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BIOENGINEERED HYDROGEL-BASED SCAFFOLD FOR MUSCLE REGENERATION

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Introduction. Injuries, tumor resections, and degenerative diseases can lead to extensive skeletal muscle defects, and consequently, to functional impairment and severe disability. Over the past few decades, various approaches to skeletal muscle tissue engineering have been developed. However, there remains a high demand for new effective methods and materials that promote functional regeneration, which could be used in the clinical treatment of muscle defects.
Objective. Development of a bioengineered polymer hydrogel-based scaffold for extensive skeletal muscle defects.
Materials and methods. Bioengineered scaffold samples were obtained by hydrogel cryostructuring followed by lyophilization. The microstructure and morphology of the scaffold were analyzed using a Tescan VEGA III scanning electron microscope. The mechanical properties, tensile strength, and relative elongation were analyzed using an Instron ElectroPuls E3000 testing instrument. The cytotoxicity of the scaffold was studied using fluorescence microscopy on a culture of murine fibroblasts Balb/3T3 clone A31 and NCTC clone 929 culture. The statistical significance of differences was determined using a one-way analysis of variance (ANOVA) followed by the Holm–Šídák multiple comparison test, with the critical significance level α set at 0.05.
Results. A modified scaffold was developed, representing a layered construct based on a combination of natural polymers, collagen, and sodium hyaluronate, with the addition of crosslinking agents to form layers with varying resorption rates and proteolytic degradation profiles. The scaffold samples were found to possess a multilayered porous spongy structure. The core layer (1.2 ± 0.1 mm thick) exhibited high — uniform and interconnected — porosity with nearly spherical pores ranging from 100–150 μm in size and a polymer framework wall thickness of less than 5 μm. The outer layer (300–500 μm thick) had a denser, lamellar structure with a polymer framework wall thickness of less than 2 μm and slit-shaped pores up to 200 μm in length and 50 μm in width. Compared to the core layer, significantly lower pore interconnectivity was observed, a channel structure was virtually absent, and pores were mostly isolated from one another by polymer walls. Tensile strength was 50 ± 0.5 kPa, and relative elongation was 26 ± 6%. Sterilization had no effect on the strength and elongation parameters. The absence of a cytotoxic effect of the obtained construct was demonstrated: no statistically significant differences in the number of dead cells compared to the control were detected at any of the observation time points (24 and 96 h).
Conclusions. Scaffold samples with a porous structure were obtained using a combination of layer-by-layer casting/molding methods with stepwise freezing and structure control, followed by lyophilization. For the developed construct, an ethylene oxide sterilization protocol was established. The sterilization process was found to have no effect on the strength and elongation parameters. The developed methods for obtaining biocompatible constructs based on polymer hydrogels and their modification techniques will make it possible to obtain devices with a high degree of biocompatibility for enhanced tissue regeneration.

Keywords: bioengineered scaffold; collagen; sodium hyaluronate; albumin; hydrogel; cytotoxicity; mechanical properties

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БИОИНЖЕНЕРНЫЙ СКАФФОЛД НА ОСНОВЕ ГИДРОГЕЛЯ ДЛЯ РЕГЕНЕРАЦИИ МЫШЦ

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Введение. Травмы, резекции опухолей и дегенеративные заболевания могут привести к обширным дефектам скелетных мышц и, следовательно, функциональным нарушениям и тяжелой инвалидности. В последние десятилетия были разработаны различные стратегии тканевой инженерии скелетных мышц, однако по-прежнему наблюдается высокий спрос на создание новых

