
MATERIALS FOR ENSURING HUMAN LIFE ACTIVITY
AND ENVIRONMENT PROTECTION

A Biomedical Cell Product for the Regeneration of Articular Cartilage: Biocompatible and Histomorphological Properties (an Experimental Model of Subcutaneous Implantation)

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Abstract—An in vivo study of the biological safety of a biomedical cell product for regeneration of articular cartilage with subcutaneous implantation of cell-engineered cartilage tissue was carried out. A comparative study of the biological safety of the cell-engineered construct of human articular cartilage tissue (CEC hAT) and the biocompatible BMCH matrix in an implantation test did not show significant differences between the control and experimental groups. The results demonstrated that the cell component of the cell-engineered construct of hAT is not a risk factor for its implantation for up to 28 days. During the study, some signs of formation of articular tissue were observed at the site of subcutaneous implantation of the CEC hAT. Considering high proliferation and differentiation activity of the CEC hAT previously confirmed after its cultivation for 42 days, it is possible to suggest the potential of using CEC hAT for regeneration of injured cartilage as an injection form of biomedical cell product.

Keywords: microstructured gel, collagen-content matrix, human adipose tissue-derived mesenchymal stromal cells, chondrogenic differentiation, tissue-engineered construct, articular cartilage, subcutaneous implantation, biocompatibility

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INTRODUCTION

Osteoarthritis (OA) is a disease of peripheral and/or central (vertebral) joints with destruction of articular cartilage, degenerative changes in epiphyses of articulating bones, and formation of subcartilaginous osteocystomas and marginal osteophytes [1]. The disease is based on attrition, thinning, and loss of articular cartilage with its amortization function being lost. At later stages of the disease, degenerative-dystrophic changes could appear in the subcartilaginous bone tissue near the joint. The disease often leads to a significant decrease in the quality of life and disability of the patient, in particular, if hip and knee joints are damaged. This is due to restriction of the joint movement and sharp pain upon movement or activity.

The incidence of osteoarthritis in Russia occupies a leading position among the diseases of joints, and the disease is found in almost all age groups [1]. The prevalence of OA in Russia is more than 20 per 1000 people in the age group of 18 years and older. Every year in Russia, about 600 000 new cases of OA are recorded. It has been predicted that by 2020 the incidence of OA in

the population could reach 57%, a tendency toward an increase in the incidence of the disease being observed in the age group younger than 45 years.

Independently of the etiology of the disease, the damage of the cartilaginous tissue of joints is the main factor in the pathogenesis of OA. The absence in articular cartilage of blood supply and a low level of metabolism due to a small number of cells per unit volume of the tissue result in complete reparative regeneration of the cartilage being possible only if there are small areas of damage [2].

The treatment mode of the cartilage based on transplantation of autologous cartilage corpuscles has been widely applied in clinical practice [3]. However, this method has drawbacks, the main ones being traumatic biopsy of a healthy region of the cartilage; the possibility of dedifferentiation of cartilage corpuscles in vitro, including fibroblast-like rearrangement [4]; and incomplete restoration of the cartilaginous tissue. In connection with this, as an alternative to this method, variants of replacement of cartilage corpuscles by mesenchymal stromal cells (MSC), which are present in all organs and tissues of the human body and

have multilinear potential of differentiation in adipogenic, osteogenic, and chondrogenic lineages, were considered [5].

One of the promising approaches is stimulation of restorative processes of the cartilage damaged by introduction in situ of a biomedical cell product in the form of CEC hAT including the following components [6]:

- mesenchymal stromal cells isolated from endogenous sources in the patient (autologous cells) or from donors (allogenic cells) capable of formation of functioning extracellular matrix (ECM);

- an appropriate biocompatible biodegradable carrier (matrix, scaffold) for cell transplantation and temporary bioartificial matrix;

- bioactive molecules (cytokines, growth factors) having a biostimulating effect on the cells of the damaged tissue.

Adipose tissue (AD) is of greatest interest as a source of MSCs owing to the simplicity of cell isolation technology, sufficient cell yield, and minimal traumatism for the patient. It has been shown that AD MSCs are adhesive to the surface of a culture plastic and fibroblast-like; in regard to a set of surface antigens, they correspond to the MSC phenotype and are capable of multilinear differentiation into mesenchymal lineages [7, 8].

Recently, to obtain a tissue equivalent of cartilaginous tissue, tissue-engineered constructs using different three-dimensional polymer matrices have been developed [9, 10].

Earlier, we performed comparative studies of restorative capacity of human adipose-tissue-derived MSCs (hAT MSCs) with a different degree of chondrogenic differentiation (monolayer, 3D culture (microspheres), and undifferentiated MSCs) upon cultivation on a BMCH matrix [14]. The matrix for the CEC AT was chosen to be represented by an injection form of a biopolymer microstructured collagen-containing hydrogel (BMCH) from a range of the heterogeneous composition of the implantable gel Sphero®GEL (ZAO Biomir Service, Krasnoznamensk, Russia) registered within the territory of Russia. The implantable material Sphero®GEL is produced from extracts of embryonic and postnatal tissues of animal origin [11] and represents a unique complex of almost all high- and low-molecular components of extracellular matrix, providing its high biocompatible and biostimulating properties. An injection form of Sphero® GEL is extremely convenient for development of minimally invasive technologies in replacement and regenerative surgery [12, 13].

