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Bacteriostatic effects of cell-free matrix lyophilisates and hydrogel from human umbilical cord

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ABSTRACT

The bacteriostatic effects of human umbilical cord-derived matrices and hydrogels were examined. The use of biomimetics based on the extracellular matrix of extraembryonic organs, including the human umbilical cord, is promising for regenerative medicine and tissue engineering. Cell-free products from the extracellular matrix of various human organs and tissues are resistant to intentional bacterial contamination. Two acellular scaffolds prepared using different human umbilical cord decellularization protocols and two derived hydrogels were evaluated for their bacteriostatic properties. Two clinical cases of the use of lyophilisates of umbilical cord-derived hydrogels were described. The compositions of human umbilical cord-derived acellular matrices and hydrogels were studied using biochemical analysis techniques. The sensitivities of *Staphylococcus aureus* and *Escherichia coli* to umbilical cord-derived matrices and hydrogels were assessed using culture techniques, and metabolic activities of bacteria were also examined. Human umbilical cord-derived acellular matrices and hydrogels consist of collagens and contain proteins and glycosaminoglycans. A significant bacteriostatic effect of hydrogels against *Escherichia coli* was detected during the first 16 h of incubation, regardless of the type of detergents used for their preparation. The matrices did not show a bacteriostatic effect, which indicates that the hydrolysis of structural components contributes to the release of substances with bacteriostatic activities. The effect was presumed to be due to the influence on the level of metabolic activity of microorganisms. The use of powdered lyophilized hydrogels derived from human umbilical cord as an adjunct to autodermal graft in the treatment of infected deep wounds in two volunteer patients promoted healing without infections. In general, the use of hydrogel lyophilisates from acellular human umbilical cord as an additional treatment allows for the engraftment of skin autografts and promotes the healing of extensive deep wounds at risk of infection.

Keywords: tissue engineering: bacteriostatic effect; hydrogel; acellular matrix from human umbilical cord; extraembryonic organs; lyophilisate; decellularization; autodermograft; regenerative medicine.

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Бактериостатические эффекты лиофилизатов бесклеточных матрикса и гидрогеля из пуповины человека

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АННОТАЦИЯ

Исследованы бактериостатические эффекты матриксов и гидрогелей из пуповины человека. Известно, что использование биомиметиков на основе внеклеточного матрикса внеэмбриональных органов, в том числе пуповины человека, перспективно для нужд регенеративной медицины и тканевой инженерии. Бесклеточные продукты из внеклеточного матрикса разных органов и тканей человека устойчивы к преднамеренному бактериальному заражению. Два бесклеточных матрикса, изготовленных с использованием разных протоколов децеллюляризации пуповины человека, и два гидрогеля на их основе были оценены на предмет наличия бактериостатических свойств. Описаны два клинических случая применения лиофилизатов гидрогеля из пуповины. Исследован состав бесклеточных матриксов и гидрогелей из пуповины человека с помощью биохимических методик анализа. Чувствительность *Staphylococcus aureus* и *Escherichia coli* к матриксам и гидрогелям из пуповины оценивали, используя культуральные методики, также исследована метаболическая активность бактерий. Заметим, что бесклеточные матриксы и гидрогели из пуповины человека состоят из коллагенов и содержат белки и гликозаминогликаны. Обнаружено достоверное бактериостатическое действие гидрогелей в отношении *Escherichia coli* в течение первых 16 ч инкубации, независимо от вида детергента, использованного для их приготовления. Матриксы не показали бактериостатического эффекта, что позволяет предположить, что именно гидролиз структурных компонентов способствует высвобождению веществ с бактериостатической активностью. Эффект обусловлен, предположительно, влиянием на уровень метаболической активности микроорганизмов. Применение порошкообразного лиофилизата гидрогеля из пуповины человека в качестве дополнения к аутодермотрансплантату при лечении инфицированных глубоких ран у двух пациентов-добровольцев способствовало заживлению без инфицирования. В целом применение лиофилизатов гидрогеля из бесклеточной пуповины человека в качестве дополнительного лечения позволяет обеспечить приживление кожных аутотрансплантатов и создает условия для заживления обширных глубоких, склонных к инфицированию, ран.

Ключевые слова: тканевая инженерия: бактериостатическое действие; гидрогель; бесклеточный матрикс из пуповины человека; внеэмбриональные органы; лиофилизат; децеллюляризация; аутодермотрансплантат; регенеративная медицина.

Как цитировать

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人脐带组织无细胞基质和水凝胶冻干物的抑菌作用

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摘要

对来自人类脐带的基质和水凝胶的抑菌作用进行了研究。众所周知，以胚胎外器官（包括人类脐带）的细胞外基质为基础的生物仿生学很有希望满足再生医学和组织工程学的需求。来自不同人体器官和组织的细胞外基质的无细胞产物对有意的细菌感染具有抵抗力。研究人员采用不同的方法对人脐带进行脱细胞处理，制成了两种无细胞基质，并对基于这两种基质的两种水凝胶的抑菌特性进行了评估。介绍了脐带水凝胶冻干物的两种临床应用。使用生化分析技术研究了人脐带无细胞基质和水凝胶的成分。利用培养技术评估了 *Staphylococcus aureus* 和 *Escherichia coli* 对脐带基质和水凝胶的敏感性，同时还研究了细菌的代谢活动。需要注意的是，来自人类脐带的无细胞基质和水凝胶由胶原蛋白组成，并含有蛋白质和糖胺聚糖。在培养的最初 16 小时内，无论使用哪种洗涤剂制备水凝胶，都能发现水凝胶对 Escherichia coli 有可靠的抑菌作用。基质没有抑菌作用，这表明是结构成分的水解促进了具有抑菌活性的物质的释放。这种影响可能是由于对微生物代谢活动水平的影响。使用人脐带水凝胶冻干粉作为自体皮移植的添加剂，治疗两名志愿者的深部感染伤口，促进了伤口愈合，且未发生感染。一般来说，使用无细胞人类脐带水凝胶冻干物作为额外治疗，可以确保皮肤自体移植的接合，并为容易感染的大面积深层伤口的愈合创造条件。

关键词：组织工程；抑菌作用；水凝胶；人脐带无细胞基质；胚外器官；冻干物；脱细胞；自体皮肤移植；再生医学。

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INTRODUCTION

One of the main tasks of tissue engineering is the construction of bioequivalents using extracellular materials, cells, and growth factors to replace tissues and organs lost caused by disease, tumor, or injury. Decellularized native tissue products help create an optimal microenvironment for recipient cells and are bioactive and biodegradable [1]. They are a source of growth factors and biologically active molecules and can be used as the basis for repopulating cells in vitro or for repopulating the recipient's cells at the injury site [1, 2]. The use of umbilical cord-derived extracellular matrix for tissue engineering and regenerative medicine has particular advantages caused by its composition, homologous origin, and lack of undesired immune reactions when implanted in the recipient [1–3].

