ANIMAL PATHOLOGY, MORPHOLOGY, PHYSIOLOGY, PHARMACOLOGY AND TOXICOLOGY

ПАТОЛОГИЯ ЖИВОТНЫХ, МОРФОЛОГИЯ, ФИЗИОЛОГИЯ, ФАРМАКОЛОГИЯ И ТОКСИКОЛОГИЯ

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Development of a Methodology for Obtaining Connective Tissue Bioequivalent from Rabbits

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Abstract

Introduction. The potential of cell therapy and tissue engineering technologies in veterinary medicine is quite high. However, the use of these technologies in the Russian Federation is currently limited due to the absence of standardized protocols for cell isolation, donor selection and creation of tissue equivalents. Development of a methodology for obtaining connective tissue bioequivalent is particularly relevant for clinical veterinary medicine, as connective tissue constitutes up to the half of the body weight and ensures the normal functioning of skin, mucous membranes, and internal organs of animals. The aim of this study is to develop a methodology for obtaining connective tissue equivalent from rabbits.

Materials and Methods. The study was conducted at Don State Technical University (DSTU) from November 13, 2023 to March 17, 2025. The objects of the study were multipotent mesenchymal stem cells (MMSCs) and fibroblasts from adult male rabbits. Enzymatic methods were used to isolate MMSCs from the greater omentum and fibroblasts from the animal skin. Stable cell lines were obtained, and their differentiation potential was studied *in vitro* during myogenic and lipogenic induction. Connective tissue equivalents were created using 3D extrusion bioprinting, their morphological properties were studied by means of light, confocal, and electron microscopy.

Results. Application of the sets of factors during induction ensured the adipogenic and myogenic differentiation of MMSCs. Adipogenic differentiation came along with formation of lipid droplets, while myogenic differentiation — with formation of myotubes. 3D bioprinting enabled creation of connective tissue equivalents with maintained cell viability, developing intercellular contacts, and active secretion for at least 72 hours.

Discussion and Conclusion. A new approach to obtaining connective tissue equivalents from rabbits was developed by optimizing MMSCs isolation and differentiation techniques. The resulting constructs demonstrated morphological and functional activity, thus, confirmed their potential for using in clinical veterinary medicine for regeneration of connective tissue and for experimental studies.

Keywords: tissue engineering, cell culture, mesenchymal stem cells, MMSCs, tissue equivalent, bioequivalent, rabbit, lipoblasts, fibroblasts, transmission electron microscopy

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Оригинальное эмпирическое исследование

Разработка методики получения биоэквивалента соединительной ткани кролика

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Аннотация

Введение. Клеточная терапия и тканевая инженерия обладают значительным потенциалом применения в ветеринарии, однако использование данных технологий в Российской Федерации в настоящее время ограничено из-за отсутствия стандартизированных протоколов выделения клеток, подбора доноров и создания тканевых эквивалентов. Особую актуальность для клинической ветеринарии имеет разработка методики получения биоэквивалента соединительной ткани, ведь она составляет до половины массы тела и является основой для нормального функционирования кожи, слизистых оболочек и внутренних органов животных. Цель исследования — разработать методику получения тканевого эквивалента соединительной ткани кролика.

Материалы и методы. Исследование проведено на базе ДГТУ в период с 13 ноября 2023 г. по 17 марта 2025 г. Объектом исследования выступили мультипотентные мезенхимальные стволовые клетки (ММСК) и фибробласты взрослых самцов кролика. Ферментными методами были выделены ММСК из большого сальника и фибробласты из кожи животных. Получены стабильные культуры клеток, исследован их дифференцировочный потенциал при миогенной и липогенной индукции *in vitro*. С применением экструзионной 3D-биопечати созданы эквиваленты соединительной ткани, морфологические свойства которой изучены с помощью световой, конфокальной и электронной микроскопии.

Результаты исследования. Индукция наборами факторов обеспечила дифференцировку ММСК в адипо- и миогенном направлении. Адипогенная дифференцировка сопровождалась образованием липидных капель, миогенная — формированием миотрубочек. 3D-биопечать позволила сформировать эквиваленты соединительной ткани с сохранением жизнеспособности клеток, развитием межклеточных контактов и активной секрецией в течение не менее 72 ч.

