ASSESSING BIODISTRIBUTION OF BIOMEDICAL CELLULAR PRODUCT BASED ON HUMAN CHONDROCYTES FOLLOWING IMPLANTATION TO BALB/C NUDE MICE

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Despite the prospects of the approach to cell therapy of cartilage damage in humans involving autologous chondrocytes, similar technologies are just beginning to be introduced into medical practice in the Russian Federation. In this regard, the development of biomedical cell products (BCPs) for cartilage tissue repair is quite topical, while the use of organoid technology is the most close to the native tissue conditions. According to requirements of legislation of the Russian Federation, it is necessary to assess biodistribution characterizing migration potential of the cells, their tropism for body tissues following implantation within the framework of preclinical trials. The study was aimed to assess biodistribution of novel BCP based on human chondrocytes in the form of chondrospheres after subcutaneous implantation in Balb/c nude mice. Implantation to 12 mice was performed during the first phase, along with administration of saline to 12 control animals. Weighting and follow-up were conducted for 90 days. Then mice were withdrawn from the experiment to collect samples of organs and tissues for histological analysis of the implant, estimation of its viability, integration. During the second phase biodistribution was assessed by PCR in order to detect human DNA in the organ and tissue samples. Chondrospheres successfully integrated in the tissues surrounding the inoculation zones and formed cartilage tissue. No significant ($p < 0.05$) changes in weight were reported. No human DNA found in chondrosphere implantation zones was detected in the samples collected from other organs and tissues. BCP demonstrated no biodistribution across other tissues and organs of mice 90 days after implantation, which suggested that the product developed was safe.

Keywords: biomedical cellular product, chondrocytes, biodistribution, preclinical trials, chondrospheres

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ИССЛЕДОВАНИЕ БИОРАСПРЕДЕЛЕНИЯ БИОМЕДИЦИНСКОГО КЛЕТОЧНОГО ПРОДУКТА НА ОСНОВЕ ХОНДРОЦИТОВ ЧЕЛОВЕКА ПРИ ИМПЛАНТАЦИИ МЫШАМ ЛИНИИ BALB/C NUDE

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Несмотря на перспективность подхода клеточной терапии повреждений хряща человека с помощью аутологичных хондроцитов, подобные технологии только начинают внедрять в медицинскую практику в Российской Федерации. В связи с этим разработка биомедицинских клеточных продуктов (БМКП) для восстановления хрящевой ткани достаточно актуальна, а использование органоидных технологий наиболее приближено к условиям нативной ткани. Согласно требованиям законодательства РФ, в рамках доклинических исследований необходимо изучение биораспределения, характеризующего миграционный потенциал клеток, их тропность к тканям организма при имплантации. Целью работы было исследовать биораспределение нового БМКП на основе хондроцитов человека в виде хондросфер после подкожной имплантации мышам линии Balb/c nude. На первом этапе осуществляли имплантацию 12 мышам, а также введение физиологического раствора 12 контрольным животным. В течение 90 дней проводили взвешивание и наблюдение, а затем выводили мышей из эксперимента для получения образцов органов и тканей для гистологического анализа импланта, оценки его состоятельности, интеграции. На втором этапе изучали биораспределение методом ПЦР для выявления ДНК человека в образцах тканей и органов. Хондросферы успешно интегрировались в окружающие ткани зоны инокуляции, формировали хрящевую ткань. Статистически значимых ($p < 0,05$) изменений в весе не зафиксировали. В образцах из зоны имплантации хондросфер была выявлена ДНК человека, которую не обнаруживали в других органах и тканях. БМКП через 90 дней после имплантации демонстрировал отсутствие биораспределения в другие ткани и органы мышей, что свидетельствует о безопасности разрабатываемого продукта.

Ключевые слова: биомедицинский клеточный продукт, хондроциты, биораспределение, доклинические исследования, хондросферы

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Advances of recent years in the development of various approaches to cell therapy for cartilage tissue damage enable treatment of some joint disorders, including disorders associated with human cartilage lesions of multifactorial etiology [1]. Implantation of autologous chondrocytes is considered to be an effective and promising method to treat cartilage tissue damage with minimal risk of adverse events [2]. The principle of this procedure consists in scaling up and culturing chondrocytes obtained from the biopsy specimen of the patient's articular cartilage fragment with subsequent transplantation of cells immediately into the defect [2]. The researchers became more and more interested in cell therapy since the first report of implantation of autologous chondrocytes into the damaged area of human articular cartilage [3]; new methods to modify and optimize this approach appeared over the decade [1]. The use of biomedical cell products (BCPs) based on autologous chondrocytes was superior to more conventional methods, arthroscopic debridement and microdamage, in terms of efficacy [4–7]. Today, a number of BCPs are through various phases of pre-clinical and clinical trials [1, 2, 6, 8, 9]; some products are approved for treatment of the human cartilage focal lesions [11–14].