The umbilical cord contains significant amounts of extracellular matrix components such as collagen types I, III, IV, and V, hyaluronic acid, and sulfated glycosaminoglycans [1, 2–4]. Acidic and basic fibroblast growth factors, insulin-like growth factor I, platelet-derived growth factor, epidermal growth factor, and transforming growth factors have been identified in the composition of Warton's jelly [2, 3, 5]. The biological characteristics of the extracellular matrix, along with the noninvasive nature of the procedure for obtaining the umbilical cord and the absence of ethical restrictions, contribute to the feasibility of utilizing the human umbilical cord to create a natural three-dimensional product. This product cannot only fill extensive defects mechanically but can also stimulate the regeneration of damaged tissues [1, 6].

Studies have published encouraging results of the treatment of infected wounds using biomaterials derived from extraembryonic organs, such as the umbilical cord [2, 6–8]. One of the defining properties of a medical device for wound treatment is its interaction with endogenous and exogenous microflora and effective bacteriostatic action [9, 10]. The nature of this phenomenon and specific candidates for the role of antimicrobial agents are not yet fully understood. Several simultaneous mechanisms are assumed to be involved. The bactericidal and bacteriostatic actions of tissue-engineered products against bacteria, microscopic fungi, and infected cell lines in laboratory experiments conducted in vitro and in vivo have been studied [11].

This study aimed to determine the bacteriostatic activity of decellularized umbilical cord-derived matrices and hydrogels and identify the influence of enzymatic hydrolysis of the decellularized matrix on the bacteriostatic effect.

MATERIALS AND METHODS

All studies were conducted at the Research Center of the Kirov Military Medical Academy (VMA) in 2020–2023 in accordance with the principles approved by the Ethics Committee of the VMA (Protocol No. 230 dated December 17, 2019) and after obtaining the ethical opinion on the possibility of conducting a clinical trial from the Ethics Council of the Clinical Research Center "Medical Technologies" (Protocol of the first ethical review dated June 6, 2022). The statistical significance of differences in quantitative indicators of bacteriostatic activity in the experiment was considered an inclusion criterion.

Preparation of human umbilical cord-derived decellularized matrices and hydrogels. Previously, we [12] produced decellularized products by decellularization and additional enzymatic treatment. In this study, lyophilized products made from decellularized Warton's jelly of the human umbilical cord were used. With the informed consent of the mothers, human umbilical cord samples were obtained from healthy preterm infants. After the removal of the vessels aseptically, the umbilical cords were minced using a Bosch (Germany) blender and homogenized using an automated tissue homogenization station (gentleMACS™ Dissociator, Miltenyi Biotec, Germany). Umbilical cord tissues were decellularized in a thermostat orbital shaker "ES-20/60" (Biosan, Latvia) at 180 rpm and 25°C by two methods: 1) in 0.05% sodium dodecyl sulfate (SDS) solution (Biolot, Russia) or 2) in 0.1 N sodium hydroxide solution (NaOH) for 24 h and washed. If NaOH was used, neutralization was performed with 0.1 M hydrochloric acid (HCl) solution to pH 7.4. In this way, two types of decellularized matrix (SDS and NaOH) were obtained, which were lyophilized using a laboratory freeze dryer "VaCo 5 II" (Zirbus Technology, Germany).

The matrices were enzymatically treated with pepsin in an acidic medium at a ratio of 10 mg matrix per 1 mL of pepsin solution to prepare hydrogels. One milligram of pepsin (P/1120/46, Thermo Fisher Scientific, Germany) was dissolved in 1 mL of 0.01 N HCl with pH 2.0. Fermentation was carried out at 180 rpm for 72 h at room temperature. The action of pepsin was stopped by adjusting the acidity of the solution to pH 7.4 with 0.1 N NaOH. The products were lyophilized. Two hydrogel types were prepared: SDS and NaOH hydrogels (Fig. 1).

All products were stored, hermetically sealed at –20°C, and sterilized in the microbiological safety box "Laminar-C" (Lamsystems, Russia) for 15 min before use.

Agarose gel electrophoresis. Deoxyribonucleic acid (DNA) was extracted from the matrix, hydrogel, and umbilical cord samples (n = 15 0.002 g each) using the DNA-DU-250 kit (Biolabmix, Russia). The size of residual DNA fragments was analyzed by ethidium bromide staining. For this purpose, 10 μL of the extracted DNA and molecular weight standard (100–1500 nucleotide pairs) (SibEnzyme, Russia) were loaded into wells of 1% agarose gel (Sigma-Aldrich, USA) with 5 μL of glycerol (Ekos, Russia). Electrophoresis was performed in a 7 × 7 cm horizontal Mini-Sub Cell GT chamber (Bio-Rad, USA) in a simple Tris-acetate buffer at 70 V for 1 h.

The results were recorded by a gel visualization system with ChemiDoc XRS+ software (Bio-Rad).

Protein concentration determination. Lyophilized samples of matrices, hydrogels, and native umbilical cord (n = 9) were dissolved in 8 M urea (Biolot, Russia). Colorimetric analysis of protein content was performed by the Lowry method using the KliniTest-BL kit (ECOservice, Russia) and Bradford method using the Servicebio kit (China). Optical density measurements were performed in 96-well plates (Rosmedbio, Russia) on a FlexA-200 spectrophotometer (Allsheng, China) at the wavelengths specified in the instructions.