Обсуждение и заключение. Разработан новый подход к получению тканевых эквивалентов соединительной ткани кролика благодаря оптимизации методов выделения и дифференцировки ММСК. Сформированные конструкты продемонстрировали морфофункциональную активность, что подтверждает перспективность их применения в клинической ветеринарии для регенерации соединительной ткани и в экспериментальных исследованиях.

Ключевые слова: тканевая инженерия, культура клеток, мезенхимальные стволовые клетки, ММСК, тканевой эквивалент, биоэквивалент, кролик, липобласты, фибробласты, трансмиссионная электронная микроскопия

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Introduction. Nowadays, cell technologies based on the use of cell cultures and tissue equivalents are being actively implemented in veterinary medicine. This can be explained by the need to reduce the ethical burden within the experimental research, by improvement of the *in vitro* experimental models, as well as by development of the regenerative medicine and implementation of the human medicine achievements into veterinary medicine [1, 2]. The benchmark for the development of this scientific field is implementation of the 3R bioethical principles — Replacement, Reduction, Refinement [3] — aimed at optimisation of the use of animals in experiment to stimulate transition to *in vitro* models [4]. Cell cultures and tissue equivalents used as experimental models represent practical implementation of these principles enabling not only to replace animals in research and reduce their number, but also to enhance accuracy and reproducibility of results through standardization [5].

A tissue equivalent (bioequivalent) is a bioengineered construct — an analogue of a tissue or organ. The key properties of a tissue equivalent are its cellular composition, the composition and properties of the intercellular substance, and a specific histotypic architecture [6].

Connective tissue constitutes approximately 50% of animal body weight and performs critical functions: supporting-motor, reparative and metabolic. Its cells influence the differentiation of epithelial and muscle cells and angiogenesis. Connective tissue is involved in the most functions of the body in the normal condition, and is always involved in pathological processes and participates in regeneration [7].

Therefore, the development and clinical implementation of connective tissue bioequivalents for the temporary or permanent replacement of defects and damages of native tissues is highly requested in the regenerative medicine.

Since 2025, the U.S. Food and Drug Administration (FDA) has officially recommended the use of *in vitro* platforms for toxicologal studies and evaluation of the efficacy of new pharmacological agents emphasising that cell models, organoids, and tissue equivalents more accurately reproduce the molecular and cellular mechanisms of pathogenesis, reduce inter-experimental variability, and increase the predictive capacity of the data obtained [8, 9]. The integration of *in vitro* systems at the preclinical stage of drug development makes it possible to reduce the number of rejections at later stages of clinical trials and optimize risk management when testing new therapeutic strategies [5, 9]. Modern research confirms that the use of cell cultures provides reproducible and statistically reliable data using smaller groups of animals compared to the traditional *in vivo* models, which minimizes bioethical risks and increases the rationale of scientific approaches [5]. Alongside, the direction of modifying cellular systems is developing to create highly accurate models of specific pathologies, including infectious, oncological and metabolic ones, which enables in detail study of the molecular mechanisms of pathogenesis and search for new targets for potential medicinal compounds [10, 11].

In veterinary regenerative medicine, the use of multipotent mesenchymal stem cells (MMSCs) is being actively implemented for the treatment of osteoarthritis in horses and spinal injuries in dogs and cats [12]. The clinical efficacy of MMSCs has been verified in studies in dogs, where intraarticular administration of autologous MMSCs improved the function of a joint and reduced pain [13, 14]. Similar results have been obtained in the treatment of horses with ligament injuries [14–16]. Stem cell therapy proved to be efficient in the treatment of acute and chronic skin lesions in dogs [12].

3D bioprinting technology is getting improved, this enables creation of multilayer tissue constructs from cells and biocompatible polymers that are similar in architecture and mechanical properties to the native body tissues. The use of bioprinted implants for the restoration of bone defects in pets improves osseointegration and accelerates healing [17]. 3D bioprinted tissue constructs are used in screening and research within antitumor and radiation therapy in animals. Phantoms made from tissue equivalents can be used to measure and verify radiation dose distribution, ensuring the accuracy of radiation treatment plans for pets and calibrating equipment [18].

