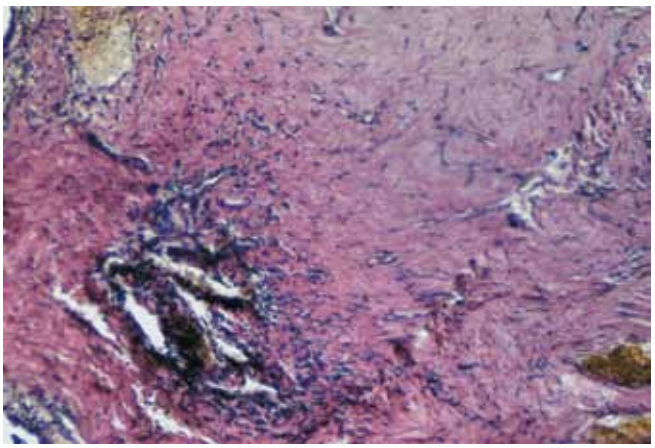

Fig. 4. Orderly arranged bundles of collagen fibers in the orbital stump of the 1st group of animals on the 21st day after evisceroenucleation with implantation of a tissue-engineering construction from titanium nickelide and suspension of autologous blood mononuclear cells. Stained with hematoxylin and pikrofuksin by the method of Van-Giezon. ×300

(4386.3 ± 498.1 cells per 1 mm^2 section) and a large number of newly formed vessels were found around the titanium nickelide implant (Table).

In animals of group 2 (6 rats), on the 21st day after the operation, a loose connective tissue with thin collagen fibers disordered around the titanium nickelide implant was found in the cavity of the eyeball stump. Between bundles of collagen fibers, diffuse mononuclear infiltration (2020.6 ± 562.8 cells per 1 mm^2 section) and a small number of newly formed vessels were found (Table, Fig. 5).

In animals of group 3 (6 rats) on the 21st day after the operation, adipose tissue was detected in the cavity of the eyeball stump, insignificant mononuclear infiltration was observed between the lobules (106.1 ± 43.5 cells per 1 mm^2 section) together with loose connective tissue (Fig. 6). Around the Alloplant biomaterial implant, single moderately full-blooded vessels were found (Table).

According to morphometry, in the cell composition of the eyeball stump in rats of all 3 groups throughout the experiment (21 days), mononuclear leukocytes population prevailed. Moreover, in animals of the 1st group with an implant from a tissue-engineering construct, the cells number in this population during the experiment was statistically significantly higher than that in animals of the remaining groups.

Indeed, on the 7th day after the operation, the number of mononuclear leukocytes in the eyeball stump in rats of the group 1 was 3.3 times ($p_U = 0.034$) higher than in animals of group 2 with the titanium nickelide implant and 56.1 times ($p_U = 0.0002$) higher in animals of group 3 with the Alloplant biomaterial implant. On the 14th

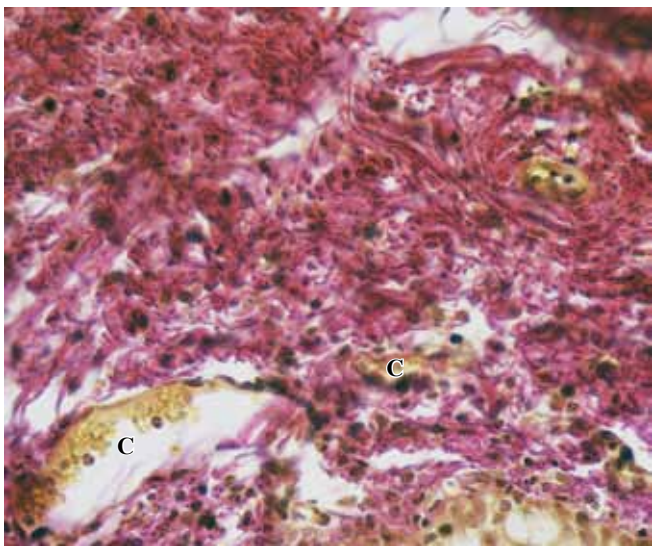

Fig. 5. Loosely arranged bundles of collagen fibers and moderately full-blooded vessels in the orbital stump of the 2nd group of animals on the 21st day after evisceroenucleation with implantation of titanium nickelide. C – vessels. Stained with hematoxylin and pikrofuksin by the method of Van-Giezon. ×400