Protein electrophoresis in a polyacrylamide gel (100 × 80 mm, 1 mm thick) was performed in a Mini-PROTEAN Tetra Vertical Electrophoresis Cell unit (Bio-Rad). The solutions for the preparation of lower and upper gels were prepared in accordance with the prescribed methodology. The solutions were prepared using distilled water (5.9 and 3.4 mL), 30% acrylamide/bisacrylamide (5 and 0.83 mL, Bio-Rad), 1.5 M Tris (pH 8.8 and 6.8, 3.8 and 0.63 mL), and 10% SDS (0.15 and 0.05 mL). A total of 0.15 and 0.05 mL of 10% ammonium persulfate (Central Drug House, USA) and 0.006 and 0.05 mL of tetramethylethylenediamine (Bio-Rad) were used for polymerization, respectively. The matrix, hydrogel, and umbilical cord samples were dissolved in 6 M urea, mixed with a 1:1 application buffer (Servicebio, China), and incubated at 100°C for 3 min. Subsequently, 15 μL of the prepared samples were placed in the gel wells. The uncolored Precision Plus Protein standards (Bio-Rad) were used as reference samples to determine the molecular weight. Electrophoresis was conducted at V per 1 cm^2 of gel. After the migration of the samples into the lower gel, the voltage was increased to 15 V/cm^2. After extraction, the gel was stained for 1 h in a solution of Coomassie R-250 dye (Diaem, Russia), followed by washing in a solution containing 10% ethanol and 5% glacial acetic acid. The results were recorded using the ChemiDoc XRS+ gel visualization system (Bio-Rad).

The amount of sulfated glycosaminoglycans was determined using 0.5% Alcian Blue 8GX solution (Lenreactiv, Russia) in 0.1 M HCl (pH 1.5). Then, 20 μL of the test sample was added to 200 μL of the dye solution and incubated overnight. After centrifugation, the supernatant was removed using a Gyrozen Mini microcentrifuge (China). The optical density of the extracted 250 μL of 6 M HCl was determined on a FlexA-200 spectrophotometer (Allsheng, China) at 620 nm in a 96-well plate. Glycosaminoglycan concentrations were determined using a calibration curve based on known concentrations of chondroitin sulfate (Sigma-Aldrich).

*The amount of collagen i*n matrices, hydrogels, and native umbilical cord samples was determined spectrophotometrically by oxidation of hydroxyproline after hydrolysis (10 mg sample per 2 mL of 6 M HCl; 110°C, 48–72 h; n = 9). Concentrations were determined using a calibration curve constructed from known concentrations of hydroxyproline (AppliChem, Germany). Hydroxyproline was oxidized by adding 1 mL of chloramine T (Diaem, Russia). Then, 1 mL of 3.15 M perchloric acid ($HClO_4$, Lenreaktiv) was added and shaken. Subsequently, 1 mL of 20% para-dimethylaminobenzaldehyde (Diaem, Russia) was added at 60°C for 20 min, cooled, and added with 5 mL of ethyl Cellosolve (Lenreaktiv). The optical density was measured at a wavelength of 550 nm using a UV-1800 spectrophotometer (Shimadzu, Japan). The collagen content of the sample was calculated by converting the measured hydroxyproline with a coefficient of 0.135 [13].

Fourier transform infrared spectroscopy. The absorption spectra of the lyophilized matrices, hydrogels, and umbilical cord samples (n = 15) were recorded on a Bruker Alpha spectrometer (Germany) with a spectral resolution of 2 cm^{-1} for 45 scans each at different sections of the lyophilized samples. Measurements were performed at 21 ± 1°C in an air-conditioned room.

Microbiological objects. Staphylococcus aureus, B-6646 (ATCC 6538P). and Escherichia coli K-12 B-3254 (KS-507) were obtained from the National Bioresource Center, All-Russian Collection of Industrial Microorganisms of the Kurchatov Institute – State Research Institute of Genetics and Breeding of Industrial Microorganisms. Lysogeny broth (LB) according to Miller's method (Rosmedbio, Russia) was prepared as per the manufacturer's instructions. Microorganism cultures in the logarithmic growth phase were grown for 16–18 h in LB at 37°C in a dry-air TC-1/20 thermostat (Russia). Subsequently, 500 μL of the suspension was transferred into 15 mL of LB and cultured for 2–3 h more, with optical densities of 0.49 ± 0.05 and 0.37 ± 0.04 for *S. aureus* and *E. coli*, respectively. Before the experiment, 50 ± 10 mg of lyophilized sterile samples of all four products were rehydrated in 500 μL of sterile deionized water.

Moreover, 15 mL of sterile liquid LB medium was supplemented with 500 mg of a hydrated sample and 500 μL of a bacterial suspension of microorganisms in the logarithmic growth phase (n = 8). The tubes were not supplemented with any samples to monitor bacterial growth, and product sterility was not evaluated with the addition of microorganisms. The growth of the bacterial suspension was recorded at 16, 24, and 40 h after inoculation. Nephelometric measurements of the optical density of the *S. aureus* (570 nm) and *E. coli* (600 nm) suspensions were performed on a UV-1800 spectrophotometer (Shimadzu, Japan).

The number of microorganisms at each time point was determined using the serial dilution method. To this end, bacterial suspensions were diluted in 1:2, 1:10, 1:100, and 1:1000 ratios in sterile nutrient medium LB. Then, 1 mL of the corresponding bacterial suspension was plated on a Petri dish containing dense sterile agar (agar concentration 10 g/L). After 1 day, the number of colonies was counted, and the colony-forming unit (CFU) content in 1 mL was recalculated.

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Resazurin was used to examine the effect of decellularized human umbilical cord-derived matrices and hydrogels on the metabolic activity of microorganisms. In the presence of actively metabolizing living objects, blue resazurin is reduced to pink and fluorescent resorufin. The assay was conducted using bacteria in the stationary growth phase. Thereafter, the microbial suspension was cooled to 4°C after 16–18 h of cultivation. Cultivation was conducted in sterile glass tubes at ratios of LB, hydrated sample, and microorganisms in the stationary growth phase (n = 8) comparable with those described above. After incubations of 16, 24, and 40 h post-inoculation, 300 µL from each tube was transferred to the wells of a 96-well flat-bottom plate (Rosmedbio, Russia). Subsequently, 20 µL of a 600 micromolar phosphate-buffered resazurin solution (Biocompass-S, Russia) was added. After a 30-min incubation period at 37°C with a shaking speed of 300 rpm on a PST-60 tablet thermoshaker (Biosan, Latvia), the fluorescence intensity was quantified on a Victor X5 multifunctional tablet analyzer (Perkin Elmer, USA) at a wavelength of 595 nm and excitation of 560 nm.