In veterinary medicine, three-dimensional tissue equivalents allow creating the personalized animal tumor models using various cells and biomarkers for the selection of targeted chemotherapy and immunotherapy in the treatment of oncological diseases [12]. Also, tissue equivalents of skin and connective tissue, constructed from the appropriate cell cultures and matrix, can be used to treat animal burns and are a promising direction in the treatment of wounds in animals in general [19, 12]. Significant experience in this field has been accumulated worldwide [20, 12], however, in the Russian Federation, there is still no production of affordable tissue equivalents with cells for the treatment of wounds in both humans and animals, since standardized protocols for cell isolation, donor selection, and obtaining bioequivalents have not been yet developed. Thus, the aim of the study is to develop a methodology for creating connective tissue equivalents, which performs vital functions in the body, from rabbit biomaterials.

Materials and Methods. The study was conducted from November 13, 2023, to March 17, 2025, at the Faculty of Bioengineering and Veterinary Medicine, DSTU (Rostov-on-Don). All cell culture experiments were performed in the Cell Technologies Laboratory; the laboratory animals were maintained and handled in the faculty's vivarium in accordance with the acting regulations.

Adult male Soviet Chinchilla breed rabbits (*Oryctolagus cuniculus domesticus*) served as donor animals (n=3). To isolate MMSCs, 2 g of adipose tissue fragments were collected from the greater omentum via percutaneous biopsy; abdominal skin biopsies were used to isolate fibroblasts. To isolate MMSCs, fragments of greater omentum adipose tissue were incubated for 60 min in a 0.2% collagenase solution in Dulbecco's phosphate-buffered saline (DPBS) at 37°C with constant stirring. After filtering the resulting suspension through a cell strainer, the cell fraction was pelleted by centrifugation. The pellet was transferred to culture flasks containing DMEM/F12 medium supplemented with 10% fetal bovine serum (FBS) and 1 ng/ml recombinant basic human fibroblast growth factor-2. The isolated cell culture was subcultured for five passages and then cryopreserved.

Fibroblasts were isolated from skin biopsies. After antiseptic treatment, the biopsies were aseptically excised with scissors and transferred to a trypsin solution preheated to 37°C for 15 minutes to dissociate the cells. The cells were then pelleted by centrifugation, placed in culture flasks containing DMEM medium supplemented with 10% FBS, antibiotics, and antifungals, and incubated at 37°C with 5% CO2. Taking advantage of fibroblasts' ability to rapidly adhere to the flask surface, to separate them from other cell types, the flasks were washed after 4 hours to remove unattached cells and replaced with fresh nutrient medium. The procedure was repeated after 2 hours, after which the isolated cells were cultured to a subconfluent monolayer. Fibroblast subculture was performed without the addition of antibiotics or antifungals.

MMSCs were subjected to lipogenic and myogenic differentiation. The first was used to obtain lipoblasts, which

are necessary for the formation of a bioequivalent of connective tissue. Myogenic differentiation served as a marker of the multipotency of the isolated stem cells.

DMEM medium containing 4 mg/l glucose, 10% FBS, 1 μmol dexamethasone, 100 μmol indomethacin, 500 μmol 3-isobutyl-1-methylxanthine, and 10 μg/ml insulin were used as adipogenic differentiation inducers [21]. The cells were exposed to the inducers for 10 days, after which they were maintained in standard DMEM medium. As a negative differentiation control, MMSCs were cultured in complete DMEM medium without inducers. The formation of lipid droplets in the cell cytoplasm served as a differentiation marker [22].

DMEM medium containing 4 mg/l glucose, 10% FBS, 10 μmol 5-azacytidine, and 50 μmol hydrocortisone were used to induce myogenic differentiation. The cells were exposed to inducers for 15 days, after which they were maintained in standard DMEM medium. As a negative differentiation control, MMSCs were cultured in complete DMEM medium without inducers. Cell fusion to form myotubes was considered a marker of myogenic differentiation [23].

An improved AI-based algorithm, YOLOv8S-seg, developed and described previously by us [24], was used to count cells at all stages of culture.