высокоэффективных методов и материалов, способствующих функциональной регенерации, которые могли бы быть использованы при терапии мышечных дефектов в клинической практике.
Цель. Разработка медицинского изделия (биоинженерного скаффолда) на основе полимерного гидрогеля для заживления обширных дефектов скелетных мышц.
Материалы и методы. Образцы биоинженерного скаффолда получены путем криоструктурирования гидрогеля с последующей лиофилизацией. Микроструктуру и морфологию скаффолда анализировали при помощи сканирующего электронного микроскопа Tescan VEGA III. Анализ механических свойств, прочность на разрыв и относительное удлинение проводили при помощи испытательной машины Instron ElectroPuls E3000. Цитотоксичность скаффолда была изучена с помощью флуоресцентной микроскопии на культуре мышиных фибробластов Balb/3T3 клон A31 и культуре NCTC клон 929. Статистическую значимость отличий определяли с помощью одностороннего дисперсионного анализа ANOVA с последующим множественным сравнением Холма – Сидака, критический уровень значимости α приняли равным 0,05.
Результаты. Разработан модифицированный скаффолд, представляющий собой слоистую конструкцию на основе комбинации натуральных полимеров, коллагена и гиалуроната натрия с добавлением сшивающих агентов для формирования слоев с различными сроками резорбции и протеолитической деградации. Выявлено, что скаффолды обладали многослойной пористой губчатой структурой: средний слой (толщиной 1,2 ± 0,1 мм) имел крупную равномерную взаимосвязанную пористость, форма пор была близка к сферической, размер 100–150 мкм, толщина стенок полимерного каркаса менее 5 мкм. Верхний слой (толщиной 300–500 мкм) имел более плотную слоистую структуру, толщина стенок полимерного каркаса менее 2 мкм, форма пор щелевидная размером до 200 мкм в длину и 50 мкм в ширину. В сравнении с центральным слоем наблюдали значительно меньше взаимосвязанности пор, также практически отсутствовала канальная структура, а поры в основном были изолированы друг от друга стенками полимера. Прочность на разрыв составила 50 ± 0,5 кПа, относительное удлинение 26 ± 6%; стерилизация не оказывала влияния на показатели прочности и относительное удлинение. Показано отсутствие цитотоксического действия полученной конструкции: статистически значимых различий в количестве погибших клеток относительно контрольных условий на всех сроках наблюдений (24 и 96 ч) выявлено не было.
Выводы. Пористая структура образцов скаффолда была получена с помощью комбинации методов послойной заливки / формования с пошаговой заморозкой и контролем структуры с последующим лиофильным высушиванием. Подобран протокол стерилизации разработанной конструкции с помощью этиленоксида, а также продемонстрировано отсутствие влияния процесса стерилизации на показатели прочности и относительное удлинение. Разрабатываемые методы создания биосовместимых конструкций на основе полимерного гидрогеля и способы их модификации позволят получать изделия с высокой степенью биосовместимости для улучшения регенерации тканей.

Ключевые слова: бионженерный скаффолд; коллаген; гиалуронат натрия; альбумин; гидрогель; цитотоксичность; механические свойства

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Соответствие принципам этики: исследование не требовало заключения локального биоэтического комитета. В работе использовали эмбриональные мышиные фибробласты CCL 1 (NCTC клон 929) и CCL 163 (Balb/3T3 клон A31), полученные из Российской коллекции клеточных культур (ИНЦ РАН, Санкт-Петербург).

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INTRODUCTION

The development of innovative tissue-engineered constructs is a priority direction of modern regenerative medicine. Skeletal muscle regeneration is a complex process that depends on the migration of various cell types into the wound bed, signaling molecules, mechanical signals from the extracellular matrix, and its physicochemical properties. Over the past few decades, various strategies for skeletal muscle tissue engineering have been developed. However, there remains a high demand for new, more effective methods and materials capable of promoting the restoration of skeletal muscle structure and functional regeneration, which could be used in the clinical treatment of muscle defects [1].