It was shown that the greatest proliferative and differentiation potential of hAT MSCs in vitro was observed upon their chondrogenic differentiation directly during cultivation in the BMCH matrix. On the basis of the results obtained, the optimal composition of the biomedical cell product CEC AT hAT MSC

was chosen in the second and third passages seeded into the chondrogenic medium on the BMCH matrix at a concentration of 2×10^6 cells/mL of hydrogel. After cultivation of the CEC AT with the composition chosen for 42 days under standard conditions, a significant increase in the cell mass, cell invasion into the BMCH, and an increase in the amount of the ECM produced by the cells were observed. Histochemical and immunohistochemical analyses revealed a significant amount of type II collagen inherent in the cartilaginous tissue and active synthesis of glycosaminoglycans (GAG). In some preparations, lacune-like areas, groups of several cells surrounded by the ECM resembling isogenous cell groups of the native cartilage, were found. We note that we did not succeed in obtaining viable cell-engineered constructs of the cartilaginous tissue using as a cell component hAT MSCs predifferentiated in chondrogenic lineage in a monolayer or 3D culture by a microsphere method [14, 15]. The results obtained coincided with data of other researchers. In [16], it was shown that hAT MSCs cultivated in a monolayer were not capable of GAG synthesis even in the presence of chondrogenic induction medium. In [17], it was found that MSCs cultivated in a collagen hydrogel produced a greater amount of GAG than upon cultivation of the cells in the form of high density three-dimensional aggregates. Moreover, direct differentiation of MSCs in a hydrogel makes it possible to obtain lacune-like areas typical of cartilaginous tissue, in contrast to cell cultivation by the microsphere method.

Despite the demonstrated high biocompatible properties of the BMCH (Sphero®GEL), a matrix component of the cell-engineered construct for regeneration of articular cartilage, preclinical studies of the biomedical cell product CEC hAT require the necessary evaluation of its biological safety.

The purpose of this study is to investigate in vivo the biological safety of the biomedical cell product for regeneration of articular cartilage upon subcutaneous implantation of the cell-engineered construct of cartilaginous tissue into experimental animals (male DBA mice).

MATERIALS AND METHODS

Production and Cultivation of hAT MSC

MSCs were obtained from samples of human subcutaneous adipose tissue according to the conventional method described earlier in [7]. Briefly, adipose tissue was washed from blood with phosphate buffered saline (PBS) supplemented by antibiotics and, then, minced to a homogeneous mass, which then was treated with a type IA collagenase solution (600 units/g) (Sigma, #C98891, United States). The enzyme was inactivated with growth medium (GM) for human MSCs, MesenPRO RS™, without glutamine with growth supplements MesenPRO RS™ Growth Supplement

(Gibco® by Life Technologies™, United States). The cells were precipitated by centrifugation for 15 min at 400g. The cellular pellet was filtered through a 70–100 µm mesh filters, resuspended in GM, and plated in culture flasks (Corning-Costar, United States). The cultivation was performed under standard conditions, at 37°C and in a $5 \pm 1\%$ CO₂ humid atmosphere. The cell culture medium was replaced every 3 days. The culture was visually assessed by an inverted microscope (Micros MC700, Austria). The hAT MSCs of the second and third passages were used for the experiments.

BMCH Matrix

A BMCH matrix as in previous studies in vitro [10] was represented by an injection form of a biopolymer microstructured collagen-containing hydrogel from a range of heterogeneous composition of implantable gel Sphero®GEL (Sphero®GEL trademark; registration certificate no. FSR 2012/13033 from February 1, 2012; ZAO Biomir Service, Krasnoznamensk, Russia). The injection form of the BMCH from the range of Sphero®GEL had the following characteristic properties: average microparticle size of 145.79 ± 0.09 µm; modulus of elasticity of 1170 ± 12 Pa; viscosity modulus of 62.9 ± 7.9 Pa; a swelling factor not lower than 86.6 ± 3.0 wt %; and time of bioresorption of the BMCH of up to 9 months.

CEC hAT Samples for Introduction to Laboratory Animals

To obtain a CEC of the human cartilaginous tissue, hAT MSCs were taken from a culture plastic by trypsinization and mixed layer by layer in 15 mL sterile centrifuge tubes (Corning-Costar, United States) with the BMCH matrix heated to 37°C for 30 min at hydrogel concentration of 2×10^6 cells/mL. Two hours later, chondrogenic induction medium (STEMPRO® Chondrogenesis Differentiation Kit (Invitrogen, United States)) was added to the tubes. Then, the CEC AT was incubated in the chondrogenic induction medium for 7 days at 37°C in a humid atmosphere containing $5 \pm 1\%$ CO₂. Before implantation, the CEC AT was carefully washed with a Hanks solution (Paneko, Russia) to remove the differentiation medium.

