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Experience in the production and clinical application of the cell-based medicinal product Easytense® for the repair of cartilage defects of the human knee

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ABSTRACT

INTRODUCTION. The current cell-based cartilage repair methods, such as autologous chondrocyte transplantation, are not sufficiently effective, and the surgery is painful and traumatic. Therefore, there is a need for a more effective cell therapy product with a minimally invasive surgical procedure for its implantation into the patient.AIM. This study aimed to develop a manufacturing technology for the production of an autologous cell-based medicinal product (CBMP) comprising three-dimensional structures (3D-spheroids) based on chondrocytes isolated from the patient's cartilage tissue, as well as to evaluate its clinical efficacy.

AIM. This study aimed to develop a manufacturing technology for the production of an autologous cell-based medicinal product (CBMP) comprising three-dimensional structures (3D-spheroids) based on chondrocytes isolated from the patient's cartilage tissue, as well as to evaluate its clinical efficacy.

MATERIALS AND METHODS. Autologous chondrocytes isolated from the patient's cartilage biopsy were propagated in monolayer culture to obtain the required number of cells. Subsequently, the chondrocytes were cultivated on plates with a non-adhesive coating to form 3D spheroids. All CBMP production steps were performed under aseptic conditions in cell culture isolators. The authors used phase-contrast microscopy and immunohistochemical staining with specific fluorescence-labelled antibodies to characterise chondrocyte phenotypes at different stages of cultivation. Genetic stability was controlled by karyotyping. The efficacy of Easytense® was evaluated in a clinical trial using specialised functional tests and the Magnetic Resonance Observation of Cartilage Repair Tissue (MOCART) score. The primary efficacy endpoint was a change in the overall score on the Knee Injury and Osteoarthritis Outcome Score (KOOS).

RESULTS. A manufacturing technology without using animal sera, growth factors, cytokines, or other additives was developed for the production of the autologous CBMP Easytense®. Karyological data confirmed that the chondrocytes retained genetic stability for 3 passages in monolayer culture. When cultured as 3D spheroids, the chondrocytes produced cartilage extracellular matrix proteins (type II collagen, aggrecan), thus acquiring the ability to repair damaged cartilage. The clinical trial demonstrated a statistically significant improvement in knee cartilage 12 months after the transplantation of 3D spheroids derived from autologous chondrocytes. The mean change in the overall KOOS score was 23.8±15.9.

CONCLUSIONS. The clinical trial results indicate that Easytense® is highly effective for cartilage repair. Based on these results, the CBMP has been granted marketing authorisation and introduced into clinical practice in the Russian Federation. Easytense® has the potential to replace endoprosthetics and expensive surgeries abroad.

Keywords: Easytense®; cell-based medicinal product; CBMP; knee cartilage; chondrocytes; autologous chondrocytes; spheroids; transplantation; genetic stability; quality control of CBMPs; clinical trials of CBMPs

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Опыт производства и клинического применения биомедицинского клеточного продукта Изитенс® для восстановления повреждений хрящевой ткани коленного сустава человека

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РЕЗЮМЕ

ВВЕДЕНИЕ. Существующие клеточные методы восстановления хрящевой ткани, такие как аутологичная трансплантация хондроцитов, недостаточно эффективны, а хирургическое вмешательство болезненно и травматично. Поэтому требуется разработка более эффективного продукта для клеточной терапии с малоинвазивной хирургической процедурой его имплантации пациенту.

ЦЕЛЬ. Разработка технологии производства аутологичного биомедицинского клеточного продукта (БМКП), содержащего трехмерные структуры (3D-сфероиды) на основе хондроцитов, выделенных из хрящевой ткани пациента, и оценка клинической эффективности его применения.

МАТЕРИАЛЫ И МЕТОДЫ. Аутологичные хондроциты, выделенные из биоптата хрящевой ткани пациента, культивировали в монослойной культуре для получения необходимого количества клеток. Дальнейшее культивирование хондроцитов проводили с использованием неадгезивного покрытия для формирования 3D-сфероидов. Все этапы производства БМКП проводили в асептических условиях в изоляторах клеточных культур. Для характеристики фенотипа хондроцитов на разных этапах культивирования использовали фазово-контрастную микроскопию и иммуногистохимическое окрашивание с применением специфических флуоресцентно меченных антител. Генетическую стабильность контролировали кариологическим методом. В рамках клинического исследования (КИ) проводили оценку эффективности БМКП Изитенс® при помощи специализированных функциональных тестов и по Шкале оценки восстановления хрящевой ткани по результатам магнитно-резонансной то-

мографии (MOCART). Основным критерием эффективности было изменение общего балла по Шкале оценки исхода травмы и остеоартроза коленного сустава (KOOS).
РЕЗУЛЬТАТЫ. Разработана технология производства аутологичного БМКП Изитенс® из собственных клеток пациента без использования сыворотки животных, ростовых факторов, цитокинов и других добавок. С использованием кариологического метода подтверждено сохранение генетической стабильности хондроцитов на протяжении 3 пассажей культивирования в монослойной культуре. При 3D-культивировании в виде сфероидов хондроциты продуцируют характерные для матрикса хрящевой ткани белки (коллаген типа II, аггрекан), приобретая таким образом способность к восстановлению поврежденного хряща. В ходе КИ продемонстрировано статистически значимое улучшение состояния хрящевой ткани коленных суставов через 12 мес. после трансплантации 3D-сфероидов из аутологичных хондроцитов. Среднее изменение общего балла KOOS составило 23,8±15,9.
ВЫВОДЫ. Продемонстрирована высокая эффективность БМКП Изитенс® для восстановления хрящевой ткани. На основании результатов клинических исследований данный БМКП зарегистрирован на территории Российской Федерации, введен в клиническую практику и может стать альтернативой эндопротезированию и дорогостоящим операциям за рубежом.

Ключевые слова: Изитенс®; биомедицинский клеточный продукт; БМКП; хрящевая ткань коленного сустава; хондроциты; аутологичные хондроциты; сфероиды; трансплантация; генетическая стабильность; контроль качества БМКП; клинические исследования БМКП

Для цитирования: Зоричева А.С., Звонова Е.А., Агапова Л.С., Лыкова М.С., Маркова О.А., Леонов В.С. Опыт производства и клинического применения биомедицинского клеточного продукта Изитенс® для восстановления повреждений хрящевой ткани коленного сустава человека. *БИОпрепараты. Профилактика, диагностика, лечение.* 2024;24(2):172–187. https://doi.org/10.30895/2221-996X-2024-24-2-172-187

Финансирование. Работа выполнена в рамках клинических исследований биомедицинского клеточного продукта Изитенс®, спонсируемых АО «ГЕНЕРИУМ».
Потенциальный конфликт интересов. Существует потенциальный конфликт интересов в связи с аффилиацией авторов данной научной работы с АО «ГЕНЕРИУМ». Однако при написании рукописи авторы руководствовались соображениями научной ценности полученного материала и заявляют о беспристрастности оценки полученных данных.