Muscle regeneration depends on the presence of a heterogeneous population of resident muscle stem cells (also referred to as satellite cells), the presence of interstitial cells, and the formation of blood vessels *de novo*. This process is mainly controlled by extracellular matrix proteins and associated factors (TGF-β, IL-4) [2]. The transplantation of allogeneic muscle cells is currently the clinical approach of choice for healing muscle tissue defects. However, this therapy has significant limitations due to the risk of rejection reactions, the presence of various pathologies in the donor, and an insufficient number of donor cells [3]. Furthermore, the problem of delivery and retention of cells in muscles remain unresolved, which hinders sustained regeneration necessary for adequate functional recovery [4, 5]. Therefore, the implementation of alternative strategies is required. Scaffolds based on biomaterials are a powerful tool for restoring the cellular microenvironment and ensuring the regeneration of skeletal muscle,

whose contractile strength fully corresponds to healthy tissue [5–8].

In order to induce skeletal muscle regeneration, it is necessary to mimic the native microenvironment of cells, not only by recreating the extracellular matrix through proper selection of biomaterials and methods for obtaining 3D scaffolds, but also by incorporating biologically active compounds into the composition. In terms of their micro- and nanostructure and physicochemical properties, modern scaffolds represent an artificial extracellular matrix for cells, replicating the properties of native tissue. Additionally, biologically active agents, such as proteins and growth factors, incorporated into the scaffold support cell adhesion and proliferation, inducing cells to synthesize their own extracellular matrix. To ensure mechanical, structural, physicochemical, and biological properties optimal for skeletal muscle regeneration, a number of multifunctional biomaterials have been developed. Such materials are capable of performing several important functions, such as influencing cell migration and proliferation [9], delivering drugs [10], and regulating stem cell differentiation [11].

One of the most promising types of constructs for skeletal muscle regeneration are scaffolds derived from hydrogels. Hydrogels are 3D crosslinked hydrophilic polymer matrices with a high content of water, which mimic the native aqueous environment of the body [7, 12] and structurally resemble the natural extracellular matrix. Importantly, hydrogel-based constructs can be produced in various shapes [4].

Biocompatible materials used to obtain hydrogel-based scaffolds must possess the following characteristics: degradation rates and mechanical properties corresponding to native muscle tissue; absence of toxic effects and a low risk of inflammation; and the ability to support cell migration, adhesion, and proliferation [13, 14].

A wide range of materials, both natural and synthetic, can be used for skeletal muscle regeneration. Synthetic polymers such as poly-L-lactic acid (PLA) [15, 16], poly(L-lactide-co-glycolide) (PLGA) [16], polycaprolactone [17–20], polyethylene glycol (PEG) [21–23] and their copolymers [24], as well as various polyurethanes [25–28] have found wide application for muscle tissue engineering. Among the advantages of synthetic polymers are the relative simplicity of their processing and structuring, the possibility to control mechanical properties and the release rate of biologically active compounds. However, such materials may exhibit not only low biocompatibility but also produce toxic degradation products or lack biodegradability [6, 29]. Current strategies for developing highly effective constructs for healing muscle defects are based on creating scaffolds from natural materials due to their combination of biocompatibility and biodegradability, resulting in the formation of non-toxic compounds [7]. Components of the extracellular matrix, such as collagen [30, 31], fibrin [32, 33], hyaluronic acid [34], etc., most often serve as such materials. Polymers of natural origin, e.g., alginate [35–37], silk fibroin, and gelatin [12, 30, 38] are also widely used.

Despite the advantages of natural polymers, creating constructs on their basis is associated with numerous challenges, such as insufficient mechanical strength and elasticity, low solubility, lack of stability under physiological conditions, and difficulty in structuring the materials. To date, transplantation of muscle autografts remains the only clinically proven approach for repairing extensive skeletal muscle defects. Currently, only one material — decellularized porcine skin (XenMatrix™ AB Surgical Graft, BD, USA) — is undergoing clinical trials. Therefore, the search for new materials and the development of approaches to create effective constructs for the regeneration of skeletal muscle defects remain urgent priorities in tissue engineering.

The aim of this research was to develop a bioengineered scaffold based on a polymer hydrogel for managing extensive skeletal muscle defects.