Subcutaneous Implantation of the CEC AT in Laboratory Animals

All the experiments with animals (male DBA mice) were in accordance with principles of humane treatment of animals as provided by international recommendations and observing the bioethical standards and requirements of the International Committee on Science.

Animals and their keeping. Male DBA mice were obtained on October 3, 2012, from the Andreevka farm of laboratory animals of the Scientific Center for Biomedical Technologies of the Russian Academy of Medical Sciences with veterinary certificate no. 0329456. The mice were kept under standard conditions in polycarbonate cages. The temperature and humidity of the room were recorded and documented once a day. The animals were acclimated to the laboratory vivarium for 14 days. During this period, all the animals remained healthy.

Experimental conditions. The CEC AT was implanted in the male DBA mice subcutaneously in the region of the midline of the back. The experiment duration was 28 days.

Formation of groups and procedures with animals. The animals were distributed into groups randomly. Body weight was the criterion, so that an individual value of the weight would not differ from the average value more than by 10%. The following groups were formed: a control group ($n = 7$) with the BMCH implanted and experimental group ($n = 10$) with the CEC hAT implanted.

A day before the beginning of the study, immunocompetent mice of both groups were depressed using a Sandimmun immunosuppressive preparation (content of active substance of Cyclosporine of 50 mg/mL) at a dose of 5 mg/kg and deprived of food.

The implantation was performed using general anesthesia provided by intramuscular injections of a Zoletil veterinary preparation (registration number PVI-2-1.9/01425, France) at a dose of 15 mg/kg.

The surgical procedure was performed under sterile conditions. The hair coat in the region of the surgery was removed by shaving. This region was treated with an antiseptic solution (70% alcohol solution). The CEC AT and biopolymer heterogeneous hydrogel without cells were implanted in the region of the midline of the back. The skin was cut, and a pocket was made by blunt dissection. The pocket base was located at a distance of more than 10 mm from the cutting line. One implant of 0.5 mL of the BMCH was put into the pocket. After the surgery, the wound was sutured using MONOCRYL® Plus resorbable suture material (Johnson & Johnson, United States).

During the study, each of the animals was observed every day with the following being noted:

- wound state (healing time; the presence of infection, hematoma, necrotic tissues; healing type; implant migration);

- habit view (hair coat, eyes, ears, limbs, teeth); condition and behavior (activity, gait, temperament, eating);

- physiological functions (breathing, salivation, urination, excreta).

During the experiment, the body weight was recorded once a week.

Study of the biological material. The animals were removed from the experiment in two stages, 14 and 28 days after the implantation. The mice were subjected to euthanasia using an excess of etherization after 18 h of starvation.

Blood for clinical analysis was sampled into special Yunivet tubes (Eiliton, Russia) with an EDTA anticoagulant. The analyses were performed on a Medonic hematology analyzer (Boule Medical A.B., Sweden) for veterinary medicine. Special serum-separating tubes with beads were used for biochemical analyses of the blood. The biochemical analyses were carried out by uniform methods on Stat fax 1904+ and Stat fax 4500+ biochemical photometers (Awareness Technology, United States) using UTS standard reagent kits (Unimed, Russia).

Indices of the blood clinical analysis were studied including red blood cell concentration (RBC), hematocrit (HCT), platelet concentration (PLT), hemoglobin concentration (HGB), and white blood cell concentration (WBC) taking into account differentiation in the three populations of lymphocytes (LYM), mid-sized leukocytes (MID), and granulocytes (GRAN).

Indices of the blood biochemical analysis were determined including alkaline phosphatase (AP) activity and content of glucose, triglycerides, urea, crude protein, and potassium.

An autopsy was carried out immediately after the death according to the full postmortem scheme. The animals were subjected to necropsy, including inspection of the outer surface of the body; injection place; and skull, thoracic, and abdominal cavities and their content.

Morphometric estimation of the parameters of the organs of the animals was carried out using a Vibra balance (Shinko Denshi CO Ltd, Japan) with further calculation of absolute (organ weight) and relative weight (organo-somatic index) of organs and their standard deviations.

For histological studies, tissue in the region of the implantation of the BMCH matrix and CEC hAT was taken and fixed for 4 h in a 10% formalin solution.

For all quantitative data, group means (M) and standard errors (m) were calculated. Statistical significance of differences was assessed according to Student's t -test (p). The difference was considered to be reliable at $p < 0.05$.

Histological and Immunohistochemical Methods of Estimation

Preparation of paraffin sections. Tissue samples taken in the region of implantation of the BMCH matrix and CEC hAT and fixed in the 10% formalin solution were washed in running water, dehydrated in ascending ethanol concentrations (70%, 80%, 90%, 96%, 100%), degreased in a mixture of absolute ethanol and chloroform or xylene, and embedded in paraf-

fin with beeswax being added. Cross sections with a thickness of 4–5 μm were obtained using a Leica microtome (RM 3255 model, Germany).

Staining of paraffin sections with hematoxylin and eosin, alcian blue, and according to the Mallory technique. The histological preparations were dewaxed and stained with hematoxylin and eosin, with alcian blue, and according to the Mallory technique using conventional methods.