A 3D bioprinting method was used to create connective tissue equivalents. To prepare the bioink, sodium alginate powder was sterilized under ultraviolet light in a biological safety cabinet for 40 minutes. The components were then dissolved in calcium- and magnesium-free DPBS at a concentration of 30 mg/ml. Three ml of the resulting hydrogel was transferred to a 5 ml syringe, and fibroblasts and lipoblasts were added at a 1:1 ratio, at a rate of 2 x 10^6 cells per 1 ml of hydrogel, by mixing them between two syringes containing the hydrogel, connected by an adapter.

The resulting bioink was transferred to the injector of a 3D bioprinter assembled on the basis of Anet A8 (*Anet*, China) and modified in-house as described previously in [21], and the printing process was carried out according to a given digital model with the following parameters: object dimensions 30 × 40 × 3 mm, layer height — 0.2 mm, number of perimeters — 0, number of solid layers at the bottom — 0, number of solid layers at the top — 0, fill — 8%, fill type — rectilinear, fill angle — 90°, fill speed — 10 mm/s, nozzle diameter — 0.2 mm, rod diameter — 1.2 mm, extruder feed multiplier — 1.3. Printing was carried out in a microbiological safety cabinet at room temperature in Petri dishes. After printing, the resulting scaffolds were filled with CaCl2 solution (100 mg/ml) for 10 min to stabilize the alginate. After hardening, the scaffolds were transferred to Petri dishes with DMEM medium and placed in a CO2 incubator at 37 °C for 72 h with the medium changed every 12 h.

After 72 hours of incubation, samples were collected from random sections of the tissue equivalents for microscopic analysis and prepared accordingly. The morphological properties of the resulting tissue equivalents were examined using an Olympus BX53 light microscope (*Olympus*, Japan), an Infinity Line confocal laser scanning microscope (*Abberior Instruments*, Germany), and a JEM-1011 transmission electron microscope (*Jeol*, Japan).

Results. The cell isolation and differentiation protocols used allowed us to obtain stable cell cultures that retained their phenotype and growth dynamics during subculture for 25 passages for fibroblasts and lipoblasts and 12 passages for myosatellite cells (Fig. 1).

The appearance of the Ca2+-stabilized construct after 3D bioprinting is shown in Fig. 2.

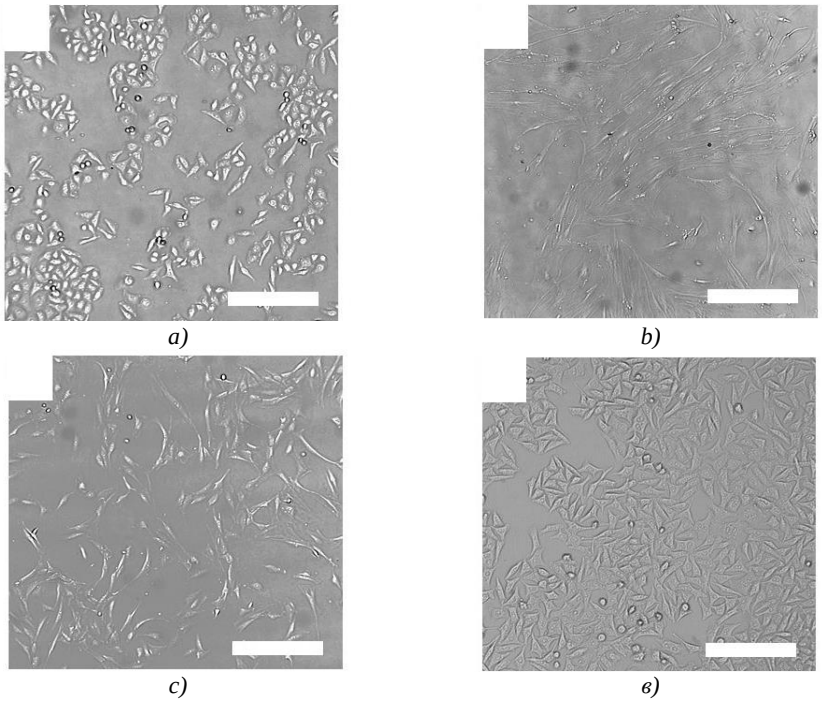


Fig. 1. Light microscopy of the obtained cell cultures, scale bar represents 200 μm: *a* — MMSCs; *b* — myosatellite cells; *c* — fibroblasts; *d* — lipoblasts

Fig. 2. Appearance of the 3D-bioprinted construct stabilized by Ca^{2+}

Figure 3 shows a micrograph of semi-thin sections of a fragment of the construct after 72 hours of cultivation. It contains round and spindle-shaped cells uniformly distributed within the matrix, with elongated processes and cytoplasmic outgrowths.