MATERIALS AND METHODS

Bioengineered scaffold preparation

The core layer of the scaffold was formed by a mixture of 2% collagen (Belkozin Luga Plant, Russia), 1% sodium hyaluronate prepared in 0.9% NaCl (Platina, Russia), and a 10% albumin solution (Ivanovo Regional Blood Transfusion Station, Russia) in a volume ratio of 70:20:10, respectively. The middle layer consisted of 2% collagen, 1% sodium hyaluronate, and 10% albumin solution in a volume ratio of 30:35:35. The outer layer of the scaffold consisted of a 2% collagen solution, a 1% hyaluronic acid solution, and a 10% albumin solution in a volume ratio of 10:80:10, respectively. A solution comprising 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) (Macklin LLC, China) and N-hydroxysuccinimide (NHS) (Sigma-Aldrich, USA) in a 4:1 ratio was used as a crosslinking agent. To form the scaffold, the biopolymer hydrogel mixture was foamed and poured into molds printed using 3D prototyping. The samples were then stepwise frozen and dried in a freeze dryer (Labconco, USA).

Sterilization

Gas low-temperature sterilization with ethylene oxide was chosen as the sterilization method, in accordance with GOST ISO 11135-2017.[1]

Each scaffold sample was individually sealed in a double gas-permeable package — Tyvek film (DuPont de Nemours, Inc., Delaware, USA). Sterilization was carried out using a Steri-Vac 8XL (224 L) gas sterilizer/

[1] GOST ISO 11135-2017 “Interstate Standard. Sterilization of health-care products. Ethylene oxide. Requirements for the development, validation and routine control of a sterilization process for medical devices”.

aerator (3M Health Care, St. Paul, USA). Sterilization program No. 1 according to GOST ISO 11135-2017 was used: sterilization temperature — 37 °C, sterilization exposure duration — 180 min, ethylene oxide concentration — 100%. To determine the exact concentration in the sterilizer chamber, the gas cylinder was weighed before and after sterilization. Based on the calculations, the ethylene oxide concentration inside the chamber was taken to be no less than 730.0 ± 30.0 mL/L. Aeration of the samples after ethylene oxide sterilization with sterile air was carried out for 12 h.

Scanning electron microscopy

The microstructure and morphology of the sample surface and cross-sections were studied using a Tescan VEGA III microscope (Scanning Electron Microscopy (SEM), Brno, Czech Republic), equipped with an energy dispersive spectroscopy (EDS) system (INCA Energy, Oxford Instruments, Abingdon, UK) for chemical composition analysis. The samples were pre-coated with gold using a Q150R Quorum Technologies (Lewes, UK) system. Images of the material surfaces were obtained at a pressure of 7.3×10^{-2} Pa in the column and 1.5×10^{-1} Pa in the chamber.

Mechanical properties of the obtained scaffold

To determine the tensile strength characteristics, the obtained scaffolds were used to cut dumbbell-shaped specimens with the dimensions shown in Fig. 1. The specimens were prepared simultaneously under identical conditions. Care was taken to ensure smooth surfaces without visible defects. Prior to testing, the prepared specimens were immersed in physiological saline (0.9% NaCl solution) at a temperature of 37 ± 2 °C for half an hour followed by their placement in the grips of the testing machine. During mounting, care was taken to prevent slippage and avoid damage to the specimen at the gripping points.

The tensile strength and elasticity of the obtained materials were studied using an Instron ElectroPuls E3000 testing machine (Instron, USA) at a loading rate of 5.0 mm/min, using five specimens in each series. The experiments were conducted in physiological saline (0.9% aqueous NaCl solution) at a temperature of 37 ± 2 °C. Prior to testing, the specimens were kept for half an hour in physiological saline at a temperature of 37 ± 2 °C following by their mounting in the holders. During the experiment, the specimen was stretched along its main longitudinal axis, measuring the sustained load with an error not exceeding 1% of the measured value.