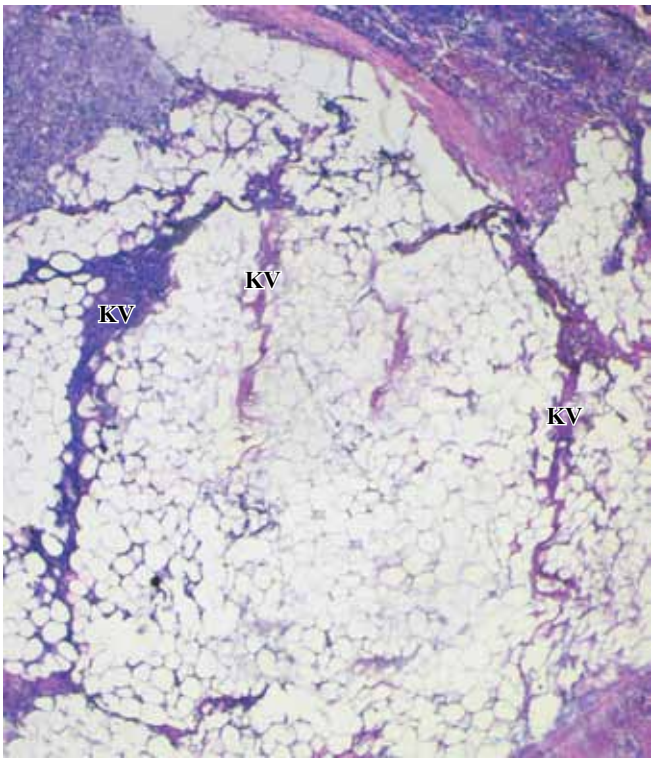

Fig. 6. Loose collagen fibers between the segments of adipose tissue in the orbital stump of the 3rd group of animals on the 21st day after evisceroenucleation with implantation of biomaterial "Alloplant". KV – collagen fibers. Stained with hematoxylin and eosin. ×200

day, the number of cells of this population in the eyeball stump in rats of the group 1 exceeded that in rats of group 2 by 1.9 times ($p_U = 0.01$), while in rats of group 3 – by 87.7 times ($p_U = 0.0001$), on the 21st day – 2.2 times ($p_U = 0.02$) and 41.3 times ($p_U = 0.0002$), respectively. The discovered regularity is explained both by the direct introduction of a suspension of autologous mononuclear leukocytes of blood into the tissue-engineering structure during the formation of the eyeball stump in group 1 rats and by the additional migration of cells of this population due to the inducing effect of exogenously introduced mononuclear cells.

The specific volume of connective tissue in the eyeball stump in animals of group 1 with the tissue-engineering implant, from the 7th day after the operation and throughout the experiment, has also been statistically significantly higher than that in animals of the other groups. On the 7th day the operation, this indicator in rats of group 1was 7.9 times ($p_U = 0.048$) higher than in animals of group 2 with a titanium nickelide implant and 15.8 times ($p_U = 0.039$) higher than in animals of group 3 with the Alloplant biomaterial implant (Table). On the 14th day after the operation, the volume of connective tissue in the eyeball stump in animals of group 1 reached the highest value compared to that in animals of other groups.

On the 21st day after the operation, in animals of group 1, morphometry revealed decrease in the stroma volume of the eyeball stump by 1.2 times ($p_U = 0.0019$) compared with the value on the 14th day (Table), which is due to the maturation of connective tissue in the eyeball stump. In animals of group 2, on the contrary, the stroma volume of the eyeball stump on the 21st day reached the highest value both in comparison with the initial data and in comparison with those in the rats of the other groups (Table). However, in the connective tissue of the eyeball stump, all animals of group 2 (6 rats) under light microscopy showed signs of immaturity. In animals of group 3, although the stroma volume of the eyeball stump on the 21st day after the operation exceeded that on the 14th day by 26.5 times; nevertheless, its level was of the least importance compared to rats of the other groups (Table).

The numerical density of newly formed vessels in the eyeball stump in animals of group 1, from the 14th day after the operation and till the end of the experiment (21st day), was statistically significantly higher than that in rats of the other groups (Table). Moreover, on the 21st day, in rats of group 1, this indicator was 4.0 times ($p_U = 0.001$) higher than in animals of group 2 and 9.8 times ($p_U = 0.0003$) higher than in animals of group 3 (Table).

DISCUSSION

Mononuclear leukocytes of blood, due to the synthesis and secretion of a large number of cytokines (interleukins – IL-1α, IL-1β, IL-6, IL-10, tumor necrosis factor, and vascular endothelial growth factor) are known to inspire the migration of mononuclear cells, PML and fibroblasts, accelerate proliferation of fibroblasts and endotheliocytes, affect the complement system and collagen production [14, 15, 16]. Probably, due to the functional cooperation of mononuclear leukocytes exogenously introduced into the structure of a titanium nickelide implant placed in the rat eyeball stump and additionally migrating cells under their influence which ensure the development of an inflammatory-reparative reaction, and the rapid change in cell phases occurs accelerating the transition of inflammation to the repair phase [7, 15]. At the same time, there is a rapid (within 21 days) maturation of connective tissue in the of the stump and accelerated neovasculogenesis starting at the 14th day.

It should be noted that the use of titanium nickelide as the basis of the implant, due to the porous structure of the material, significantly facilitates the germination of the implant with fibrovascular tissue. This, in turn, ensures its strong retention in the stump cavity of the eye of the experimental animal and significantly reduces the risk of the implant exposure and rejection [15, 16]. During the *in vivo* experiment with a titanium nickelide implant (36 rats, 36 eyes) no complications (infection, implant rejection) were revealed in the postoperative period. According to published data [1, 2, 4], the risk of

postoperative complications with orbital implants from various synthetic materials varies from 4 to 38%.

In addition, the titanium nickelide implant, due to the elastic properties of the material, provides a stable form of the eyeball stump.

CONCLUSION

Implantation of titanium nickelide tissue-engineered construct and a suspension of autologous blood mononuclear leukocytes into the scleral sac after evisceroenucleation in an *in vivo* experiment leads to accelerated maturation of the connective tissue and intensive vascularization in the eyeball orbital stump. This ensures strong fixation of the implant and reduces risk of rejection. The results are promising for further clinical studies.

The authors declare no conflict of interest.

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