Three-dimensional reconstruction of a SYTOX® Green-stained tissue equivalent fragment after 72 h of cultivation (Fig. 4) demonstrates the integrity of cell nuclei and normal chromatin density

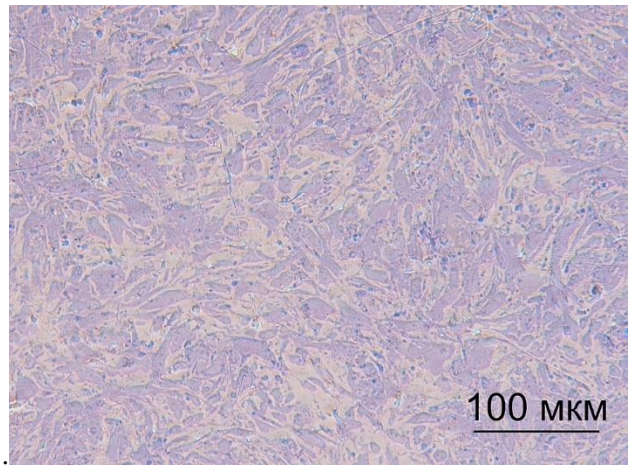


Fig. 3. Semi-thin section of tissue equivalent after 72 hours of cultivation, stained with methylene blue

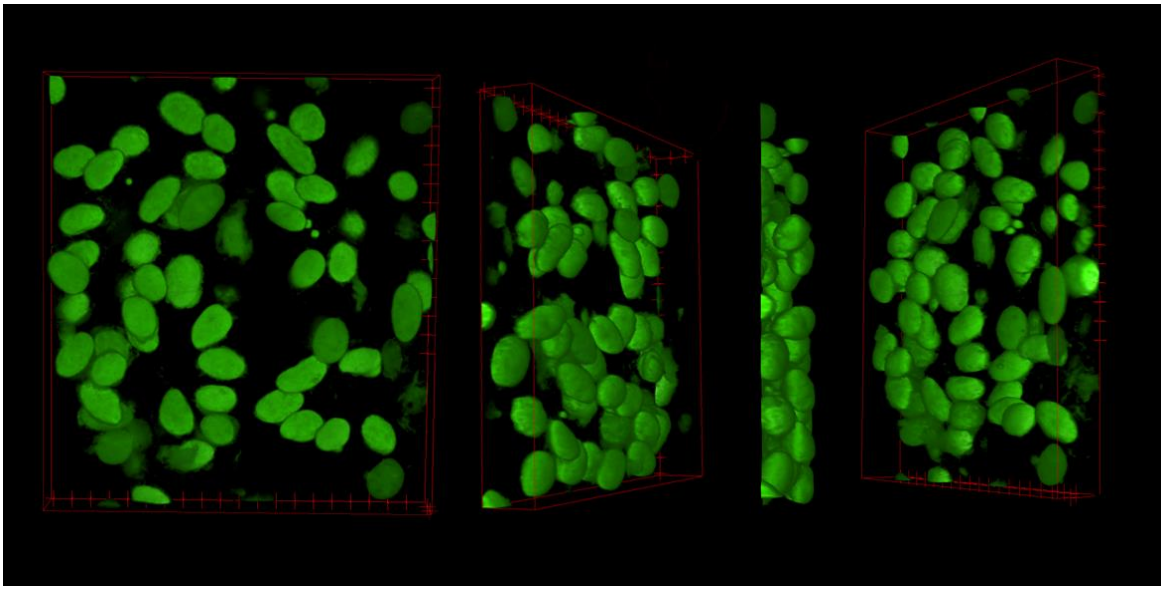


Fig. 4. Confocal laser scanning microscopy of a fragment of the construct stained with SYTOX® Green. Three-dimensional reconstruction was performed using ImageJ (Rasband, 1997–2018)