The tensile strength at break was calculated using formula (1):

$$\sigma = \frac{F \times 1000}{S} \times 100\%, \quad (1)$$

where F is the tensile load at which the specimen failed (H); S is the cross-sectional area of the specimen (mm^2); σ is tensile strength at break (kPa).

An identical approach was used to study elasticity. Dumbbell-shaped specimens were also cut from the scaffold samples, with the dimensions indicated in Fig. 1. The specimens were prepared simultaneously under identical conditions following visual assessment of their integrity.

The relative elongation at break was calculated using formula (2):

$$\varepsilon = \frac{l_2^* - l_2}{l_2} \times 100\%, \quad (2)$$

where l_2 is the initial length of the narrow section of the specimen (mm); l_2^* is the length of the narrow section of the specimen at the moment of rupture (mm); ε is relative elongation at break (%).

To determine the bulk compression modulus, defined as the ratio of the stress increment to the corresponding increment in relative compressive strain, rectangular specimens (80 ± 2 mm x 10 ± 2 mm) were cut from the obtained scaffolds. The specimen thickness was 4 ± 0.5 mm. The specimens were prepared simultaneously under identical conditions. During specimen preparation, care was taken to ensure smooth surface free of visible defects.

Evaluation of the cytotoxic effect of the developed materials on CCL 1 (NCTC clone 929) and CCL 163 (Balb/3T3 clone A31) cells using fluorescence microscopy

Cytotoxicity study of the obtained scaffold samples was carried out in accordance with GOST ISO 10993-5-2011.[2] This method is based on determining the ratio of

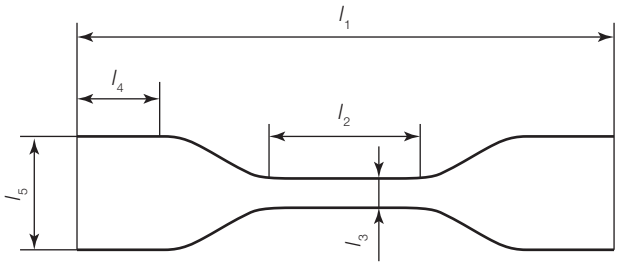

Figure prepared by the authors based on their own data

Fig. 1. Dumbbell-shaped specimen for tensile mechanical testing: l_1 — total length (60 ± 5 mm); l_2 — length of the narrow section (20 ± 0.5 mm); l_3 — width of the narrow section (10 ± 0.5 mm); l_4 — length for grip attachment (10 ± 0.5 mm), l_5 — total width (20 ± 0.2 mm); thickness of the specimen — 4 ± 0.5 mm

[2] GOST ISO 11135-2017 "Interstate Standard. Sterilization of health-care products. Ethylene oxide. Requirements for the development, validation and routine control of a sterilization process for medical devices".

live to dead cells cultured on the material by staining the cells with fluorescein diacetate (stains the cytoplasm of live cells green) and propidium iodide (stains the nuclei of dead cells red), using fluorescence or scanning confocal microscopy.

The cells were cultured in a mixture of DMEM/F12 media (Sigma-Aldrich, USA) supplemented with 10% fetal bovine serum (HiMedia Laboratories, South America), 40 μg/mL gentamicin sulfate (Sigma-Aldrich, USA) at 37 °C under conditions of 5% CO_2 in the gas mixture. For analysis, cells were seeded into wells of a 96-well plate (SPL Life Sciences, South Korea) containing samples of the developed materials, at a concentration of 5×10^4 cells/mL. Following 24 and 96 h of incubation on the developed material, the cells were stained in L-15 medium (Sigma-Aldrich, USA) with 1% fetal bovine serum (HiMedia Laboratories, South America), containing 1 μg/mL fluorescein diacetate (Sigma-Aldrich, USA) and 2 μg/mL propidium iodide (Sigma-Aldrich, USA), for 25 min at 37 °C. Following incubation, the cells were washed twice with L-15 medium supplemented with 1% fetal bovine serum. Analysis of live and dead cells was performed using a Nikon Eclipse Ti-E microscopic station (Nikon, Japan) and a TCS SP5 scanning confocal microscope (Leica, Germany). More than 500 cells were evaluated for the analysis.