INTRODUCTION

The main reasons for the limited regenerative capacity of cartilage are the low number of chondrocytes in the tissue and their low proliferative potential. Cartilage tissue has virtually no ability to regenerate after trauma or disease (osteochondritis dissecans, osteoarthritis, etc.).

The most common type of cartilage damage is knee injury; its symptoms (pain, swelling, stiffness, and limited range of motion) significantly reduce patients' quality of life. Although many surgical approaches exist to restore damaged articular cartilage, this condition requires new technological solutions.

In recent years, cell-based methods for cartilage repair, such as autologous chondrocyte transplantation (ACT), have been a promising direction for treatment development. The ACT method is based on the isolation of the patient's own cells, which are cultured *in vitro* and then delivered to the site of cartilage damage, where the cells integrate with the recipient tissue and fill the damaged area. ACT is believed to be significantly superior to previously used therapies [1–3].

Despite progress in the development of cell-based cartilage repair technologies, many questions remain unanswered. One of the main concerns associated with ACT is the dedifferentiation of chondrocytes in culture, which is manifested by phenotypic changes and a reduced ability to express extracellular matrix proteins (aggrecan, type II collagen, etc.). When transplanted, such dedifferentiated chondrocytes form fibrous cartilage with inadequate biomechanical properties for physical stress instead of hyaline cartilage [4]. This stimulates the search for new culture conditions to induce chondrocyte redifferentiation *in vitro*. The literature describes several methodological approaches to induce chondrocyte redifferentiation in culture, including the use of growth factors [5–7], inhibitors and inducers of various signalling pathways [8], and genetic modification of cells with regulated gene expression [9, 10]. Many studies have shown that the use of three-dimensional matrices to culture chondrocytes promotes redifferentiation into functionally active chondrocytes [11, 12]. At the same time, foreign material in the graft

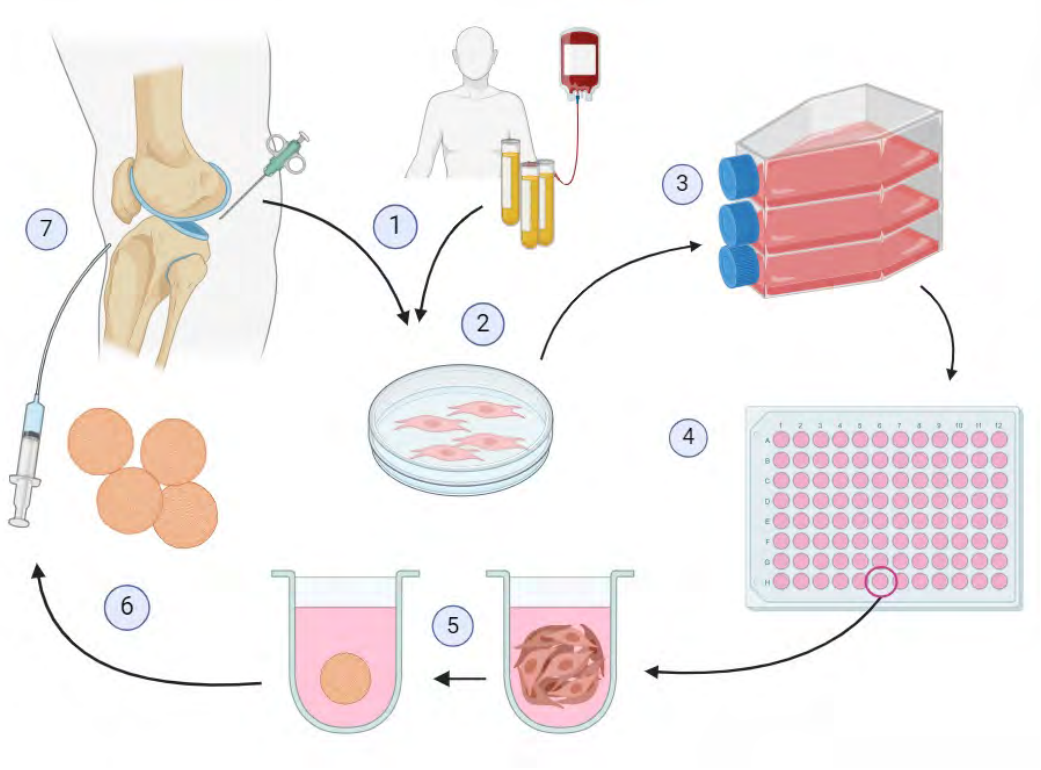

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Fig. 1. Scheme for the production of the cell-based medicinal product (CBMP) from autologous human chondrocytes formed as spheroids. 1, harvesting of a cartilage biopsy and autologous serum; 2, isolation of chondrocytes from the biopsy material; 3, cultivation of the chondrocytes in monolayer culture; 4, transfer of the chondrocytes to a 96-well plate with a non-adhesive coating to obtain spheroids; 5, formation of chondrocyte spheroids; 6, production of the finished CBMP dosage form; 7, transplantation of the CBMP into the patient.
Рис. 1. Схема получения биомедицинского клеточного продукта (БМКП) на основе аутологичных хондроцитов человека в виде сфероидов. 1 — отбор биоптата хряща, получение аутологичной сыворотки крови; 2 — выделение хондроцитов из биоптата; 3 — культивирование хондроцитов в монослойной культуре; 4 — пересев хондроцитов в 96-луночный планшет с неадгезивным покрытием для получения сфероидов; 5 — формирование сфероидов из хондроцитов; 6 — изготовление готовой формы БМКП; 7 — трансплантация препарата БМКП пациенту.

composition may cause immune and inflammatory reactions. Often, the use of three-dimensional matrices precludes minimally invasive implantation and complicates the surgical intervention. The current step in ACT technology development is the production of three-dimensional cell cultures without the use of matrices or other stimulation [13–15].