For statistical data processing, the analysis of variance (ANOVA) with Bonferroni post hoc analysis was performed in Statistica 7.0. Quantitative data are presented as means (M) and standard deviations (SD). Differences were considered significant at $p < 0.05$.

RESULTS AND DISCUSSION

Lyophilized human umbilical cord-derived matrices and hydrogels are porous sponges that can be crushed if necessary (Fig. 1).

Matrices and hydrogels produced by different technologies are decellularized as confirmed by agarose gel electrophoresis (Fig. 2). Large amounts of DNA are visualized in the native umbilical cord preparation (Fig. 2, *e*). Matrices and hydrogels prepared with different detergents showed acceptable levels of cell and DNA removal (Fig. 2, *b, c, f,* and *g*).

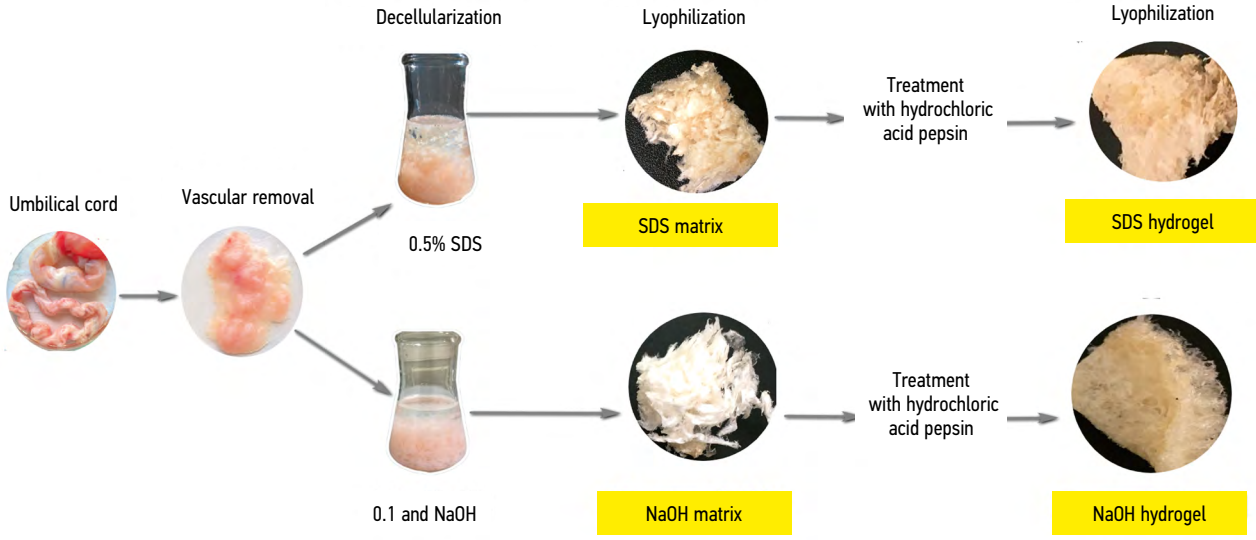


Fig. 1. Manufacturing scheme and appearance of human umbilical cord-derived matrices and hydrogels
Рис. 1. Схема изготовления и внешний вид матриксов и гидрогелей из пуповины человека

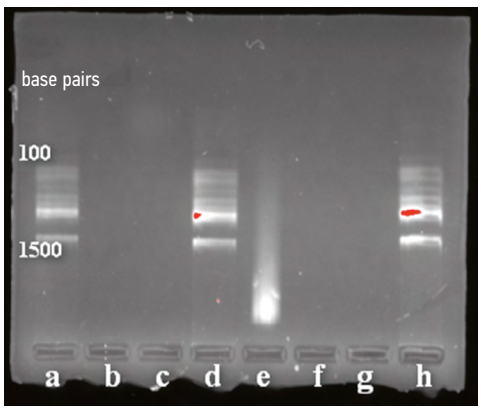


Fig. 2. Electrophoresis in agarose gel: *a*, *d*, *h* — DNA molecular weight marker; *b* — SDS matrix; *c* — NaOH matrix; *e* — native umbilical cord; *f* — SDS hydrogel; *g* — NaOH hydrogel
Рис. 2. Электрофорез в геле агарозы: *a*, *d*, *h* — ДНК-маркер молекулярного веса; *b* — матрикс SDS; *c* — матрикс NaOH; *e* — нативная пуповина; *f* — гидрогель SDS; *g* — гидрогель NaOH

Lyophilizates of decellularized matrices and hydrogels and native umbilical cord biomaterials were examined by Fourier transform infrared spectroscopy. The products were predominantly collagen and contained glycosaminoglycans (Fig. 3).

The Fourier transform infrared spectroscopy spectra of decellularized matrices and hydrogels revealed the main peaks in the spectral regions of 1685 and 1570 cm^{-1} (amide I and amide II), respectively, which are inherent to collagen [3]. The qualitative removal of cells and DNA did not affect the structure of collagens. Furthermore, it allowed the preservation of glycosaminoglycans in the composition of matrices and hydrogels. The spectra showed absorption bands between 1170 and 800 cm^{-1}, inherent to the absorption of the (C–O) and (C–O–C) groups of carbohydrate fragments of collagen and glycosaminoglycans [3]. For a more detailed assessment of the composition of the matrices and hydrogels, biochemical studies of the protein, collagen, and glycosaminoglycan contents were performed in comparison with native human umbilical cord (Table).

Because decellularized matrices and hydrogels contain proteins with different compositions and properties, they were determined by two methods. The main mass of the matrices and hydrogels consisted of collagens (see Table). Given that the matrices were prepared by removing cells from Warton's jelly, their collagen content was significantly higher than that of the native umbilical cord. Owing to the hydrolysis of collagens by pepsin during hydrogel preparation, the collagen content was reduced compared with that in matrices.

Among organs or tissues in the human body, the umbilical cord contains the highest amounts of glycosaminoglycans [2, 3]. The umbilical cord is rich in sulfated glycosaminoglycans [14]. Sulfated glycosaminoglycans persisted and their amounts even slightly increased after decellularization of the umbilical cord (Table) [2, 3]. Their amount slightly decreased compared with the matrix material during hydrogel production.