Transmission electron microscopy data reveal the ultrastructure of cells within the tissue equivalent and their intercellular interactions (Figs. 5–6). Electron diffraction patterns reveal two cell types with ultrastructural characteristics of fibroblasts and lipoblasts. Both cell types are spindle- or triangular-shaped, with branched, thin processes directed primarily along the cell bodies. In addition to these processes, the cells have irregularly shaped cytoplasmic outgrowths. A high cell density within the construct is also noted, indicating an increase in their number over 72 hours of cultivation, as such a density is unattainable at the initial concentration used during printing (2 × 106 cells per 1 ml of hydrogel). The cells within the construct are tightly adjacent to each other and are in contact with the membranes of their cell bodies and processes (Fig. 5). The cells contain regularly shaped nuclei with finely dispersed chromatin, with one or two large nucleoli visible within the nuclei—a sign of fibroblast activity [25–27]. In some cases, the cell nuclei had invaginations and protrusions of the karyolemma. The fibroblast cytoplasm is rich in organelles, reflecting a high level of intracellular metabolism. Numerous mitochondria, rough endoplasmic reticulum, the Golgi complex, lysosomes, centrioles, and multivesicular bodies are visible within the cytoplasm. The lipoblast cytoplasm contains numerous inclusions in the form of rounded osmophilic droplets, further confirming the lipogenic differentiation of MMSCs. Cells have a well-developed network of endoplasmic reticulum, which occupies most of the cytoplasm and is visualized as stacks of convoluted tubes and elongated cisterns studded with ribosomes on the side facing the cytoplasm. Ribosomes not attached to the reticulum are also evenly distributed throughout the cytoplasm.