The Russian Federation has approved the first cell-based medicinal product (CBMP) Easytense® (GENERIUM JSC). Its clinical trial (CT) programme included a prospective, open-label, multicentre CT to evaluate the efficacy and safety of its transplantation in patients with knee defects measuring 1–10 cm^2. The CBMP comprises spheroids of autologous human chondrocytes bound by a matrix produced *de novo* by these cells *(Fig. 1)*. A major advantage of this CBMP is its fully autologous nature. The only starting materials used are the patient's own tissue biopsy and serum. No components of animal origin may be used in the cultivation of chondrocytes or in the formation of spheroids. The cell culture process that is free of additives (growth factors, inhibitors, etc.) provides this CBMP with advantages in efficacy and safety over other chondrocyte-based options. Another advantage of the product is its ability to attach to the surface of the damaged cartilage without any external help. As a result, the treatment is minimally invasive and does not require additional fixation stages, thereby reducing the duration of the surgical intervention and hospital stay, decreasing pain, and improving postoperative rehabilitation.

The research presented in this paper aimed to develop a manufacturing technology for the production of the autologous cell-based medicinal product comprising three-dimensional structures (3D-spheroids) based on chondrocytes isolated from the patient's cartilage tissue, as well as to evaluate its clinical efficacy.

MATERIALS AND METHODS

All steps of Easytense® production were performed under aseptic conditions in cell culture isolators (Comecer, Italy) equipped with CO_2 incubators, centrifuges, and microscopes [16].

Isolation of chondrocytes from cartilage biopsy and their in vitro cultivation

Chondrocyte isolation from human cartilage biopsy involved mechanical grinding and collagenase treatment (Nordmark, Germany) with constant stirring. After centrifugation, the precipitated chondrocytes were incubated at 37 °C and 5% CO_2 in culture flasks with a 1:1 mixture of αMEM medium and Ham's F12 medium (Corning, USA) supplemented with 2 mM L-glutamine (Corning, USA) and 10–15% autologous serum. The culture medium was refreshed every 3–4 days. Subconfluent cells (80–90%), as determined visually using an inverted microscope (Nikon, Japan), were dissociated with TrypLE™ (Gibco, USA). The next cell passage was performed at a density of 5,000–7,000 cells/cm^2. Cells were counted using a haemocytometer (INCYTO, Republic of Korea) and Trypan blue (Sigma-Aldrich, USA).

Formation of spheroids on a non-adhesive surface

To form the graft, passage 1 or 2 chondrocytes were transferred into the wells of a 96-well plate with a non-adhesive coating (Corning, USA), where the cells formed three-dimensional spheroids. The spheroids were cultured at 37 °C and 5% CO_2 in αMEM–Ham's F12 medium supplemented with 2 mM L-glutamine and 10% autologous serum. The culture medium was refreshed every 3–4 days.

Determination of the proliferative activity and population doubling time of cultured chondrocytes

The assessment of the proliferative activity of cultured chondrocytes involved calculating the population doubling level (PDL) and the population doubling time (PDT). This required a consideration of the cultivation time, the number of cells at the monolayer dissociation stage and at the next passage. The PDL was calculated using the following formula (1):

$$PDL = \frac{\ln (N/N_0)}{\ln 2}, \quad (1)$$

where N_0 is the initial number of cells and N is the number of cells at time T.

PDT was calculated using following formula (2):

$$PDT = \frac{T}{PDL}, \quad (2)$$

where T is the cultivation time.

Additionally, the proliferative activity of chondrocytes was assessed using an immunocytochemical assay based on detecting the expression of the Ki-67 proliferation marker [17]. Microscopy was used to calculate the number of cells positively stained with antibodies against Ki-67 and the total number of cells in several fields of view, and then the percentage of mitotically active cells was calculated.

Karyological analysis

For karyology, metaphase chromosome preparations were obtained by a standard procedure. Individual chromosomes were identified by Mitelman's differential Giemsa staining technique [18]. The analysis was performed using an Axio Imager Z2 microscope (Carl Zeiss, Germany) and Ikaros software (MetaSystems, Germany). An immersion lens (×100) was used for chromosome analysis. At least 50 metaphases were counted at passage numbers exceeding the number required for CBMP production. The karyotype interpretation procedure used the criteria of the International System for Human Cytogenomic Nomenclature[1].

Immunohistochemical staining

Cultured chondrocytes were fixed in 4% formaldehyde to analyse the expression of chondrogenic markers. Spheroids were embedded in Tissue-Tek® O.C.T. Compound medium (Sakura Finetek, Japan) and frozen. Afterwards, 8 μm sections were obtained using a CM1520 cryostat (Leica, Germany), transferred to a warm glass slide, and fixed in cold acetone (–20 °C).

The fixed samples were stained with primary antibodies for 12–15 hours at 4 °C. The study used antibodies against the following proteins: Sox9 (1:200; Abcam, UK), aggrecan (1:10; R&D Systems, USA), type II collagen (1:100; Abcam, UK), type I collagen (1:200; Abcam, UK), and Ki-67 (1:200; Abcam, UK). When the primary antibodies were washed off, the samples were incubated with the appropriate fluorochrome-conjugated secondary antibodies (Alexa Fluor 488, Alexa Fluor 546; 1:500; Life Technology, USA) for 1 hour at room temperature. After washing, the samples were coated with Vectashield mounting medium (Vector Laboratories, USA) containing 4',6-diamidino-2-phenylindole (DAPI) dye for nuclear staining. The stained samples were analysed using the Axio Imager Z2 microscope with appropriate fluorescence filters.

[1] ISCN 2016: An international system for human cytogenomic nomenclature. Reprint of Cytogenetic and Genome Research. 2016;149(1–2).

The photograph is taken by the authors / Фотография выполнена авторами

Fig. 2. Cell culture isolator used for the production of the cell-based medicinal product Easytense®.
Рис. 2. Изолятор клеточных культур, используемый для производства биомедицинского клеточного продукта Изитенс®.

Clinical trials and statistical analysis

Easytense® has been studied in the HACS-KCD-III Prospective, Comparative, Open-Label, Multicentre Clinical Trial of the Efficacy and Safety of Transplantation of GNR-089 (Spheroids of Human Autologous Matrix-Bound Chondrocytes) (GENERIUM JSC, Russia) in Patients with Knee Defects of 1–10 cm^2. The CT protocol was approved by the Ministry of Health of Russia (CBMP Clinical Trial Authorisation No. 1 of 12 March 2021), the Ethics Council of the Ministry of Health of Russia (Meeting Minutes No. 1 of 10 August 2020), and the independent local ethics committees of the clinical sites. The CT was conducted at 8 clinical sites in Russia in accordance with the current Good Clinical Practice[2] and the ethical principles for medical research involving human subjects set forth in the Declaration of Helsinki of the World Medical Association[3].

