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PROSPECTS FOR APPLICATION OF BOVINE PERICARDIAL SCAFFOLD FOR CARDIAL SURGERY

A. A. Sokol
D. A. Grekov
G. I. Yemets
N. V. Shchotkina
A. A. Dovghaliuk
N. M. Rudenko
I. M. Yemets

State Institution „Scientific — Practical Medical Center of Pediatric Cardiology and Cardiac Surgery of the Ministry of Health of Ukraine“, Kyiv

E-mail: cardiotissue@gmail.com

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The aim of the study was to estimate the properties of the scaffold obtained by decellularization with of bovine pericardium a 0.1% solution of sodium dodecyl sulfate. The experiment included standard histological, microscopic, molecular genetic, and biomechanical methods. Scaffold was tested *in vitro* for cytotoxicity and *in vivo* for biocompatibility. A high degree of removal of cells and their components from bovine pericardium-derived matrix was shown. Biomechanical characteristics were of artificial scaffold were the same same as those of the native pericardium. With prolonged contact, no cytotoxic effect on human cells was observed. The biointegration of the scaffold in laboratory animals tissues was noted, thus confirming the potential possibility of the implant application in cardiac surgery.

Key words: pericardium, scaffold, decellularization, cardioimplant, tissue engineering.

Today, cardiovascular diseases are the leading cause of death in the world. According to WHO statistics, heart diseases kill more than 17 million people each year, which is 31% of all deaths [1]. Congenital heart defects occur with a frequency of about 9 % [2]. To date, cardiac surgery is performed in almost all congenital heart defects, where in most cases a complete anatomical correction is performed using artificial implants. The total postoperative mortality from these surgeries in the world‘s leading clinics is less than 3%. However, the use of artificial prostheses has a number of disadvantages that significantly impair the quality of patients life in the postoperative period. About a third of patients those get operated require repeated surgery at various times in the long term. Patients usually need lifelong anticoagulation therapy. A promising direction in overcoming the above problems may be the use of biological implants. However, they also have a number of unresolved issues, such as complete or partial biodegradation and calcification after implantation [3].

In the world medical practice, bioimplants made of xenogenic tissues, for example from the pericardium of pigs, horses, cattle, which have the elasticity of the material close to human tissues, are increasingly used [4]. To obtain such an implant, the native material is subjected to decellularization, which is the complete elimination of donor cells and purification from antigenic molecules while maintaining the structure of the extracellular matrix. Today, scaffolds are successfully used in the clinic for tissue engineering and regenerative medicine [5–8]. Decellularized extracellular matrix made of bovine pericardium is a promising biomaterial for cardiovascular tissue repair, as the structu re of collagen-elastin components of the framework is satisfactorily preserved, and antigenic molecules are properly eliminated and thus reduces the antigenicity of such material [9–11].

Sodium dodecyl sulfate (SDS) is one of the most commonly used anionic detergents to create extracellular matrices, as it can more

efficiently washout cytoplasmic proteins and remnants of nuclear components from tissues than other detergents [12, 13]. For example, it was the main detergent used to decellularize cardiac perfusion in all rats [12]. Thus, in a number of studies, colleagues described protocols for material SDS processing in accordance with the standard requirements for complete removal of cells and elimination of at least 90% of host DNA from tissues and organs of different species [14–17].

Another detergent often used in conjunction with SDS is Triton X-100, a non-ionic detergent that can remove cellular contents and help wash residual SDS from the ECM [12, 18]. Although the use of Triton X-100 eliminates cells from the heart valve, it is less effective in clearing the myocardium and aortic wall of cellular residues [19].

Unfortunately, today there is no ideal biomaterial that would meet all the requirements of cardiac surgery and has athrombogenicity, elasticity, durability, minimal antigenicity, lack of immunogenicity and cytotoxicity, and strength, i.e. was close to the characteristics of natural tissues. Therefore, the search for methods of biotechnological transformation of xenotissue, their development and improvement, which could provide high quality material obtaining, that will significantly improve the life quality of children with congenital heart disease, reduce the number of repeated cardiac surgeries and the cost of treatment.