The live cells number (LCN) after incubation on the developed material was calculated as a percentage relative to the control using formula (3):

$$LCN = \frac{LCN_m}{LCN_c} \times 100\%, \quad (3)$$

where LCN_m is the live cells number after incubation on the developed material; LCN_c is the live cells number under control conditions (not incubated on the developed material).

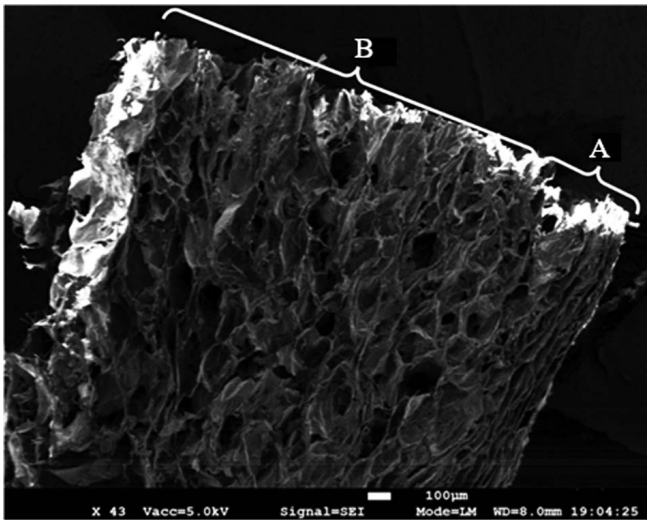

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Fig. 2. Microstructure of the bioengineered scaffold sample: A — outer layer; B — core layer (SEM, magn. ×43)

Statistical data processing

Statistical analysis was performed using the Python 3 (version 3.9.16) and R (version 4.3.0) programming languages in the Spyder (version 5.4.1) and RStudio (version 2023.03.0+386) software environments, respectively, employing the Pandas (version 1.5.3), NumPy (version 1.23.5), SciPy (version 1.10.0), and PMCMRplus (version 1.9.10) packages. Results were presented as the mean value and standard deviation ($M \pm SD$). Experiments were carried out in at least three replicates ($n \geq 3$). The statistical significance of differences was determined using a one-way analysis of variance (ANOVA) followed by the Holm–Šídák multiple comparison test, with the critical significance level α set at 0.05.

RESULTS AND DISCUSSION

As a result of the experiments, a porous bioengineered scaffold was obtained. This material represents a layered construct based on a combination of natural polymers, collagen, and sodium hyaluronate with the addition of EDC and NHS to form layers with different resorption rates and proteolytic degradation profiles [40]. Crosslinking agents are added to improve the mechanical strength and structural stability of collagen scaffolds upon hydration, in order to extend the possibilities of their application in certain tissues.

The porous structure of the scaffold samples was obtained using a combination of layer-by-layer casting/molding of foamed polymers followed by stepwise freezing.

The microstructure analysis using SEM established the multilayered porous spongy structure of the polymer scaffold. The core layer, 1.2 ± 0.1 mm thick, exhibits high — uniform and interconnected — porosity (Fig. 2), with pores nearly spherical in shape, 100–150 μm in size, and a polymer framework wall thickness of less than 5 μm. The outer layer, 300–500 μm thick, shows a denser lamellar structure, with a polymer framework wall thickness of less than 2 μm and slit-shaped pores up to 200 μm in length and 50 μm in width. Compared to the core layer, significantly lower pore interconnectivity was observed, a channel structure was virtually absent, and pores were mostly isolated from one another by polymer walls. This architecture of the construct was achieved through cryostructuring by stepwise freezing of the sample, which is consistent with literature data [41].