The CT enrolled 104 patients aged 18 to 50 years, in accordance with the inclusion criteria, with one isolated cartilage lesion of Grade III or IV according to the International Cartilage Repair Society (ICRS) grading system [19]. Patients were divided into two groups according to the size of their chondral lesions. Group I included 52 patients with lesions from ⩾1 to <4 cm^2, and Group II included 52 patients with lesions from ⩾4 to <10 cm^2. All patients gave informed consent to participate in the CT prior to any protocol procedures. Patients received 10–70 spheroids per 1 cm^2 of cartilage lesion after debridement.

An interim assessment was performed 12 months after CBMP transplantation. Follow-up and assessment of the functional status will continue for 36 months after transplantation, as required by the CT protocol.

The efficacy of the treatment of articular cartilage lesions was evaluated using a series of specialised functional tests and the Magnetic Resonance Observation of Cartilage Repair Tissue (MOCART) score at 12 months after CBMP administration. Additionally, 9 patients of Group II underwent follow-up arthroscopy. The primary efficacy endpoint of the

[2] Integrated addendum to ICH E6(R1): Guideline for good clinical practice E6(R2). https://database.ich.org/sites/default/files/E6_R2_Addendum.pdf
[3] WMA Declaration of Helsinki: Ethical principles for medical research involving human subjects. https://www.wma.net/policies-post/wma-declaration-of-helsinki-ethical-principles-for-medical-research-involving-human-subjects/

Table 1. Main characteristics of the production process and the cell-based medicinal product
Таблица 1. Основные характеристики производственного процесса и биомедицинского клеточного продукта

Characteristics *Характеристика*	**Description** *Описание*	
Cell culture *Культура клеток*	Human autologous chondrocytes *Аутологичные хондроциты человека*	
Number of passages *Количество пассажей*	Not more than two *Не более двух*	
Culture medium *Питательная среда*	Chemically defined medium (Ham's F-12; αMEM) with autologous human serum *Химически определенная среда (Ham's F-12; αMEM) с аутологичной сывороткой крови человека*	
Production environment *Производственная среда*	cGMP grade isolator, cleanliness grade A/ISO 5 *Изолятор класса cGMP, класс чистоты A/ISO 5*	
Cell culture isolator parameters *Параметры изолятора клеточных культур*	Incubator CO_2 levels *Концентрация CO_2 в инкубаторе*	4–6%
	Incubator temperature *Температура в инкубаторе*	36–38 °C
	Laminar flow velocity *Скорость ламинарного потока*	0.36–0.54 m/s 0,36–0,54 м/с
	Chamber overpressure *Избыточное давление в камере*	Not less than 50 Pa *Не менее 50 Па*
	Maximum permitted airborne particle concentration for Grade A clean zones *Нормы концентрации аэрозольных частиц в зоне чистоты класса А*	Not more than 3,520 particles above 0.5 μm per m^3 *Частицы размером более 0,5 мкм — не более 3520/$м^3$*
		Not more than 20 particles above 5.0 μm per m^3 *Частицы размером более 5,0 мкм — не более 20/$м^3$*
	Microbial contamination limits for Grade A clean zones *Пределы микробной контаминации для класса чистоты А*	<1 CFU/settle plate in 4 h (sedimentation method) *<1 КОЕ/чашку Петри за 4 ч методом седиментации*
		<1 CFU/m^3 (aspiration method) *<1 КОЕ/$м^3$ воздуха методом аспирации*
		<1 CFU/contact plate (imprint method) *<1 КОЕ/пластину методом отпечатка*
Product *Продукт*	Spheroid suspension for intra-articular injection in 0.9% sodium chloride solution *Суспензия сфероидов для внутрисуставного введения в 0,9% растворе натрия хлорида*	
CBMP dosage *Дозировка БМКП*	10–70 spheroids per 1 cm^2 of joint cartilage defect *10–70 сфероидов на 1 $см^2$ дефекта суставного хряща*	

The table is prepared by the authors using their own data / Таблица составлена авторами по собственным данным

Note. CFU, colony-forming unit; CBMP, cell-based medicinal product.
Примечание. КОЕ — колониеобразующая единица, БМКП — биомедицинский клеточный продукт.

CT was the change in the total Knee Injury and Osteoarthritis Outcome Score (KOOS).

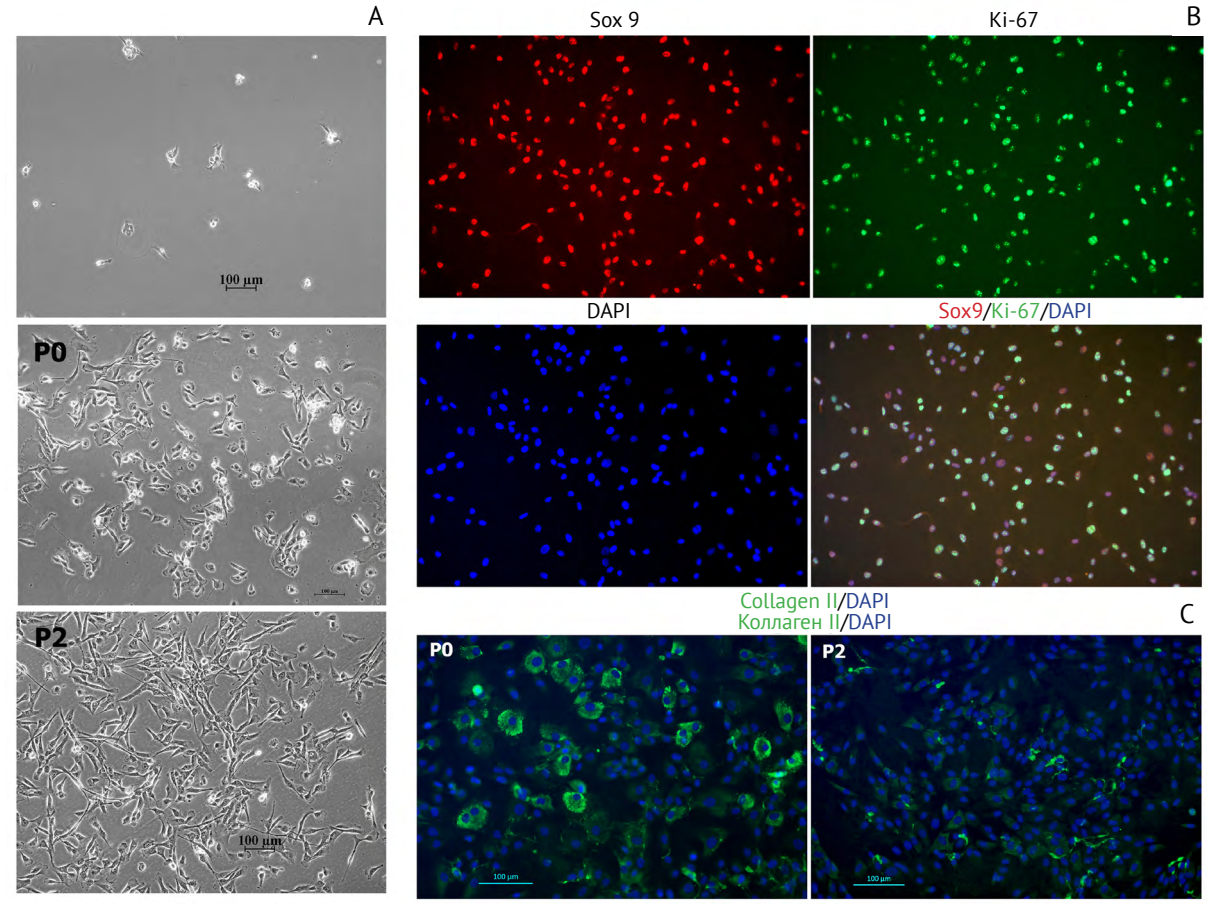
Paired *t*-tests were used to analyse the changes in the total score and the scores on the five subscales of the KOOS scale, as well as other endpoints in the form of numerical variables at different time points from baseline. The MRI data were processed using descriptive statistics.