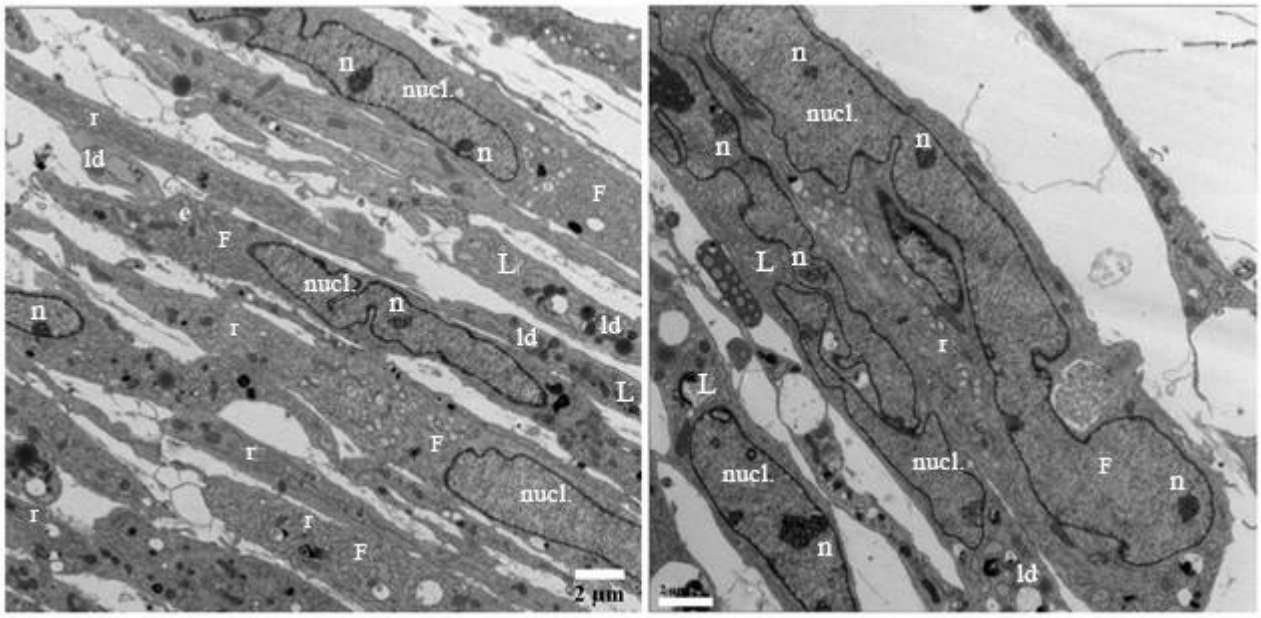
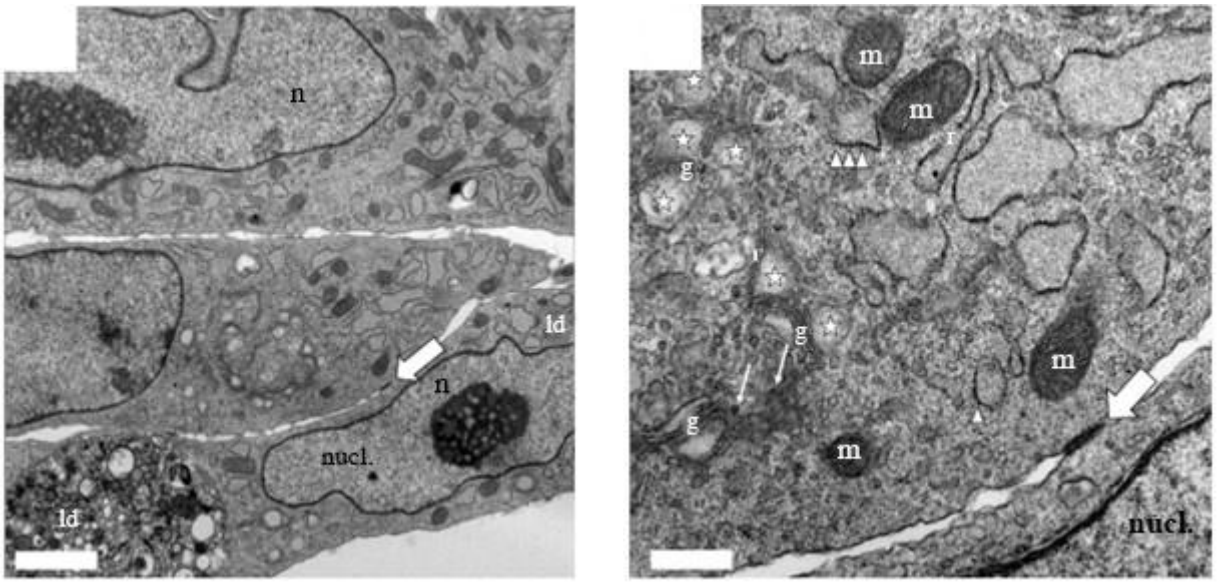


Fig. 5. Transmission electron microscopy of cells within a tissue equivalent, contrasted with osmium tetroxide, lead citrate, and uranyl acetate, scale bar represents 2 μm: F — fibroblast, L — lipoblast, nucl. — nucleus, n. — nucleolus, r —rough endoplasmic reticulum, ld— lipid droplets

Electron diffraction patterns of fibroblasts at higher magnification reveal activity of the Golgi complexes synthesizing procollagen (Fig. 6 *a*). Dictyosomes, transport vesicles, and cisternae are visible within the Golgi complexes. Some cisternae are dilated and filled with an osmium-dense, electron-dense substance resembling tangled threads, likely procollagen molecules. The contents of the vesicles gradually condense and transform into transport vacuoles, which collect into secretory granules on the side of the Golgi complex facing the plasma membrane. This pattern of Golgi complex activity also indicates high secretory activity of the cells within the construct for 72 hours.