Spheroids, the 3D structures resulting from self-aggregation of cells cultured under certain conditions, have many advantages over BCPs of other types when used for treatment of articular cartilage defects [15]. Thus, 3D conditions have a beneficial effect on proliferative activity and phenotypic stability of mature chondrocytes [9]. In contrast to suspension of autologous chondrocytes, which become capable of producing extracellular matrix (ECM) only after a certain time after transplantation, the cells comprised in spheroids can secrete the ECM components even in the phase of culturing [16, 17]. In addition, autologous spheroids do not require using third-party biomaterials, they easily integrate into the tissue in the damaged area; there is no need for systemic immunosuppression after implantation [15]. In combination, specified properties of spheroid BCPs can contribute to high-quality filling of the cartilage tissue defect. This, the efficacy of spheroids based on autologous chondrocytes has been demonstrated in animal models [17] and clinical trials [18, 19]. Spheroid BCPs have shown significantly better therapeutic effect in terms of structural cartilage defect restoration compared to the microdamage procedure [6, 7].

Spheroxтм (Co.don; Germany), one of the globally approved cell therapy drugs for restoration of human articular cartilage defects, represents a spheroid BCP. Today, the Generium company produces this drug under license from Co.don and completes phase III clinical trial in the Russian Federation [10]. At the same time, no analogues of such BCPs are available in the Russian Federation Thus, the only product that is, in fact, a technology transfer from Co.don, is currently undergoing clinical trials in the Russian Federation Given optimistic results of the cell therapy trials conducted by foreign partners, the development and introduction of such technology into research and clinical practice in the Russian Federation eems to be very topical.

The main task of working with BCPs is to maintain efficacy and meet the critical quality parameters to ensure safety and the expected effect, which should be predicted before the start of the clinical trial. That is why BCPs, as all other medications, must meet strict requirements to be approved by the authorities for further research and implementation [8]. This requires the development and implementation of appropriate assessment methods to evaluate the cell-based product before and after implantation within the framework of pre-clinical trial [19, 20]. Thus, it is necessary to identify the major risk factors, such as tumorgenicity, carcinogenicity and biodistribution, before the beginning of pre-clinical trial involving animals [21].

Biodistribution is one of the most important safety criteria characterizing migration potential of the cells comprised in BCP after implantation, along with the capabilities of forming ectopic tissue and persisting inside/outside the administration site [9, 22–24]. Biodistribution is usually assessed in immunodeficient animal models; subcutaneous implantation is preferred due to its less invasive nature [24]. Since the tested product should be as close as possible or similar to the final BCP variant based on its properties, it is advisable to avoid the use of fluorescent tags or any other approaches potentially changing the product structure and properties when assessing biodistribution [8].

The study was aimed to assess biodistribution of the Chondrosphere spheroid BCPs designed for treatment of articular cartilage lesions in humans after subcutaneous implantation to the Balb/c Nude immunodeficient mice.

METHODS

Legal regulation

The study represents a preclinical trial of novel BCP conducted in accordance with the current regulatory requirements [21, 25–27]. The study was carried out according to the approved written plan and Standard Operating Procedures. The employees, who took part in the experiment, were trained to ensure proper, humane care and use of laboratory animals.

Spheroid BCPs

The stidued BCPs represented a 3D culture of spheroids based on human chondrocytes obtained using the Aggre Well 800 microwell plate (STEMCELL Technologies; Canada) in accordance with the manufacturer's protocol. The number of cells per microwell of the plate was 4–5 × 10^3. The spheroids obtained (the cellular or tissue-engineered product is referred to as Chondrosphere) were cultured in miniature bioreactors on the 3D orbital shaker (Infors HT; Switzerland) at 37 °C and 5% CO_2 [28]. Advanced DMEM (Gibco, Thermo Fisher Scientific; USA) supplemented with 10% fetal bovine serum (FBS), 50 μM β-mercaptoethanol, 10 ng/ml bFGF (STEMCELL Technologies; Canada), 100X Glutamax (Gibco, Thermo Fisher Scientific; USA), 50X B27 (GIBCO, Thermo Fisher Scientific), 1% Insulin-Transferrin-Selenium (ITS) (PANECO; Russia), 50 μg/ml of ascorbic acid (Sigma Aldrich; USA), 5 μg/ml of plasmocin, gentamicin (PANECO; Russia) and 10 mL/L 100x solution of penicillin/streptomycin (PanEco; Russia) were used as culture medium. Spheroids were cultured for 28 days; the medium was changed every 4 days.