RESULTS AND DISCUSSION

Key features of the CBMP manufacturing process

An isolator-based technology has been developed for the production of Easytense®. *Figure 2* shows a general view of a cell culture isolator consisting of three connected units.

Since CBMP batches are not subject to terminal sterilisation, the manufacturing process should meet the stringent standards for aseptic processing. All production stages of the CBMP are performed in cell culture isolators under aseptic conditions. The isolator technology provides conditions equivalent to those in a Grade A cleanroom (according to Good Manufacturing Practice) across the entire CBMP manufacturing process, from culture through cell sampling for quality control to the product ready for implantation. Materials and consumables can be



The photographs are taken by the authors / Фотографии выполнены авторами

Fig. 3. Cultivation of chondrocytes in monolayer culture. A: attachment of isolated chondrocytes to the culture flask surface (top), cell morphology during the initial (baseline) passage (P0) and the second passage (P2) visualised by phase-contrast microscopy. B: immunocytochemical detection of Sox9, a chondrocyte marker (red), and Ki-67, a cell proliferation marker (green), visualised by immunocytochemical staining at the first passage, with nuclei counter-stained with DAPI, 4',6-diamidino-2-phenylindole (blue). C: reduction of collagen II expression in monolayer culture of chondrocytes from the baseline passage (P0) through the second passage (P2) visualised by immunocytochemical staining.

Рис. 3. Культивирование хондроцитов из биоптата хряща в монослойной культуре. А — прикрепление выделенных хондроцитов к поверхности культурального флакона (верхняя фотография), морфология клеток на первичном (нулевом) пассаже (фотография P0) и втором пассаже (фотография P2); фазово-контрастная микроскопия; В — выявление маркера хондроцитов Sox9 (красный цвет) и маркера пролиферирующих клеток Ki-67 (зеленый цвет); иммуноцитохимическое окрашивание на первом пассаже; ядра клеток докрашены красителем DAPI (синий цвет); С — снижение уровня экспрессии коллагена типа II при культивировании хондроцитов в монослое от нулевого (P0) до второго пассажа (P2), иммуноцитохимическое окрашивание.

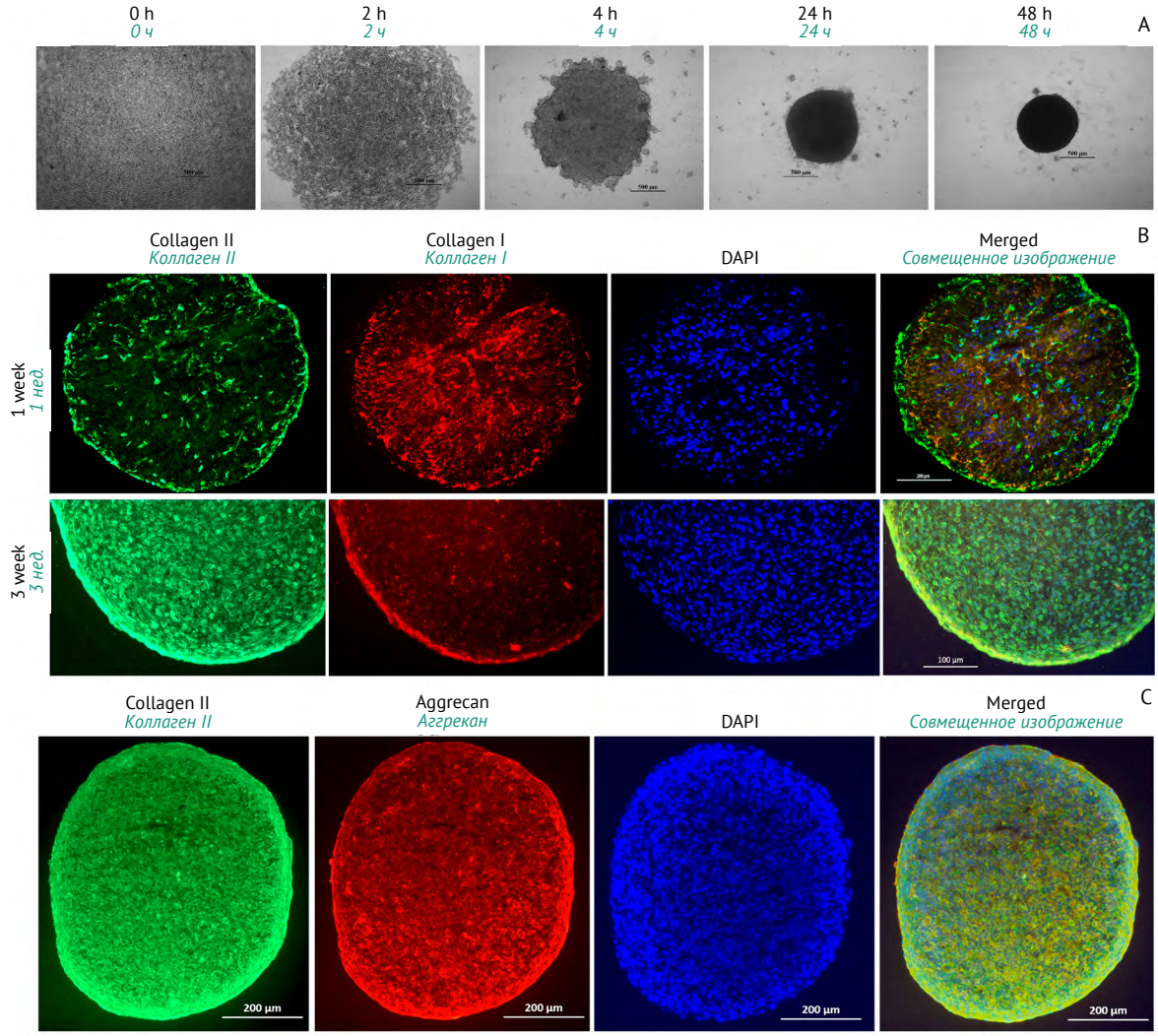
transferred into the isolator without compromising the aseptic conditions — through an airlock with hydrogen peroxide vapour bio-decontamination. This isolator-based technology eliminates direct contact between personnel and the product, thereby reducing the risk of microbial contamination and cross-contamination of the CBMP.