The main fibrous protein is collagen type I, and collagen types III, IV, V, and VI, fibronectin, and growth factors are also present in the umbilical cord [3]. The results of the separation of protein mixtures obtained by the dissolution of matrices and hydrogels and native umbilical cord, according to their electrophoretic mobility in polyacrylamide gel with subsequent Coomassie staining, are shown in Figure 4.

Table. Quantitative indicators of the composition of the human umbilical cord and products made of cell-free umbilical cord using various technologies

Таблица. Количественные показатели состава пуповины человека и продуктов из бесклеточной пуповины, изготовленных по разным технологиям

Sample	Protein		Total collagen, mg/100 mg	Sulfated glycosaminoglycans, mg/100 mg	CFU/mL
	Lowry method, mg/100 mg	Bradford method, mg/100 mg			
Human umbilical cord	35.11 ± 3.72	24.54 ± 2.32	19.36 ± 2.09	2.53 ± 0.43	0
SDS matrix	22.75 ± 4.28	16.56 ± 3.86	46.03 ± 3.21	3.68 ± 0.88	0
NaOH matrix	9.83 ± 2.35	5.96 ± 4.21	50.10 ± 4.29	3.87 ± 0.76	0
SDS hydrogel	32.54 ± 4.56	12.08 ± 1.25	39.21 ± 4.67	2.08 ± 0.85	0
NaOH hydrogel	35.50 ± 4.32	9.66 ± 3.56	37.63 ± 6.35	1.80 ± 0.38	0

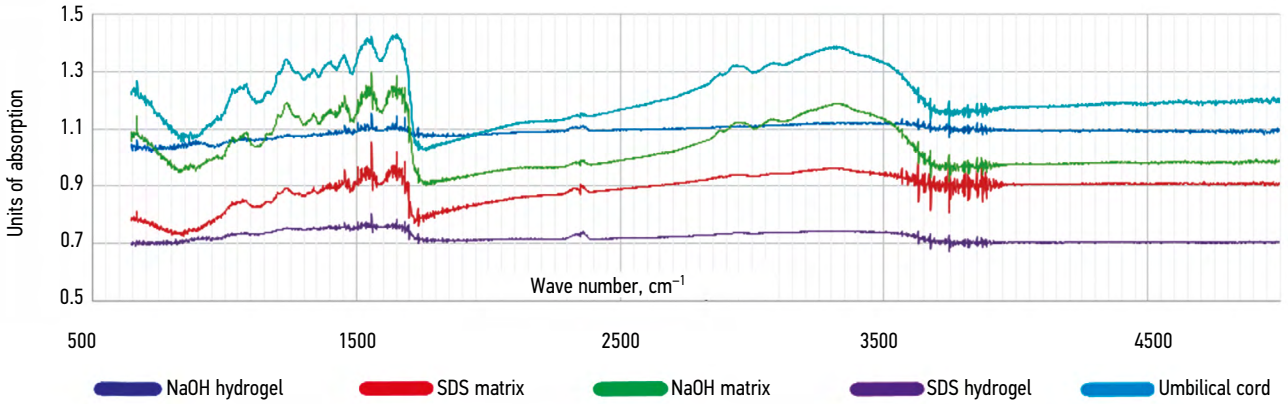


Fig. 3. Fourier-transform infrared spectroscopy spectra of native umbilical cord lyophilisates and matrices and hydrogels manufactured using different technologies

Рис. 3. Спектры инфракрасной спектроскопии с Фурье-преобразованием лиофилизатов нативной пуповины, а также матриксов и гидрогелей, изготовленных по разным технологиям

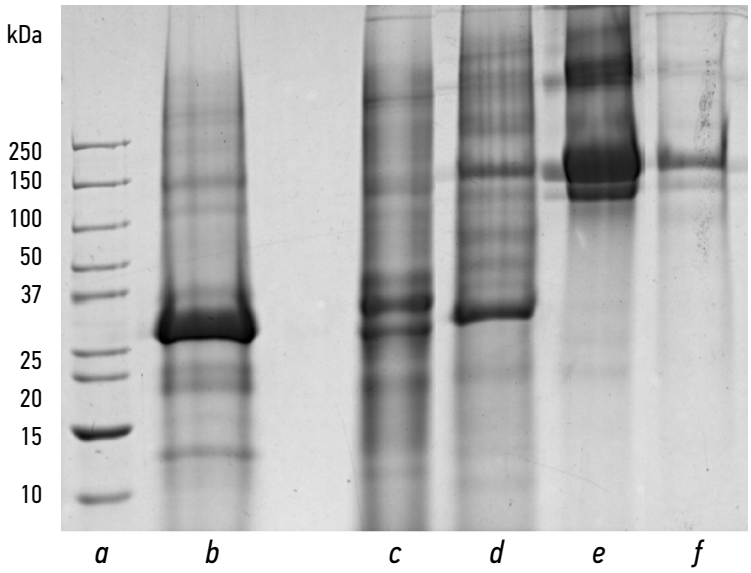


Fig. 4. Electrophoresis of samples in polyacrylamide gel: *a* — protein molecular weight marker; *b* — native umbilical cord; *c* — SDS matrix; *d* — Matrix NaOH; *e* — SDS hydrogel; *f* — NaOH hydrogel
Рис. 4. Электрофорез образцов в полиакриламидном геле: *a* — маркер молекулярного веса белка; *b* — нативная пуповина; *c* — матрикс SDS; *d* — матрикс NaOH; *e* — гидрогель SDS; *f* — гидрогель NaOH