Experimental design

Inbred Balb/c Nude immunodeficient mice were selected for safety assessment. BCPs were administered to animals (n = 12; 6 females, 6 males) by a single subcutaneous injection in the head in a dose of five spheroids in saline (groups 1, 2). In addition, 12 mice (6 males and 6 females) were used as control animals thet received subcutaneous injection of 50 μL of saline (groups 3, 4). The animals were weighted regularly, and the inoculum size was measured in the implantation area during the experiment. Then, 90 calendar days after administration 12 females and 12 males were euthanized by decapitation under inhalation anesthesia. After that specimens from the following organs and tissues were harvested: lymph nodes, thyroid,

Table 1. Primers used in the study

Name	Sequence 5'→3'	Product size
mActb-F	GAT GCA CAG TAG GTC TAA GTG GAG	121
mActb-R	CAC TCA GGG CAG GTG AAA CT	
CO1-F	CAA CCT CAA CAC CAC CTT C	269
CO1-R	CTC GTG TGT CTA CGT CTA TTC	

aorta, heart, lungs, thymus, esophagus, stomach, pancreas, small intestine, large intestine, liver, spleen, kidney, bladder, adrenal glands, brain, testes, ovary, administration site, blood, tumor.

The euthanized animal was treated with 96% ethanol. All subsequent phases of organ harvesting were accomplished under a laminar flow hood in aseptic environment.

Histological analysis

Biomaterial was fixed in the Histosafe 10% formaldehyde solution (BioVitrum; Russia) for 24 h, then washed with running water for 20 min to remove excess fixing agent and dehydrated five times with the Blic modified isopropyl alcohol (BlicMedicalProduction; Russia). Then the specimens were embedded in paraffin. The 4–5 μm histological sections were obtained using the Microm HM325 microtome (Microm; Germany). Paraffin removal was performed in accordance with the following scheme: xylene № 1 — 2 min, xylene № 2 — 2 min, 96% ethanol № 1 — 2 min, 96% ethanol № 2 — 2 min, 70% ethanol — 2 min, distilled water — 2 min. Histological sections were stained with hematoxylin and eosin (Mayer's haematoxylin, eosin 1% aqueous solution (BioVitrum; Russia)). The resulting slices were assessed using the Levenhuk 625 microscope (Levenhuk; Russia).

Genomic DNA isolation

The M-SORB-OOM kit (Sintol; Russia) was used in accordance with the manufacturer's instructions to extract genomic DNA from the organs of mice and human capillary blood to be used as positive control for human DNA. The 10–20 mg fragments of organs or 10–20 μL of capillary blood were used for extraction. Samples with no organ or tissue specimens were used as negative controls. Genomic DNA was eluted in 400 μL of elution buffer. The finite volume of the solution with isolated genomic DNA was 400 μL.

Polymerase chain reaction (PCR)

PCR was performed in the CFX96 Touch system for nucleic acid amplification (Bio-Rad; USA) using the ready-made 5X Screen Mix for PCR (Evrogen; Russia) in accordance with the manufacturer's instructions. We used primers specific for the cytochrome C oxidase subunit I (CO1) genes to detect human DNA and β-actin specific for mice (mActb) to detect murine DNA when performing the reaction (Table 1).

Amplification was performed in accordance with the following protocol:

1) 95 °C — 5 min;
2) 95 °C — 15 s;
3) 58 °C — 15 s;
4) 72 °C — 30 s.

Steps 2, 3 and 4 were repeated in 40 cycles.

Agarose gel electrophoresis

DNA electrophoresis was conducted in 1% agarose gel in Tris-Acetate-EDTA (TAE) buffer in the horizontal electrophoresis chamber (Biorad; USA). Visual detection of amplification products involved the use of 0.5 μg/mL ethidium bromide. Voltage was set as 120 V, and the run time was 20 min. The amplification products were detected with the UV transilluminator (Vilber; Germany).

Statistical analysis

The results of weight estimation in the animal subjects were processed using the Microsoft Excel (Microsoft; USA) and SPSS Statistics 17.0 (IBM; USA) software packages. The Shapiro–Wilk test was used to test the trait distribution for normality. Mann–Whitney U-test was used for comparison. Bonferroni correction for multiple comparisons was applied. The differences between groups were considered significant at $p < 0.05$. Graphs were plotted with the GraphPad Prism software (Dotmatics; USA).

Handling the remaining BCPs

BCPs not used in the experiment were autoclaved and disposed as class B waste.