Staining of paraffin sections against type II collagen. Visualization of staining of the preparations on human type II collagen was performed using a NovocastraTM Concentrated Peroxidase Detection System (RE7130-K, Leica Microsystems) according to the manufacturer's instructions. Before the staining, the dewaxed histological sections were subjected to retrieval by incubation in a 0.1% trypsin solution at 37°C for 30 min. After incubation with NovocastraTM Peroxidase Block, NovocastraTM Protein Block, optimally diluted primary antibodies against human type II collagen, secondary antibodies, and NovocastraTM Concentrated Streptavidin-HRP, the sections were washed twice in 0.5 M tris-buffered saline (TBS) (Sigma, United States) for 5 min at pH = 7.6. Then the sections were washed in distilled water, counterstained with NovocastraTM Hematoxylin (RE7107, Leica Microsystems), dehydrated in ascending ethanol concentrations, incubated with xylene, and mounted in Canada balsam. Reaction with peroxidase produces a visible brown precipitate at the antigen site.

Visual assessment of the histological preparations was performed using an Olympus CX41 optical microscope (Japan) at initial magnification $\times 200$. The preparations were photographed using a Nikon CoolPix 900 camera (Japan).

RESULTS AND DISCUSSION

Morphological Changes of CEC AT in vivo (Experimental Model of Subcutaneous Implantation)

Integral indices. Throughout the experiment, all the animals were alive. No deviations in the behavior (increased or decreased activity) and state of the mice of the experimental group (CEC hAT implantation) were observed when compared to the control group (BMCH matrix implantation). Breathing in the mice of the experimental group as in those of the control group had normal rhythm and was unhindered; salivation was without pathologies; and frequency, amount, and color of urine and consistency of fecal masses were within the physiological norm. The body weight of the mice throughout the investigation did not differ significantly ($p < 0.05$) between the groups (Table 1).

Assessment of wound healing. All the mice (except for one animal in the control group and one animal in the experimental group) had smooth viable tightly contacting wound edges, and necrotic and microflora nidi were absent, which indicates wound healing by

Table 1. Dynamics of a change in the body weight of mice ($M \pm m$, g)*

Group no.	Animal group	Animal weight, $M \pm m$, g				
		Background	7 days	14 days	21 days	28 days
1	Control (BMCH matrix)	23.2 ± 0.4 ($n = 10$)	24.5 ± 0.8 ($n = 9$)	23.3 ± 0.9 ($n = 9$)	26.0 ± 2.0 ($n = 5$)	27.0 ± 1.5 ($n = 5$)
2	CEC hAT	22.8 ± 0.3 ($n = 10$)	23.6 ± 0.6 ($n = 9$)	23.7 ± 0.4 ($n = 9$)	26.1 ± 1.0 ($n = 5$)	26.3 ± 0.6 ($n = 5$)

* There were no significant differences according to Student's *t*-test ($p > 0.05$) between indices of the control and experimental groups.

Table 2. Mean indices of clinical analysis of blood ($M \pm m$)*

Blood corpuscles	Period of the study, days	<i>n</i>	BMCH matrix	CEC hAT
Red blood cells, $10^{12}/L$	14	4	6.10 ± 0.17	5.90 ± 0.93
	28	5	6.60 ± 1.94	8.60 ± 0.74
Platelets, $10^9/L$	14	4	346.0 ± 42.4	303.6 ± 66.5
	28	5	407.5 ± 6.1	417.2 ± 5.2
Hematocrit, %	14	4	24.3 ± 1.9	22.3 ± 4.1
	28	5	33.1 ± 6.1	37.7 ± 3.5
Hemoglobin, g/L	14	4	111 ± 17	105.4 ± 17.9
	28	5	160.3 ± 1.5	145.6 ± 7.2
White blood cells (WBC), $10^9/L$	14	4	4.80 ± 0.16	4.70 ± 0.56
	28	5	11.9 ± 2.1	13.10 ± 2.23
Lymphocytes (LYM), %	14	4	76.5 ± 4.5	78.50 ± 1.35
	28	5	71.6 ± 7.5	68.80 ± 1.20
Monocytes (MID), %	14	4	9.7 ± 1.3	7.30 ± 1.55
	28	5	12.60 ± 2.85	13.10 ± 1.28
Granulocytes, %	14	4	12.8 ± 4.2	12.00 ± 1.36
	28	5	15.90 ± 4.65	18.10 ± 0.46

* There were no significant differences according to Student's *t*-test ($p > 0.05$) between indices on the 14th and 28th day of the experiment and between indices in the control and experimental groups.

primary intention. From three to four days after the implantation, the formation of a connective-tissue scar was observed and epithelization was almost completed. The two mice with poor wound healing mentioned (one in each of the groups) had inflammation at the site of the implantation, and tissue alterations prevailed in the wounds. The formation of granulation tissue was noted after 6–7 days. During the inflammation, the implants migrated outside. These animals were withdrawn from the experiment.