For subsequent experiments, an ethylene oxide sterilization protocol for the developed construct was selected. This method is considered optimal for polymeric implantable devices [42].

Studies were conducted to evaluate the mechanical properties of the obtained scaffold: tensile strength was 50 ± 0.5 kPa, and relative elongation was 26 ± 6%. Sterilization was found to have no effect on the strength and elongation parameters (Fig. 3). It should be noted that the obtained values of mechanical strength

indicators ensure the feasibility of surgical manipulations, which is especially important for conducting further *in vivo* studies [43].

In vitro cytotoxicity testing is a critical initial step in evaluating the safety of any medical device. It enables early identification and exclusion of potentially hazardous materials, thereby minimizing risks to living organisms and reducing the number of subsequent animal trials. In the present study, assessment of the cytotoxic effect of porous bioengineered scaffold samples established the absence of their cytotoxicity (Fig. 4). No statistically significant differences in the number of dead cells relative to the control were detected at any of the observation time points (24 and 96 h). Cytotoxic components, such as degradation products and residual monomers, can induce cellular stress that may lead to chronic inflammation and fibrosis rather than *de novo* tissue regeneration. Skeletal muscle tissue has a limited capacity for self-repair in cases of volumetric damage [44]. The absence of cytotoxicity suggests that the implant is unlikely to suppress the proliferation and migration of myoblasts and satellite cells necessary for the formation of new muscle fibers [45].

The obtained results formed the basis for an application for state registration of intellectual property, resulting in the granting of a patent for the invention “Method for obtaining a bioengineered scaffold based on a hydrogel” [46].

CONCLUSION

A porous bioengineered scaffold based on a composition of natural polymers has been developed. The porous

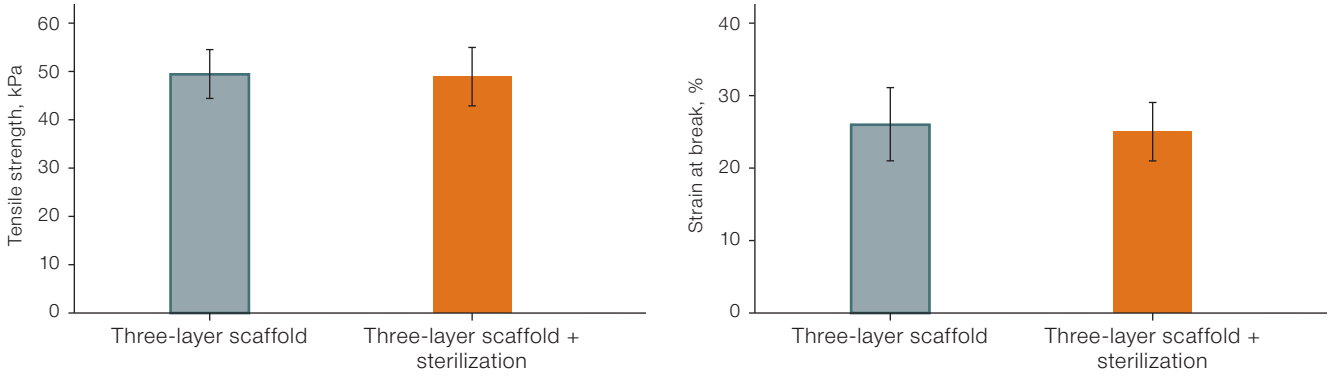

Figure prepared by the authors based on their own data

Fig. 3. Tensile strength and relative elongation of bioengineered scaffold samples