RESULTS

Morphometric analysis

Regular weighting for 90 days revealed no significant differences in body weight between the groups receiving BCPs and control groups (Fig. 1).

There were no significant differences in the animals' general health between the experimental and control groups. The animals remained active and showed normal feeding behavior.

Histological analysis

After histological staining of speciments from the BCP implantation area we observed stable cartilage tissue with the large number of chondrocytes and the emerging lacunae (Fig. 2). Cell migration from the implantation area was minimal.

Detection of human DNA in murine tissues and organs

The analysis of whole blood samples, mirine organ and tissue specimens revealed human DNA in the chondrosphere injection area only (Table 2). No traces of the tested BCP were detected in other tissues and organs of male and female mice (LOQ < 0.001 ng of DNA). Thus, the BCP biodistribution pattern was optimal for the recommended administration route.

DISCUSSION

Obtaining 3D spheroid BCPs based on autologous human chondrocytes using the organoid technique is considered to be a rather promising direction of the development of products for cell therapy of large focal hyaline cartilage defects [28]. Despite the fact that the composition of the product we are

developing now is similar to that of the product by Generium, it is obtained using a modified technique, which requires safety testing. According to the current standards, the study of BCP pharmacokinetics includes assessment of biodistribution characterizing migration potential of the cells comprised in the construct [27]. Previously, the Spheroxтм product researchers assessed biodistribution of their invention implanted in immunodeficient animals as part of registration activities after consulting with the regulator [8]. The analysis by PCR showed that there were no human DNA in the tissues and organs distant from the subcutaneous implantation site. Thus, it seems reasonable to assess biodistribution of BCPs designed for impantation in humans using the discussed approach within the framework of safety testing.

Our study was aimed to assess biodistribution of the spheroid BCP designed for treatment of human articular cartilage lesions in immunodeficient mice. As far as we know, this is the first large-scale preclinical trial of BCP based on autologous chondrocytes in the Russian Federation.

Balb/c Nude mice were used to assess biodistribution. These immunodeficient mice are widely used in the trials of xenografts, including that based on human chondrocytes [29–30]. We used subcutaneous implantation of spheroids, since this procedure is less invasive, expandable and easier to implement — for example, compared to implantation in the small rodent's joint. A single BCP dose was calculated based on the estimated therapeutic dose for humans in accordance to the cartilage tissue defect size: 10–70 spheroids per 1 cm^2 of damaged tissue [8]. In mice, the dose was five spheroids per animal.

To assess stable cartilage tissue formation in the BCP administration site, the injection sites were examined using histological analysis. We observed cartilage tissue development 90 days after implantation, which was indicative of successful integration of spheroids into murine tissues. Morphometry revealed no significant changes in body weight in the experimental groups, which suggested no systemic morbid effect. Furthermore, there was no abnormal tissue growth associated with carcinogenesis (month 3 of follow-up) or tumorgenesis.

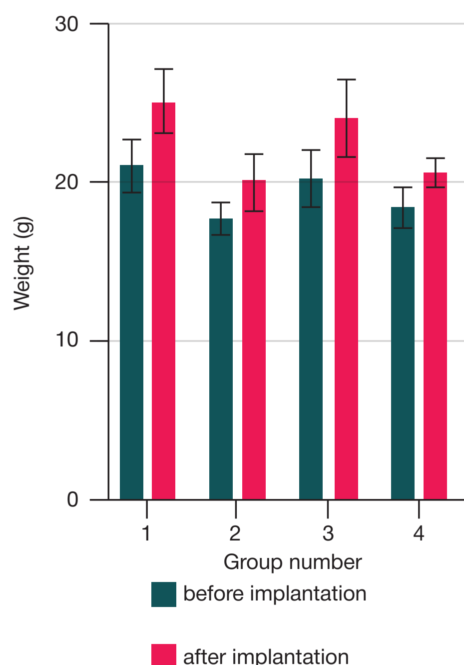

Fig. 1. Changes in the experimental animals' body weight by groups before and after the experiment. 1, 2 — groups of males and females, which underwent BCP implantation, $n = 12$; 3, 4 — groups of males and females, which received subcutaneous injection of saline, $n = 12$. * — significant intragroup differences, $p < 0.05$

To assess biodistibution, biopsy specimens of murine organs and tissues were qualitatively tested for expression of the human-specific sequence of the gene encoding cytochrome C oxidase subunit 1 (COI) 90 days after implantation. Our findings showed that a single subcutaneous administration of BCP to experimental mice resulted in the fact that human DNA was detected exclusively in the administration site, not in the other assessed tissues and organs. Thus, human DNA is related exclusively to the cells comprised in the spheroids implanted. However, in the future we plan to assess BCP biodistribution and carcinogenicity in mice throughout a longer period after implantation in order to evaluate potential delayed effects.