Clinical and Biochemical Analyses of the Blood

Table 2 gives averaged group results of the clinical analysis of the blood of the animals under study. The materials implanted did not affect hemopoiesis either 14 or 28 days after the implantation. On the 14th day of the study, the mice were found to have a significant decrease in the number of white blood cells, which

could be accounted for by preimplantation immunosuppression.

Table 3 gives the averaged data of some indices of the biochemical analysis of the blood serum. No reliable differences in the indices of enzymatic, protein, lipid, and carbohydrate metabolisms were found in the experimental group of the animals when compared to the control group.

Postmortem Data

Macroscopic study. According to the results of the macroscopic investigation of the organs studied taken 14 and 28 days after the implantation, there were no differences found between the groups.

The animals upon autopsy had clean skin and the subcutaneous tissue was developed moderately. The internal organs were arranged correctly. There was no free fluid found in the pleural and abdominal cavities.

Table 3. Mean values of biochemical analysis of blood ($M \pm m$)*

Index	Period of the study, days	<i>n</i>	BMCH matrix	CEC hAT
Urea, mmol/L	14	4	4.00 $\pm$ 0.95	4.80 $\pm$ 0.09
	28	5	4.2 $\pm$ 0.8	5.50 $\pm$ 0.12
AP, U/L	14	4	511.6 $\pm$ 67.6	526.7 $\pm$ 27.1
	28	5	593.2 $\pm$ 149.2	467.0 $\pm$ 58.8
Crude protein, g/L	14	4	56.5 $\pm$ 1.1	54.4 $\pm$ 1.9
	28	5	56.0 $\pm$ 1.6	56.0 $\pm$ 2.3
Triglycerides, mmol/L	14	4	2.00 $\pm$ 0.61	1.70 $\pm$ 0.17
	28	5	2.10 $\pm$ 0.46	2.1 $\pm$ 0.3
Glucose, mmol/L	14	4	7.70 $\pm$ 0.18	7.00 $\pm$ 0.91
	28	5	8.60 $\pm$ 1.09	8.70 $\pm$ 0.30
Potassium, mmol/L	14	4	8.8 $\pm$ 1.4	10.90 $\pm$ 0.47
	28	5	10.4 $\pm$ 0.2	9.70 $\pm$ 0.86

* There were no significant differences according to Student's *t*-test ($p > 0.05$) between indices on the 14th and 28th day of the experiment and between indices in the control and experimental groups.

Table 4. Average group indices of the weight of internal organs and OSI*

Organs	Weight of internal organs and OSI during the period of the study, days			
	14		28	
	BMCH matrix (<i>n</i> = 4)	CEC hAT (<i>n</i> = 4)	BMCH matrix (<i>n</i> = 5)	CEC hAT (<i>n</i> = 5)
Liver weight, g	1.47 $\pm$ 0.08	1.24 $\pm$ 0.05	1.72 $\pm$ 0.16	1.41 $\pm$ 0.06
OSI, %	6.10 $\pm$ 0.35	5.39 $\pm$ 0.15	6.13 $\pm$ 0.26	5.35 $\pm$ 0.14
Kidney weight, g	0.390 $\pm$ 0.031	0.370 $\pm$ 0.011	0.410 $\pm$ 0.045	0.400 $\pm$ 0.002
OSI, %	1.63 $\pm$ 0.12	1.60 $\pm$ 0.04	1.500 $\pm$ 0.084	1.540 $\pm$ 0.035
Heart weight, g	0.15 $\pm$ 0.01	0.130 $\pm$ 0.004	0.150 $\pm$ 0.015	0.140 $\pm$ 0.002
OSI, %	0.63 $\pm$ 0.04	0.550 $\pm$ 0.007	0.540 $\pm$ 0.086	0.540 $\pm$ 0.010
Thymus weight, g	0.030 $\pm$ 0.001	0.020 $\pm$ 0.003	0.040 $\pm$ 0.011	0.030 $\pm$ 0.001
OSI, %	0.110 $\pm$ 0.004	0.090 $\pm$ 0.013	0.14 $\pm$ 0.031	0.130 $\pm$ 0.006
Spleen weight, g	0.18 $\pm$ 0.01	0.160 $\pm$ 0.014	0.13 $\pm$ 0.040	0.110 $\pm$ 0.020
OSI, %	0.75 $\pm$ 0.04	0.720 $\pm$ 0.060	0.490 $\pm$ 0.175	0.480 $\pm$ 0.074
Testicle weight, g	0.180 $\pm$ 0.000	0.150 $\pm$ 0.017	0.160 $\pm$ 0.020	0.170 $\pm$ 0.007
OSI, %	0.750 $\pm$ 0.000	0.630 $\pm$ 0.064	0.600 $\pm$ 0.011	0.660 $\pm$ 0.036

* There were no significant differences according to Student's *t*-test ($p > 0.05$) between indices on the 14th and 28th day of the experiment and between indices in the control and experimental groups.

Parietal and visceral pleura and peritoneum were thin, shiny, and smooth. The subcutaneous tissue, mandibular lymph nodes, salivary glands, lungs, heart, esophagus, stomach, liver, gallbladder, pancreas, spleen, kidneys, adrenal bodies, urinary bladder, thymus, and the brain were studied visually. The condition of the tissues and organs in all the animals was within normal limits.