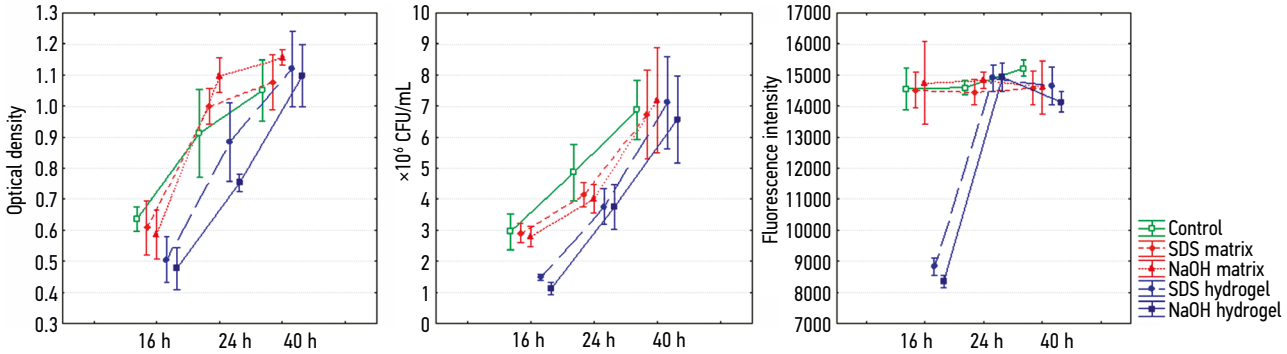


Fig. 5. Dynamics of the cultivation of *Escherichia coli* KS-507 in the presence of human umbilical cord-derived acellular matrices and hydrogels and in control (*M* ± *SD*)
Рис. 5. Динамика культивирования *Escherichia coli* KS-507 в присутствие бесклеточных матриксов и гидрогелей из пуповины человека и в контроле (*M* ± *SD*)

Thus, decellularized human umbilical cord-derived matrices and hydrogels prepared by different decellularization protocols, with sufficient cell and DNA removal, contain collagens and store glycosaminoglycans, including sulfated ones, and various soluble and insoluble proteins.

Sterile samples of human umbilical cord-derived matrices and hydrogels were used to evaluate bacteriostatic properties (Table). The analysis of the changes in optical density of nutrient media containing bacterial suspensions and samples of different decellularized matrices and hydrogels revealed that matrices produced by both technologies did not inhibit bacterial growth, whereas hydrogels had some bacteriostatic effect against *E. coli* during the first 16 h of incubation (Fig. 5).

The optical density of suspensions cultured with SDS hydrogels was lower than that of the control suspension; however, the differences were not significant (p = 0.514). The differences in optical density values between suspensions cultured with NaOH hydrogels and control were also lower; however, the differences were not significant (p = 0.077). The optical densities of *S. aureus* suspensions cultured with and without decellularized umbilical cord-derived matrices and hydrogels were not different (Fig. 6).

The CFU/mL count of suspensions also decreased in this index when *E. coli* KS-507 was incubated with SDS hydrogels, without significant differences (p = 0.304). Conversely, the reduction in the CFU/mL count of suspensions incubated with NaOH hydrogels exhibited significant differences (p = 0.024). Furthermore, differences in this index were observed exclusively during the initial 16 h of incubation (Fig. 5). The CFU/mL count of *S. aureus* suspensions during incubation with decellularized umbilical cord-derived matrices and hydrogels was different when compared with the control (Fig. 6).

M. Dubus et al. [2], T.Ž. Ramuta, and L. Tratnjek et al. [6] revealed the presence of peptides with bacteriostatic effect on matrices of decellularized extraembryonic organs and tissues. Presumably, this phenomenon was realized by the influence of such peptides on the surface structures of microbes. The disruption of the integrity of the surface structures of microorganisms is inevitably reflected in the level of their metabolic activity. Thus, the fluorescence intensity, which reflects the level of metabolic activity of the bacterial culture, may be measured. Studies of resorufin fluorescence levels of bacterial suspensions have shown a significant (p = 0.000001) effect of hydrogels prepared by both technologies on *E. coli* K-12 activity during the first 16 h (Fig. 5). No such effect of decellularized umbilical cord-derived matrices and hydrogels was found for *S. aureus* cultures (Fig. 6).

A lyophilized hydrogel prepared using 0.05% SDS solution for cord decellularization was used as an adjunctive treatment for extensive deep soft tissue injuries in two patients (clinical cases 1 and 2).

Clinical case 1. Patient X1 (33 years old) was diagnosed with a shrapnel blind wound of the upper third of the tibia with extensive soft tissue defect and fracture of the inferior pole of the patella, intra-articular fracture of the proximal metaphysis of the left tibia with displacement of the fragments, and bone defect (Fig. 7, *a*).

After surgical excision of nonviable tissues, a combined musculocutaneous plasty of the gunshot wound of the lower leg was performed with a non-free perforator muscle flap from the medial head of the calf muscle and a free split dermatome. The patient received a course of antibiotic therapy based on the pathogen detected (*Pseudomonas aeruginosa, S. aureus*, methicillin-resistant *S. aureus, E. coli,* and *Acinetobacter baumannii*). Despite the ongoing antibacterial therapy based on the sensitivity of the identified bacterial colonies to the available antibiotics, the healing of the defect was complicated by the development of the infectious process. To increase the concentration of biologically active factors stimulating collagen formation, stem cell recruitment to the injury site, and vascularization and vascular remodeling, lyophilized powdered matrices and decellularized human umbilical cord-derived hydrogels were applied (Fig. 7, *b*). On day 1 after the application of human umbilical cord-derived hydrogels, eschar formation was observed in up to two-thirds of the wound area. On day 10, the exudate became serous (Fig. 7, *c*).

The application of human umbilical cord-derived hydrogels resulted in a reduction in exudation and scab formation. In cases of extensive infectious lesions, the survival of free skin grafts can be ensured, which create conditions conducive to healing tissue defects (Fig. 7, *d*).

Clinical case 2. Patient X2 (46 years old) was diagnosed with a wound on the right tibia and foot with soft tissue defect of the lower third of the tibia and splinter fractures (Fig. 8).

The patient received surgical treatment with excision of nonviable soft tissues. On day 25 after the wound was sustained, the patient underwent skin grafting of the anteromedial defect of the lower third of the right tibia with a non-free sural flap (Fig. 8, *b*). On day 5 after the surgery, pathological greenish discharge with an unpleasant odor from the wound margins, increasing cyanosis, and dense consistency of the flap were noted. On day 9, subtotal necroectomy of the infected sural flap of the right tibia was performed. The bacteriological examination detected *P. aeruginosa*, methicillin-resistant *S. aureus*, and *Klebsiella pneumoniae*. Over the subsequent 3-week period, the patient received surgical treatment involving the excision of necrotic tissues and administered antibiotic therapy. After the results of a control microbiological study, a second surgical procedure was performed to address the soft tissue defect in the lower third of the right tibia using two split-skin autografts. The wound was then covered with an aseptic dressing and gauze swabs. On day 3 after repeated autotransplantations, lyophilized powdered decellularized human umbilical cord-derived hydrogels were applied to the defect area (Fig. 8, *c*). By the next day, the foot edema was significantly reduced, the wound surface was partially scabbed, and the pathologic discharge was significantly decreased. On day 50 after tibial osteosynthesis with the Ilizarov device, the wound was repeatedly covered with decellularized lyophilized hydrogels. One month after osteosynthesis, the condition of the defect was satisfactory (Fig. 8, *d*).