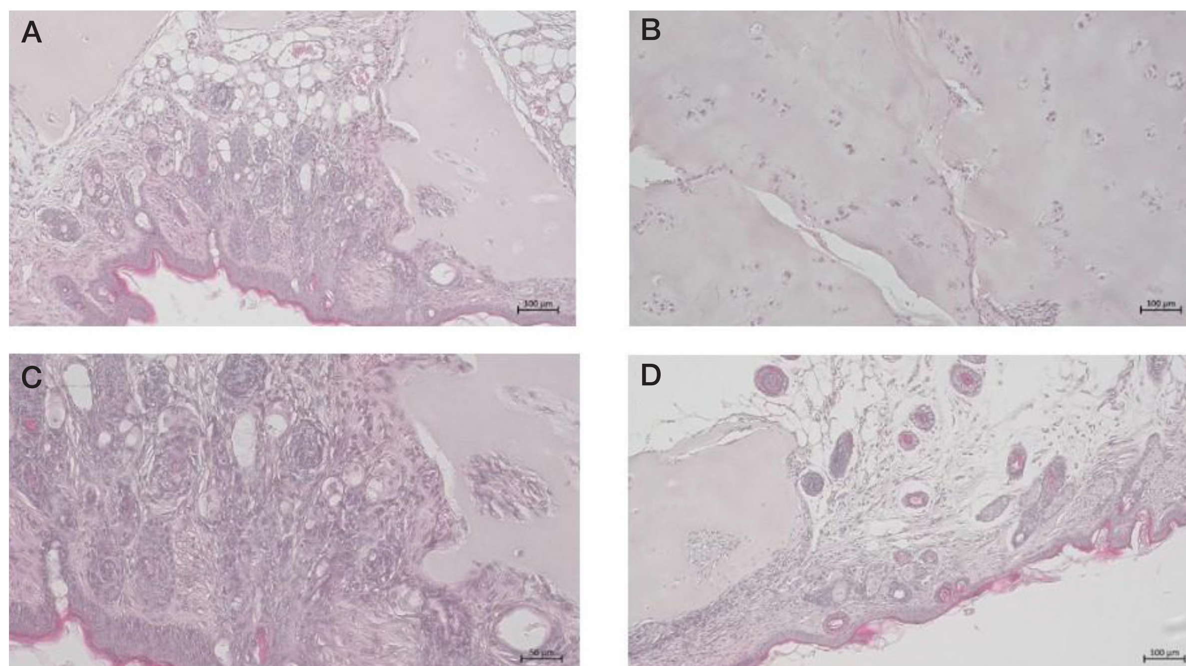

Fig. 2. Chondropheres in murine tissues 90 days after implantation. Hematoxylin and eosin stain. 10x magnification. Scale bar — 100 μm

Table 2. Human DNA detection

Organ/Tissue	Group number (*n* = 24)			
	1	2	3	4
Lymph nodes	------	------	------	------
Thyroid	------	------	------	------
Aorta	------	------	------	------
Heart	------	------	------	------
Lung	------	------	------	------
Thymus	------	------	------	------
Esophagus	------	------	------	------
Stomach	------	------	------	------
Pancreas	------	------	------	------
Small intestine	------	------	------	------
Liver	------	------	------	------
Spleen	------	------	------	------
Kidney	------	------	------	------
Bladder	------	------	------	------
Adrenal glands	------	------	------	------
Brain	------	------	------	------
Testes	------	------	------	------
Ovary	------	------	------	------
Implantation site	++++++	++++++	------	------
Blood	------	------	------	------
Tumor	------	------	------	------

Note: Positive (indicative of the presence of human DNA) qualitative data were obtained for the samples designated as "+"; 1, 2 — groups that received BCP; 3, 4 — groups that received saline.

The results obtained in this phase suggest no cell migration processes, which indicates that the product developed is safe in terms of biodistribution.

CONCLUSIONS

In this study we assessed biodistribution of BCPs in the form of chondrospheres based on human chondrocytes by subcutaneous implantation to Balb/c Nude mice. During the study we observed the development of stable mature cartilage tissue showing no signs of abnormal proliferation or cell migration outside the implantation site. Such findings allow us to conclude that the BCP developed is characterized by normal biodistribution within the administration site and successful integration into the surrounding tissies. Thus, this cell engineering product, Chondrosphere, can be recommended for further testing.

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