There was no inflammation at the site of the implantation. The implants were covered by a thin transparent capsule. No signs of rejection were observed.

Morphometric analysis of internal organs. The following organs were taken and weighed: liver, kidneys (two), heart, spleen, thymus, and testicles (two).

No reliable differences of either the absolute or relative weight (OSI) of the internal organs in the mice of the control and experimental groups at any time of the experiment were found (Table 4).

Histological Study of the Implantation Site

Fourteen days after the implantation, in the histological preparations of the samples of the BMCH matrix stained with hematoxylin and eosin, fragments of the eosinophilic biopolymer heterogeneous hydrogel surrounded by a heterogeneous cell population including epithelioid cells, polymorphonuclear leukocytes, fibroblast-like cells, macrophages, and multi-

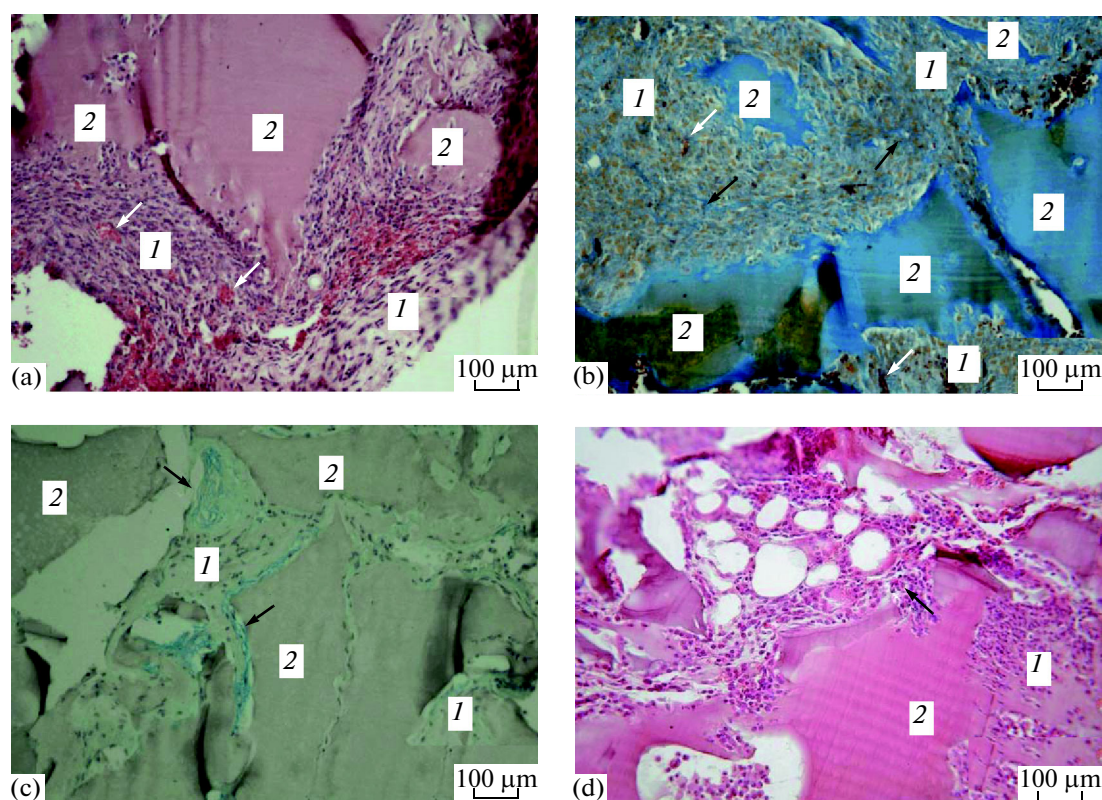


Fig. 1. Fourteen days of subcutaneous implantation of CEC hAT consisting of BMCH matrix and hAT MSC: (a) staining with hematoxylin and eosin; (b) staining against collagen according to the Mallory technique (indicated by black arrows); (c) staining with alcian blue against glycosaminoglycan (indicated by black arrows); (d) control (BMCH matrix), staining with hematoxylin and eosin. (1) Heterogeneous cell population, (2) biopolymer heterogeneous hydrogel; (a, b) capillaries are indicated by white arrows; (c) capillaries are indicated by black arrows.

nucleated foreign body giant cells and numerous capillaries were revealed in the subcutaneous tissue (Fig. 1d). Penetration of cell groups and individual cells into the hydrogel matrix was observed. The preparations of the CEC hAT samples stained with hematoxylin and eosin had active cell response to the implant observed with numerous polymorphonuclear leukocytes, macrophages, and multinucleated foreign body giant cells involved. Here, the polymorphonuclear leukocytes prevailed. The presence of numerous capillaries together with the heterogeneous cell population indicates the formation of granulation tissue. As in the control samples (BMCH matrix), the cell penetration into the BMCH accompanied by its partial resorption was observed (Fig. 1a). No edemas were observed.