The CBMP manufacturing process was validated during the manufacture of clinical batches. All the controlled process parameters met the acceptance criteria. The process provided CBMP batches that met the requirements of the finished product specification. The specification contains information about the autologous CBMP, its qualitative and quantitative composition, and its biological and other characteristics [20].

The main characteristics of the cell product and its manufacturing process are presented in *Table 1*.

Chondrocyte isolation and culture

After mechanical grinding and enzymatic digestion of the cartilage matrix from cartilage biopsy, the isolated chondrocytes were inoculated into culture flasks with medium supplemented with autologous serum. The chondrocytes were cultured under stand-



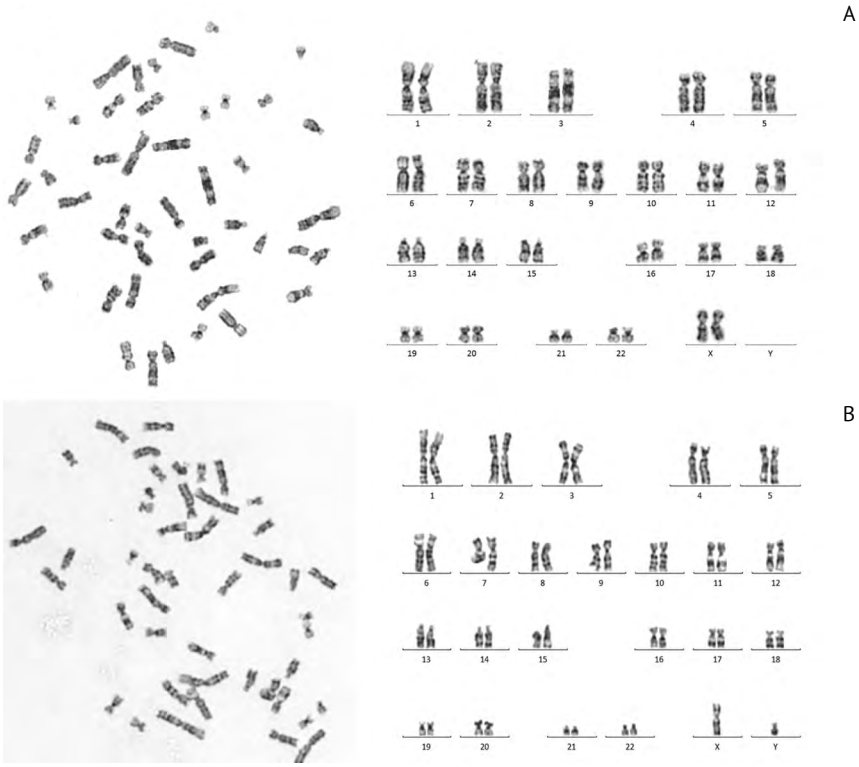
The photographs are taken by the authors / Фотографии выполнены авторами

Fig. 4. Cultivation of spheroids from chondrocytes isolated from a cartilage biopsy. A: formation of spheroids in culture plate wells with a non-adhesive coating in 0, 2, 4, 24, and 48 h, visualised by phase-contrast microscopy. B: switching from collagen I to collagen II synthesis during spheroid cultivation for 1 to 3 weeks with the expression of collagen II, a cartilage marker, increasing (green) and the expression of collagen I declining (red) by week 3, visualised by immunocytochemical staining, with nuclei counterstained with DAPI, 4',6-diamidino-2-phenylindole (blue). C: expression of the cartilage-specific markers, collagen II (green) and aggrecan (red), visualised by immunocytochemical staining, with nuclei counterstained with DAPI, 4',6-diamidino-2-phenylindole (blue).

Рис. 4. Культивирование сфероидов из хондроцитов, выделенных из биоптата хряща. А — формирование сфероидов в лунках с неадгезивным покрытием через 0, 2, 4, 24 и 48 ч; фазово-контрастная микроскопия; В — переключение синтеза с коллагена типа I на коллагена типа II при культивировании сфероидов от 1 до 3 нед.: экспрессия маркера хрящевой ткани коллагена типа II (зеленый цвет) увеличивалась к 3 нед. культивирования, тогда как экспрессия коллагена типа I (красный цвет) снижалась; иммуноцитохимическое окрашивание, ядра клеток докрашены красителем DAPI (синий цвет); С — экспрессия маркеров хрящевой ткани — коллагена II (зеленый цвет) и аггрекана (красный); иммуноцитохимическое окрашивание, ядра клеток докрашены красителем DAPI (синий цвет).

ard conditions. When released from the matrix, the chondrocytes acquired the ability to divide rapidly in culture *(Fig. 3A,* P0*)*. Their high proliferative activity was confirmed by immunohistochemistry using antibodies against the Ki-67 proliferation marker *(Fig. 3B)*. At the first passage, the percentage of cells in the active phase of the cell cycle was greater than 75%. The primary (zero) passage chondrocyte culture had the highest proliferation rate, with an average PDT value of 48±15 hours. After subculture, the rate of cell division substantially decreased to 73±13 and 81±16 hours at passages 1 and 2, respectively.