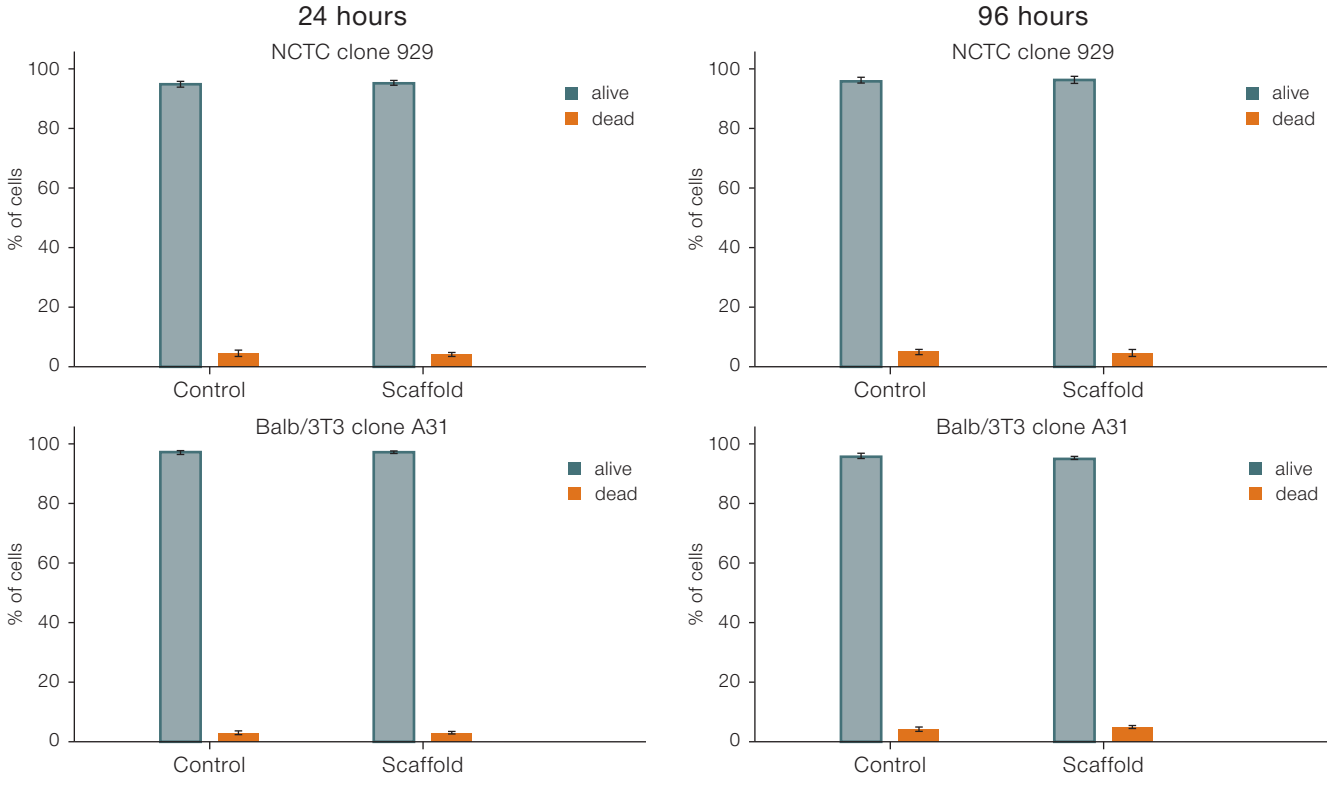

Figure prepared by the authors based on their own data

Fig. 4. Cytotoxicity assessment of the obtained bioengineered scaffold

architecture of the scaffold was achieved through a combination of layer-by-layer casting/molding methods with stepwise freezing and structure control, followed by lyophilization. An ethylene oxide sterilization protocol for the developed construct was selected. It was demonstrated that the sterilization process had no effect on strength and elongation parameters. As a result of *in vitro* tests conducted on murine fibroblast cells Balb/3T3 clone A31 and NCTC clone 929, the developed bioengineered scaffold showed no cytotoxicity. The developed methods for creating biocompatible constructs based on polymer hydrogels, along with their modification techniques, will make it possible to expand the range of medical materials available for clinical application. This will contribute to improving patients' quality of life and reducing population-level disability.

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