The staining of the experimental sections according to the Mallory technique revealed thin blue collagen fibers surrounding fragments of the BMCH matrix (Fig. 1b). Upon staining of the sections with alcian blue, it was possible to find only single loci demonstrating a positive reaction with GAG (Fig. 1c).

Unfortunately, we failed to obtain preparations of the control samples 28 days after the subcutaneous implantation.

The preparations of the experimental samples stained with hematoxylin and eosin after 28 days had fragments of the BMCH matrix surrounded by a heterogeneous cell mass with fibroblast-like cells clearly prevailing and in some regions forming a connective-tissue capsule (angiogenesis continues). More pronounced, when compared to the previous period of observation, resorption of the heterogeneous hydrogel with its replacement by scar tissue was observed with predominance of the extracellular matrix (Fig. 2a). A progressive increase in the mass of collagen fibers was found (Fig. 2b). Local cyan staining with alcian blue specific to GAG was not pronounced and was observed not in all the samples. Individual regions of the implant had a few lacune-like structures typical of the cartilaginous tissue found (Fig. 2a). The absence of toxicity and tumorigenicity in both groups indicates the biological safety of the CEC AT.

Studies devoted to morphological changes in CEC AT based on polymer hydrogels and MSCs from different sources *in vivo* give contradicting results. Thus, one group of researchers obtained evidence of the formation of cartilaginous tissue by a tissue-engineered construct, i.e., the presence of GAG and newly formed type II collagen upon subcutaneous implanta-

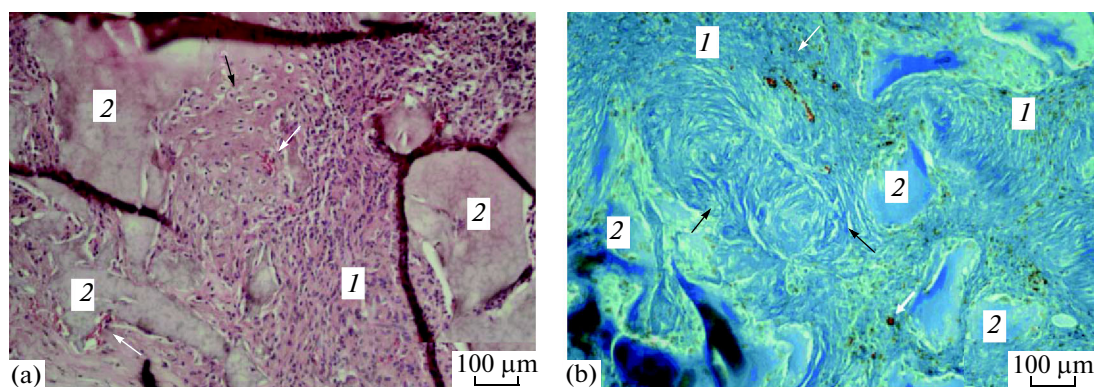


Fig. 2. Twenty-eight days of subcutaneous implantation of CEC hAT consisting of BMCH matrix and hAT MSC: (a) staining with hematoxylin and eosin (black arrows indicate lacune-like structures); (b) staining against collagen according to the Mallory technique (indicated by black arrows). (1) Heterogeneous cell population, (2) BMCH matrix; capillaries are denoted by white arrows.

tion in mice of the nude line of MSCs predifferentiated in a monolayer or 3D culture placed into a hydrogel based on silanized hydroxypropylmethyl cellulose [16]. Other authors, in contrast, found a decrease in the number of GAG and an increase in the number of type I collagen 28 days after subcutaneous implantation of hAT MSCs differentiated in the chondrogenic lineage in an alginate hydrogel [18]. This could be explained by dedifferentiation of MSCs and formation of fibrous tissue in vivo. It seems that the formation of TEC hAT depends to a large extent on the properties of the matrix used.

CONCLUSIONS

The biological safety of the CEC hAT consisting of a biodegradable microheterogeneous hydrogel matrix and mesenchymal stromal cells of human adipose tissue preliminarily cultivated for 7 days in a chondrogenic induction medium was demonstrated in the experimental model of subcutaneous implantation in DBA mice during a time period up to 28 days. Examination of the animals during the experiment, clinical and biochemical analyses of their blood, and postmortem data with the morphometric analysis of the internal organs did not reveal significant differences between the control group (BMCH matrix implantation) and experimental group (CEC hAT implantation). The result obtained indicates that the cell component of the tissue-engineered construct of hAT is not a risk factor upon implantation of the CEC hAT for 28 days. Moreover, some features of the formation of cartilaginous tissue at the site of the subcutaneous implantation of the CEC hAT studied were observed.

Taking into account high proliferative and differentiation activity of the CEC hAT upon its cultivation for 42 days [14], one can suggest that it is promising to use the CEC hAT for regeneration of damaged articular cartilage as an injection form of a biomedical cell product.