As demonstrated above, the quantity of chondrocytes required for transplantation and cartilage repair could be produced within one or two passages.



The figure is prepared by the authors using their own data / Рисунок подготовлен авторами по собственным данным

Fig. 5. Karyotype of cultured (third-passage) human chondrocytes used in the production of the cell-based medicinal product (×100 magnification). A, 46 XX; B, 46 XY.
Рис. 5. Кариотип культивируемых хондроцитов человека, использованных при производстве биомедицинского клеточного продукта, на третьем пассаже (объектив ×100). А — 46 XX; В — 46 XY.

However, chondrocytes were shown to dedifferentiate and lose their typical phenotype when passaged in monolayer culture. The cells became elongated, spindle-shaped, and fibroblast-like *(Fig. 3A,* P2*)*. Immunocytochemical staining for the main chondrogenic markers (aggrecan, type II collagen, Sox9) revealed changes in the expression level of chondrocytes in monolayer culture. The production of type II collagen, which is characteristic of hyaline cartilage, decreased, while the production of type I collagen increased *(Fig. 3C)*. These results were consistent with literature data [21, 22]. According to previous studies, transplantation of dedifferentiated chondrocytes resulted in the formation of fibrous cartilage with poor biomechanical properties [23–25], which limited the use of cells cultured as a monolayer for damaged cartilage repair. Therefore, further product development was required.

Production of spheroids

One way to redifferentiate chondrocytes is to place them in three-dimensional culture conditions promoting the formation of spheroids, which enhances the expression of key cartilage-specific protein markers [26–28]. For CBMP production, chondrocytes were inoculated into wells with a non-adhesive coating, which prevented the cells from adhering to the bottom of the wells, increased their adhesion to each other, and contributed to the formation of three-dimensional cell structures (spheroids) *(Fig. 4A)*. Chondrocyte aggregation and spheroid formation in the wells continued for 2–3 weeks without any physical, chemical, or other stimulation.

The transition to a three-dimensional system induced the redifferentiation of dedifferentiated chondrocytes, which was accompanied by the restoration of their morphological and functional properties. During week 1 of spheroid culture, the intensity of type I collagen staining remained higher than that of type II collagen, which was detected only in individual cells. Over the next 2 weeks, however, the level of type II collagen increased significantly. The intensity of type II collagen staining became uniform throughout the spheroid (both in the cells and in the intercellular space), which indicated the formation of the cartilage-specific extracellular matrix. At the same time, the level of type I collagen expression decreased, and its staining was observed mainly in the flat cells of the outer layer of the spheroids, which replicated the formation

Table 2. Causes of knee cartilage defects in the patients enrolled in the clinical trial of the cell-based medicinal product
Таблица 2. Причины формирования дефектов хряща коленного сустава у пациентов при проведении клинического исследования биомедицинского клеточного продукта

Cartilage defect cause *Причина дефекта хряща*	**Group I, defect size from ≥1 to <4 cm^2, *N* (% of the group)** *Группа I, размер дефекта от ≥1 до <4 см^2, N (% по группе)*	**Group II, defect size from ≥4 to <10 cm^2, *N* (% of the group)** *Группа II, размер дефекта от ≥4 до <10 см^2, N (% по группе)*	**Total, *N* (%)** *Всего, N (%)*
Traumatic damage *Травматическое повреждение*	12 (23,07%)	15 (28,84%)	27 (25,96%)
Osteochondritis dissecans *Рассекающий остеохондрит*	14 (26,92%)	15 (28,84%)	29 (27,89%)
Osteoarthritis *Остеоартроз*	25 (48,08%)	22 (42,31%)	47 (45,19%)
Avascular necrosis *Аваскулярный некроз*	1 (1,92%)	0	1 (0,96%)
Total: *Всего:*	52	52	104

The table is prepared by the authors using their own data / Таблица составлена авторами по собственным данным

of a layer similar to the perichondrium *(Fig. 4B)*. In 2–3 weeks, stable expression of typical cartilage proteins, type II collagen and aggrecan, was observed *(Fig. 4C)*, and type I collagen was detected at negligible levels.

The obtained results confirmed a higher level of chondrocyte differentiation in spheroids compared with that in monolayer culture. The spheroids were shown to produce proteins characteristic of the cartilage tissue matrix, thus acquiring the ability to rapidly adhere to cartilage tissue and restore damaged cartilage.

Genetic stability of cultured chondrocytes

The genetic stability of the cultured cell lines used in CBMP production is a prerequisite for ensuring the safety of cell therapy [29]. Directive 2009/120/EC of the European Commission[4] places cell line karyology and genetic stability amongst the most important parameters for evaluating the quality and safety of a cell therapy product.

The genetic stability of the cultured chondrocytes was evaluated using karyotype analysis of differentially stained metaphase chromosomes. This method can detect quantitative and large structural chromosomal abnormalities. The karyotype analysis included counting chromosomes in the cell and detecting structural abnormalities, including single and paired fragments and dicentric, ring, and atypical monocentric chromosomes. The cells had a diploid set of chromosomes and a karyotype that corresponded to a normal human karyotype (46 XX or 46 XY) *(Fig. 5)*. No structural or quantitative chromosomal abnormalities of a clonal nature were detected in cultures maintained for at least three passages.

According to the karyotyping results, chromosomes of the cultured chondrocytes remain stable at passage 3. The CBMP is produced from chondrocytes cultured *in vitro* for a maximum of two passages. Passage 3 exceeds the passage level required for production. Therefore, the karyotyping results demonstrate the genetic safety of the product.

The number of population doublings during two passages of the chondrocyte culture was 7.5–10.7 PDLs (mean: 8.9±1.24 PDLs). Therefore, the PDL value of NMT 10 can be considered as the limiting value for the chondrocyte culture in order to maintain the genetic stability.

Development of a Quality Control Strategy for the CBMP

Since the CBMP based on spheroids of human autologous matrix-bound chondrocytes is intended for administration to the patient for whom it was produced, the product has a short shelf life (72 hours) and a limited batch size. Therefore, control samples are taken at various stages of the manufacturing cycle to perform all the tests necessary to confirm the quality of the product and its

[4] Commission Directive 2009/120/EC of 14 September 2009 amending Directive 2001/83/EC of the European Parliament and of the Council on the Community code relating to medicinal products for human use as regards advanced therapy medicinal products.

compliance with Order of the Ministry of Health of Russia No. 14n[5].