This clinical observation demonstrates that the use of decellularized lyophilized hydrogels as an adjunctive treatment in autodermoplasty of extensive, deep, and infection-prone soft tissue defects helps create optimal conditions for graft engraftment.

Antibacterial activity may be an intrinsic property of native mammalian tissues, particularly those that are regularly exposed to bacterial populations, such as the skin, oral cavity, and gut. Evolution and ontogeny occur in a close relationship between the developing macroorganism with its microbiota and the diversity of the microcosm as a whole. This natural defense mechanism allows the host to limit or prevent microbial growth soon after exposure to different bacteria, before the immune system is stimulated. Peptides with antibacterial activity are thought to interact directly with the bacterial membrane, diffuse into the cytoplasm, and interfere with protein synthesis [9].

Because decellularized matrices from human and animal tissues are stroma with incorporated bioactive molecules, the fragmentation of collagen molecules and appearance of peptides with antimicrobial activity may determine the bacteriostatic effect in their clinical use [10, 15]. E.P. Brennan et al [9] showed that proteins released during the cleavage of decellularized matrix from the submucosal layer of porcine intestine and liver stroma exert variable antibacterial activities against *S. aureus* and *E. coli*. Several authors have

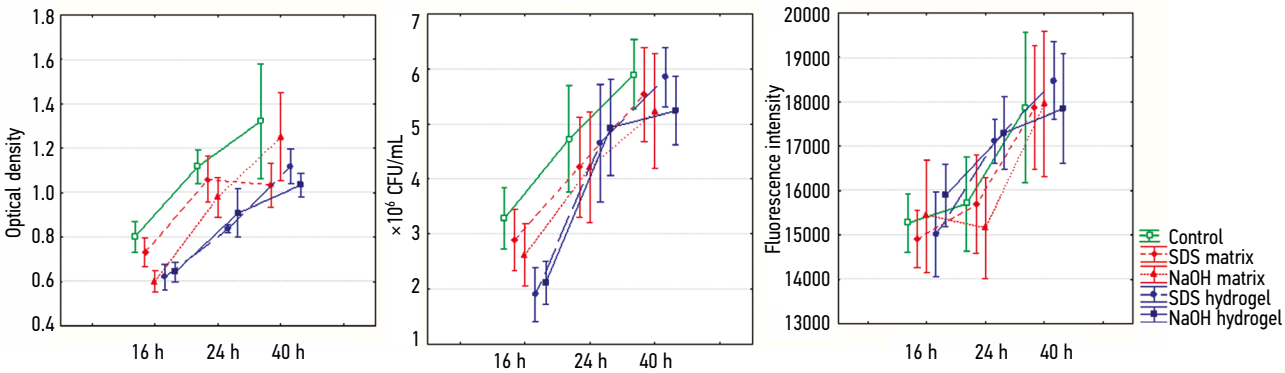
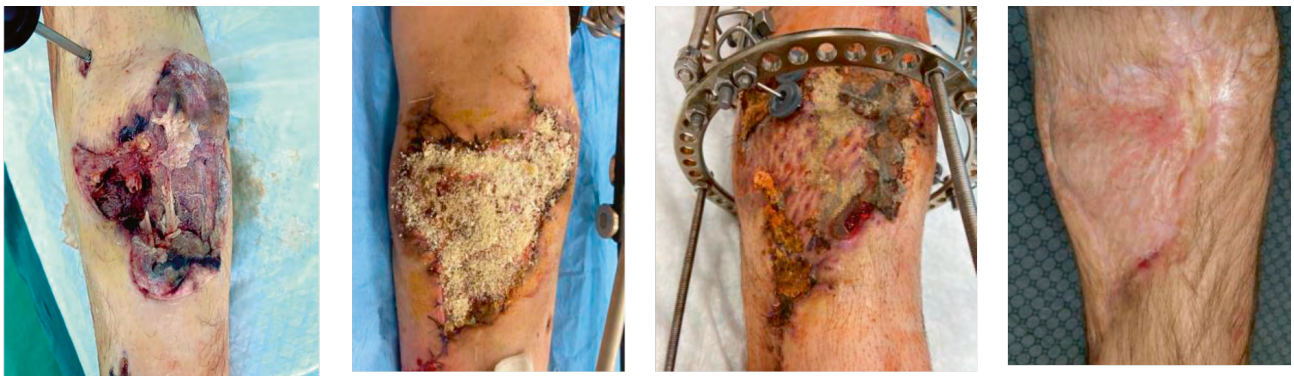


Fig. 6. Dynamics of *Staphylococcus aureus* culture in the presence of human umbilical cord-derived acellular matrices and hydrogels and in control (*M* ± *SD*)

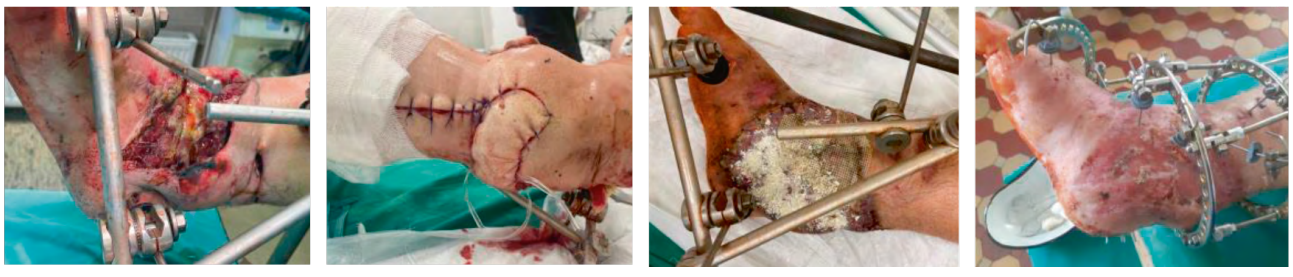
Рис. 6. Динамика культивирования *Staphylococcus aureus* в присутствии бесклеточных матриксов и гидрогелей из пуповины человека и в контроле (*M* ± *SD*)



a *b* *c* *d*

Fig. 7. Appearance of a shrapnel blind wound of the upper third of the leg with an extensive soft tissue defect and the fractures: *a* — 16 day after injury; *b* — muscle–cutaneous plastic and acellular matrix from the human umbilical cord in the wound 30 days after injury; *c* — appearance of the defect 2 months after injury with the replacement of Ilizarov apparatus; *d* — appearance of the defect 15 months after injury