Another important visualized element of intercellular interaction is the presence of tight junctions (Fig. 6 *a, b*), which appear as two osmium-filamentous seals with an intercellular cleft at the junction of the membranes of adjacent cells. A tight junction is a highly specialized, selective barrier that ensures direct contact between the proteins of two adjacent plasma membranes and allows groups of cells to function as structural units. Moreover, in the tissue equivalent sample, tight junctions were detected both between cells of the same type (fibroblast/fibroblast and lipoblast/lipoblast) and between cells of different types: for example, Fig. 6 *a, b* shows a tight junction between a fibroblast and a lipoblast.



a) *b)*

Fig. 6. Transmission electron microscopy showing signs of cell proliferation within the printed tissue equivalent after 72 hours of cultivation, contrasted with osmium tetroxide, lead citrate, and uranyl acetate, the *a* scale corresponds to 2 μm, the *b* scale to 0.5 μm. *a* — ultrastructure of the active fibroblast region. The center of the electron diffraction pattern is occupied by the Golgi complex region. Asterisks mark spherical expansions. The Golgi complex cisterns contain osmium-dense material in the form of tangled threads, which may be procollagen molecules. Vesicles with condensed contents are indicated by thin arrows: nucl. — nucleus, n — nucleolus, g — Golgi complex, m — mitochondrion, r — rough endoplasmic reticulum, ld — lipid droplets

A well-developed endoplasmic reticulum network [28], high activity of the Golgi apparatus [29], and numerous ribosomes indicate active protein synthesis processes and confirm the viability and good survival of cells within the tissue equivalent for at least 72 hours of cultivation. Transmission electron microscopy data also confirm the presence of cell differentiation markers: lipid droplets in lipoblasts and procollagen molecules in fibroblasts.

Discussion and Conclusion. This study developed a novel tissue-engineering method for producing cultured 3D connective tissue equivalents using differentiated rabbit MMSCs and 3D bioprinting. *In vitro* exposure of rabbit MMSCs to a set of inducers, including DMEM medium containing 4.5 g/l glucose, 10% FBS, 1 μM dexamethasone, 100 μM indomethacin, 500 μM 3-isobutyl-1-methylxanthine, and 10 μg/mL insulin, resulted in their adipogenic differentiation, the functional marker of which was the formation of lipid droplets. *In vitro* exposure of rabbit MMSCs to a set of inducers, including DMEM medium containing 4.5 g/l glucose, 10% FBS, 10 μM 5-azacytidine, and 50 μM hydrocortisone, induced their myogenic differentiation, the functional marker of which was myotube formation. The 3D extrusion bioprinting method ensured the formation of three-dimensional connective tissue equivalents, in which differentiated rabbit cells maintained viability and metabolic activity, forming tight junctions and secretory vesicles for at least 72 hours. Morphological analysis of the resulting bioequivalent, including confocal and transmission electron microscopy, confirmed the viability and high metabolic activity of the cells, indicating their potential to maintain the functionality of the construct.

The proposed method for creating tissue equivalents can be widely used: in clinical veterinary medicine for the treatment of connective tissue defects and injuries; in experimental veterinary medicine, as a model for studying the patterns of morphogenesis, cyto-, histo-, and organogenesis, cell differentiation and intracellular structures, intercellular interactions, regenerative processes in individual development, and their adaptation to the effects of exogenous and endogenous factors in animals at the macro-, micro-, and ultrastructural levels using morphological and other research methods; screening of new medicinal compounds; and as phantoms for developing approaches in radiation therapy and diagnostics. Furthermore, this method can be transferred to the development of cultured meat products, as it enables production of a final product with properties identical to those of productive animal tissues, including structural integrity and organoleptic properties.

A clear disadvantage of this technology is its high cost and the technological complexity of large-scale cell cultivation in standard laboratories. This high cost is primarily due to the most expensive component of the culture medium—fetal bovine serum. Despite numerous attempts to produce serum-free media and find serum substitutes, their efficiency in cultivation is currently inferior to that of serum. To reduce costs and optimize large-scale cell cultivation, it is advisable to use bioreactors, which, combined with the use of a 3D bioprinter, increases the skill requirements of operators.

The technological advantages of this method include the possibility of standardization and high reproducibility, which are ensured by the conditions of 3D bioprinting: the cell concentration in the hydrogel and their distribution within the construct will be consistent throughout reproduction, since printing is performed according to a predetermined digital template and its parameters are controlled at the hardware and software levels. The use of artificial intelligence-based algorithms for cell cultivation control significantly mitigates the influence of the human factor on the final result.

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