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REFERENCES

1. Nasonov, Ye.L. and Nasonova, V.A., *Revmatologiya. natsionalnoye rukovodstvo* (Rheumatology. Nat. Handbook) Moscow: GEOTAR-Media, 2010.
2. Melero-Martin, J.M. and Al-Rubeai, M., *In vitro Expansion of Chondrocytes. Topics in Tissue Engineering* 2007, vol. 3.
3. Jones, D.G. and Peterson, L., Autologous chondrocyte implantation, in *Cartilage Repair Strategies*, Williams, R.J., Ed., Totowa, New Jersey: Humana, 2007.
4. Marlovits, S., Hombauer, M., Truppe, M., Vecsei, V., and Schlegel, W., Changes in the ratio of type-I and type-II collagen expression during monolayer culture of human chondrocytes, *J. Bone Joint Surg., B* 2004, vol. 86, pp. 286–295.
5. Danišovič, L., Lesný, P., Havlas, V., Teyssler, P., Syrová, Z., Kopáni, M., Fujeříková, G., Trč, T., Syková, E., and Jendelová, P., Chondrogenic differentiation of human bone marrow and adipose tissue-derived mesenchymal stem cells, *Appl. Biomed.*, 2007, vol. 5, pp. 139–150.
6. Sevastianov, V.I., Perova, N.V., Nemets, E.A., Surguchenko, V.A., and Ponomareva, A.S., *Biosovmestimye materialy* (Biocompatible Materials (Textbook)), Sevastianov, V.I. and Kirpichnikov, M.P., Eds., Moscow: MIA Publ., 2011, part 2, ch. 3, pp. 237–252.
7. Egorova, V.A., Ponomareva, A.S., Bogdanova, N.B., Abramov, V.Yu., and Sevastianov, V.I., Characteristic phenotype MSC from the fatty tissue of humans by flow cytometry, *Technol. Living Syst.*, 2009, vol. 6, no. 5, pp. 40–46.
8. Zuk, P.A., Zhu, M., Mizuno, H., Huang, J., Futrell, W., Katz, A.J., Benhaim, P., Lorenz, P., and Hedrik, M.H., Multilineage cells from human adipose tissue: Implications for cell-based therapies, *Tissue Eng.*, 2001, vol. 7, pp. 211–228.

9. Liu, J., Zhao, B., Zhang, Y., Lin, Y., Hu, P., and Ye, C., PHBV and predifferentiated human adipose-derived stem cells for cartilage tissue engineering, *J. Biomed. Mater. Res., A*, 2010, vol. 94, pp. 603–610.
10. Surguchenko, V.A., The matrices for tissue engineering and hybrid organs, in *Biocompatible Materials (Textbook)*, Sevastianov, V.I. and Kirpichnikov, M.P., Eds., Moscow: MIA Publ., 2011, ch. 2(1), pp. 199–228.
11. Sevastianov, V.I. and Perova, N.V., RF Patent 2433828, 2011.
12. Sevastianov, V.I., Perova, N.V., Nemets, E.A., Surguchenko, V.A., and Ponomareva, A.S., *Biosovmestimye materialy* (Biocompatible Materials (Textbook)), Sevastianov, V.I. and Kirpichnikov, M.P., Eds., Moscow: MIA Publ., 2011, part 2, ch. 3, pp. 237–252.
13. Sevastianov, V.I., Perova, N.V., Sajkovskij, R.S., and Solov'eva, I.V., *The Use of Injectable Biopolymer Heterogeneous Hydrogels with Degenerative-Dystrophic Lesions of the Joints. Practical Manual for Doctors*, Moscow: Triada, 2012.
14. Surguchenko, V.A., Ponomareva, A.S., Kirsanova, L.A., Bubentsova, G.N., Skaletskij, N.N., and Sevastianov, V.I., On the possibility of in vitro formation of tissue-engineered construct of cartilage on the basis of cell-engineered construct composed of biopolymer hydrogel matrix and human adipose tissue-derived mesenchymal stromal cells, in *Advanced Metals, Ceramics and Composites*, Tu, H., Solntsev, K., and Zhou, R., Eds., Kunming, China: Yunnan, 2013, pp. 242–245.
15. Surguchenko, V.A., Ponomareva, A.S., Kirsanova, L.A., Skaletskij, N.N., and Sevastianov, V.I., The cell-engineered construct of cartilage on the basis of biopolymer hydrogel matrix and human adipose tissue-derived mesenchymal stromal cells (in vitro study), *J. Biomed. Mater. Res., A*, 2014, vol. 103, pp. 463–470.
16. Merceron, C., Portron, S., Masson, M., Lesoeur, J., Fellah, B., Gauthier, O., Geffroy, O., Weiss, P., Guicheux, J., and Vinatier, C., The effect of two- and three-dimensional cell culture on the chondrogenic potential of human adipose-derived mesenchymal stem cells after subcutaneous transplantation with an injectable hydrogel, *Cell Transplant.*, 2011, vol. 20, pp. 1575–1588.
17. Chung, C. and Burdick, J.A., Engineering cartilage tissue, *Adv. Drug Deliv. Rev.*, 2008, vol. 60, pp. 243–262.
18. Vinardell, T., Sheehy, E.J., Buckley, C.T., and Kelly, D.J., A comparison of the functionality and in vivo phenotypic stability of cartilaginous tissues engineered from different stem cell sources, *Tissue Eng., A*, 2012, vol. 18, pp. 1161–1170.

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