Рис. 7. Внешний вид осколочного слепого ранения верхней трети голени с обширным дефектом мягких тканей и переломами: *a* — 16-е сутки после ранения; *b* — мышечно-кожная пластика и бесклеточный матрикс из пуповины человека в ране на 30-е сутки после ранения; *c* — внешний вид дефекта спустя два месяца после ранения с установкой аппарата Илизарова; *d* — внешний вид дефекта спустя 15 месяцев после ранения



a *b* *c* *d*

Fig. 8. Right leg and foot wound with a soft tissue defect in the lower third of the leg and comminuted fractures: *a* — appearance of an irregularly shaped wound (20 × 8 cm) in the lower third of the right leg after repeated surgical treatments; *b* — 25 days after injury, the sural flap on the vascular pedicle is fixed to the defect area; *c* — 38 days after injury, repeated autoplasty with free split skin grafts. Crushed lyophilized hydrogels from the acellular human umbilical cord were applied on top of the grafts; *d* — appearance of the defect area 80 days after injury

Рис. 8. Ранение правой голени и стопы с дефектом мягких тканей нижней трети голени и оскольчатыми переломами: *a* — внешний вид раны (20 × 8 см) нижней трети правой голени после хирургических обработок; *b* — 25-е сутки после ранения, суральный лоскут на сосудистой ножке фиксирован к зоне дефекта; *c* — 38-е сутки после ранения, повторная аутопластика свободными расщепленными кожными трансплантатами. Поверх трансплантатов применен измельченный лиофилизированный гидрогель из бесклеточной пуповины человека; *d* — внешний вид зоны дефекта на 80-е сутки после ранения

identified candidate antimicrobial agents in products derived from extraembryonic tissues, including the umbilical cord [2, 6, 11]. Presumably, these agents inhibited the growth of Gram-positive and Gram-negative bacteria at micromolar concentrations and reduced endotoxin activity by binding to lipopolysaccharide [11]. M. Dubus et al. [2] used mass spectrometric analysis and demonstrated that decellularized Warton's jelly material of human umbilical cord released antimicrobial molecules involved in the innate immune response, as well as some molecules involved in bacterial agglutination.

In this study, we used three techniques to determine the sensitivity of bacterial suspensions to the action of decellularized matrices and umbilical cord-derived hydrogels during different growth periods of microbial cultures. Spectrophotometric technique and CFU counting revealed the bacteriostatic effect of hydrogels prepared by two techniques against *E. coli*. Our results of analysis using hydrogels may be a consequence of the greater fragmentation of collagen molecules during manufacturing. The technique applied using resazurin allowed us to reveal the effect of hydrogels on the metabolic activity of microorganisms. Matrices, unlike hydrogels, did not show a bacteriostatic effect, which indicates that it is the hydrolysis of structural components in vitro under the influence of pepsin that promotes the release of active ingredients. Under in vivo conditions, the implanted matrix is subjected to the action of peptidases that ensure its biodegradation. In this context, it is not unreasonable to assume a delayed release of antimicrobial agents from the matrix placed on the wound bed [9].

More complex models used in the analysis of the antimicrobial activity of decellularized matrices also allow us to evaluate their anti-adhesive effect [6, 7, 11]. The preservation of glycosaminoglycans in umbilical cord-derived decellularized products indirectly confirms the assumption of a possible decrease in bacterial adhesion [2].

CONCLUSIONS

An in vitro experiment demonstrated the bacteriostatic effect of decellularized human umbilical cord-derived hydrogels against *E. coli* strain KS-507 during the first 16 h. The bacteriostatic effect of the hydrogels did not depend on the type of detergent used for their preparation. The effect is attributed to the influence on the level of metabolic activity of microorganisms. The use of lyophilized decellularized human umbilical cord-derived hydrogels as an adjunctive treatment in two patients ensured the engraftment of skin autografts and created conditions for healing of infection-risk extensive deep wounds.

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ADDITIONAL INFORMATION

Authors' contribution. Thereby, all authors made a substantial contribution to the conception of the study, acquisition, analysis, interpretation of data for the work, drafting and revising the article, final approval of the version to be published and agree to be accountable for all aspects of the study.

The contribution of each author. A.A. Kondratenko — study of the acellularity, composition and bacteriostatic properties of umbilical cord products, statistical processing of the results, writing the article; V.E. Chernov — study of the bacteriostatic properties of umbilical cord products, writing an article; D.V. Tovpeko — production and research of the composition of products from human umbilical cord, writing an article; D.A. Volov — clinical research, article writing; N.V. Bely — research on the bacteriostatic properties of umbilical cord products, writing an article; D.A. Zemlyanoy — statistical processing of results, material support for experiments, writing the article; L.I. Kalyuzhnaya — development of the general concept, research design, material support for experiments, writing the article.

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ДОПОЛНИТЕЛЬНАЯ ИНФОРМАЦИЯ

Вклад авторов. Все авторы внесли существенный вклад в разработку концепции, проведение исследования и подготовку статьи, прочли и одобрили финальную версию перед публикацией.

Вклад каждого автора. А.А. Кондратенко — исследование бесклеточности, состава и бактериостатических свойств продуктов из пуповины, статистическая обработка результатов, написание статьи; В.Е. Чернов — исследование бактериостатических свойств продуктов из пуповины, написание статьи; Д.В. Товпеко — изготовление и исследование состава продуктов из пуповины человека, написание статьи; Д.А. Волов — клинические исследования, написание статьи; Н.В. Белый — исследование бактериостатических свойств продуктов из пуповины, написание статьи; Д.А. Земляной — статистическая обработка результатов, материальное обеспечение экспериментов, написание статьи; Л.И. Калюжная — разработка общей концепции, дизайн исследования, материальное обеспечение экспериментов, написание статьи.

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Источник финансирования. Авторы заявляют об отсутствии внешнего финансирования при проведении исследования.

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