Sterility is a critical quality parameter for CBMPs. This study controlled the parameter 4 times throughout the production process, from the sterility control of the transport medium for the cartilage tissue specimen to the retrospective sterility control of the finished product. An automated colorimetric system (BacT/ALERT® 3D Dual-T) was implemented to eliminate the possibility of errors due to the human factor and to increase the sensitivity of the analytical procedure for sterility control.

The identity and potency of chondrocyte-based medicinal products are controlled by detecting the expression of genes characteristic of human chondrocytes by reverse transcription-polymerase chain reaction followed by agarose gel electrophoresis. The expression of marker genes indicates that the cells in the finished product have the characteristics necessary to restore damaged hyaline-like cartilage tissue. The determination of viability and cell count of is spheroid formulation is performed in parallel with the packaging process of the finished product [20].

This batch quality control strategy ensures the availability of the results of all quality tests, except for the final sterility control of the finished product, which is conducted retrospectively, by the time the product is shipped to the healthcare provider. For the technical impossibility of obtaining all the results by the time of shipment, the product is released in two steps in accordance with the procedure described in Order of the Ministry of Health of Russia No. 512n[6]. At the first step, those responsible for the manufacturing and quality control of the product evaluate the records related to the manufacturing process of the CBMP batch, the monitoring results for the cleanroom environment, manufacturing conditions, and deviations from the established procedures, and the control results for the initial CBMP batch release. Upon receipt of all these results, the qualified person evaluates the completed record of the CBMP batch for the final confirmation of its compliance with the Russian marketing authorisation requirements.

Clinical use of a biomedical cell product

The clinical trial included patients aged 18–49 years; the average age was 33.5±8.7 years. The patients were divided into two groups according to the size of their chondral defect. Group I included 52 patients with defects of ≥1 to <4 cm^2, while Group II included 52 patients with defects of ≥4 to <10 cm^2. There were no significant differences between the groups in terms of the patients' age, height, weight, and body mass index (p>0.05). The causes of knee joint cartilage defects were also comparable between the groups (p>0.05) *(Table 2)*.

The analysis of changes in the total KOOS score at 12 months after CBMP administration showed a statistically significant improvement from baseline (p<0.0001). The mean change in the total KOOS score was 23.8±15.91, which was more significant in Group II (28.12±15.11) than in Group I (19.14±15.59).

The analysis of the KOOS subscale scores provided the same qualitative result as that of the total KOOS scores: the scores for each subscale at 12 months after transplantation improved relative to the baseline (p<0.0001). The mean score changes for the KOOS subscales ranged from 16.1±14.41 to 35.1±28.76.

The general MRI assessment results according to the MOCART scale also showed an improvement in the condition of knee cartilage at 12 months after transplantation in individual groups and in total (p<0.0001).

The evaluation of the articular cartilage condition during the follow-up arthroscopy in 9 patients of Group II showed a high degree of recovery; the recovered cartilage was comparable to the surrounding cartilage in 8 patients, and only 1 patient showed a recovery of 75% of the defect depth. The integration of the border zone to the adjacent cartilage was complete in 5 patients, and a demarcation line of less than 1 mm was observed in the remaining 4 patients. The overall assessment of cartilage repair in all patients was high. The cartilage condition corresponded to Grade II (nearly normal) according to the scoring system developed by L. Peterson and al. [19, 24]. These results confirmed the transplantation success of the spheroid-based CBMP.

In addition, the study analysed the proliferative activity of cultured chondrocytes as a function of patient age, sex, and diagnosis. The proliferative activity of chondrocytes did not correlate with age and sex. However, subgroups of patients with different diagnoses (traumatic cartilage injury, osteoarthritis, and osteochondritis dissecans) had statistically significant intergroup differences (p<0.0001) in the PDT. The chondrocytes isolated from the cartilage tissue of patients with traumatic cartilage lesions

[5] Order of the Ministry of Health of the Russian Federation No. 14n dated 19 January 2017, On Approval of the Specification Form for a Cell-Based Medicinal Product (CBMP).
[6] Order of the Ministry of Health of the Russian Federation No. 512n dated 8 August 2018, On Approval of the Good Practice Requirements for Working with Cell-Based Medicinal Products.

and osteochondritis dissecans had the highest proliferative activity, with the PDTs of 62.9±13.95 hours and 67.4±15.46 hours, respectively. The proliferative activity of the chondrocytes of patients with osteoarthritis was the lowest; the PDT in this group was 81.3±25.30 hours. However, the analysis of the KOOS score changes in these groups did not reveal statistically significant differences between them at 12 months after transplantation. The mean changes in the total KOOS scores ranged from 22.2±19.67 to 22.9±16.35. This indicated a lack of correlation between the proliferative activity of the cultured chondrocytes and the efficacy of the CBMP based on these chondrocytes.

The efficacy of the product was shown to be independent of the dose defined as the number of transplanted spheroids per square centimetre of the articular cartilage lesion. The change in the total KOOS score was 24.07±14.48 with transplantation of up to 30 spheroids per 1 cm^2 of cartilage defects, 21.83±16.66 with 30 to 40 spheroids/cm^2, and 18.86±20.19 with more than 40 spheroids/cm^2.

CONCLUSIONS

The manufacturing technology of the Russian product Easytense® (GENERIUM JSC, Cell-Based Medicinal Product Clinical Trial Authorisation No. 1 (БМКП No. 1)) in cell culture isolators is validated and has repeatability and robustness, which guarantees the production of the autologous CBMP that meets the specification for the finished product.

According to the results of the karyological analysis, the chondrocyte cell line maintains its genetic stability during manufacturing of the CBMP.

Clinical data have shown an improvement in the knee cartilage condition at 12 months after transplantation. According to the results, the patient's age, sex, or defect area are not limiting factors for cartilage defect treatment using autologous chondrocyte spheroid transplantation. A slight reduction in the proliferative activity of chondrocytes has been reported in patients with osteoarthritis compared with that in patients with traumatic cartilage injury and osteochondritis dissecans. It may take longer to manufacture the product for patients with osteoarthritis, but the level of proliferative activity does not affect the treatment outcome. The medicinal product is highly effective for cartilage repair at the established dose range of transplanted spheroids.

Easytense® has been authorised and implemented into clinical practice in the Russian Federation on the basis of clinical trial results. The CBMP may become an alternative to endoprosthesis surgery.

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