Materials and Methods

The procedure for tissue obtaining

The material for the study was the cattle pericardium. The pericardial sac was extracted from outbred 12–18-month-old bulls after slaughter at the TOV "Antonovsky Meat Processing Plant". All animals underwent veterinary inspection. In the process of organ removal, the rules of asepsis with the maximal available atraumaticity and taking into account the anatomical features of animals, as well as in accordance with the basic principles of bioethics and bioethical expertise, consistent with the provisions of the "European Convention for the Protection of Vertebrate Animals which are used for experimental and other purposes" (Strasbourg, France, 1985) and in accordance with the Law of Ukraine № 3447-IV "On Protection of Animals from Cruel Treatment" (2006, latest edition 2009). The biomaterial was transported to the laboratory for one hour in a sterile Hanks solution in a container on ice. Then the pericardial sac was carefully prepared, separating the serous layer from the fibrous one. Fatty appendages and excess connective tissue were removed from the latter. To wash the fragments of the isolated pericardium from the remnants of blood components, it was placed in flasks with distilled water with a volume of 1 000 ml and stirred continuously (70 rpm) for 3 hours at 4 °C.

Protocol for decellularization of the bovine pericardium

Cattle pericardial samples were decellularized as follows: a 40×40 mm pericardial sample was placed in a 200 ml bottle containing 100 ml of 0.1% solution of SDS (Sigma-Aldrich, USA) with constant shaking (200 rpm) for 40 days at 4 °C [11]. The next step was to stabilize and fix the obtained samples. We provided stabilization and fixation of all obtained samples in a solution of 70 % ethanol for 24 h at t 4 °C with constant stirring at 200 rpm. Then all fragments of the decellularized pericardium were washed with sterile NaCl solution for 24 h at 4 °C with constant stirring at 200 rpm. An additional stage of chemical decellularization was achieved using the crosslinking method: EDC/NHS solution — MES (10 mM of 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), 10 mM of N-Hydroxy-succinimide) and MES solution (pH 5.6) 6 (0.05 M of 2 — morpholinoethane sulfonic acid). Native pericardium was used as a control.

Histological staining (hematoxylin-eosin and DAPI)

Hematoxylin — eosin. Histopathological examination of tissue samples includes fixation in 10 % solution of neutral buffered formalin (pH 7.4) for at least 3 days at a temperature of 4 °C. Tissues were fixed after rapid freezing and sectioning with a cryostat (6 μm thick). Validation of cell nucleus removal was performed according to the standard hematoxylin and eosin (H&E) staining protocol. Stained samples were examined using an Olympus BX 51 light microscope.

DAPI. DAPI staining (4′,6-diamidino-2-phenylindole, dihydrochloride) was performed directly by incubating a tissue sample (luminal side up) with 25 μg/ml DAPI diluted in PBS for 2 min (in the dark). Achieving maximal tissue thickness, adventitia and middle layer were removed using the approach proposed by Jelev et al. [20]. The ECM was fixed on the plate with

forceps under a microscope. The intima layer was carefully removed by stretching it with forceps, simultaneously with cutting the edges with a scalpel, followed by restoration in PBS. DAPI stains were also applied to paraffin-embedded cross-sections (5 μm thick) following standard fixation, dehydration, embedding, cutting, dewaxing, rehydration, and staining protocols. Images were taken using an Olympus BX 40 fluorescent microscope (Tokyo, Japan).

DNA quantitative evaluation

DNA extraction was performed using Easy Blood and Tissue DNA kit (Qiagen, Germany). 10–25 mg of tissue from external materials before decellularization, or from decellularized samples, were briefly treated with proteinase K to quantify the total amount of nucleic acid, ng/mg of dry tissue, and calculate the percentage of DNA removal after decellularization. Fluorescence measurements (photons per second) were performed at room temperature in a room of 23-24 °C, using a spectrofluorometer Qubit 3.0 to count photons. The limit of DNA detection was 0.2 ng/μg of nucleic acid.

Biomechanical testing. The biomechanical properties of bovine pericardium for flaps with a size of 20×40 mm were analyzed. To determine the maximal tensile strength at break (F_{max}), the pericardial samples were loaded separately between 2 steel rods and clamped vertically in a test machine (IMADA, MX2 — 110, Japan).

In vitro cytotoxicity assessment

To determine cytotoxicity, the matrix samples were cultured in a suspension of human fibroblasts. From the obtained material by 3 fragments from each sample were cut with an area of about 8–10 mm^2. Cells were inoculated by applying 150 μl of concentrated cell suspension on decellularized pericardial samples moistened with standard growth medium (DMEM + 10% of serum). Impregnation lasted 30 minutes. The number of inoculated on fragments cells was 300 thousand. They were then transferred to a standard 6-well tissue culture plate and immersed in growth medium DMEM + 10% of serum, cultivation was performed under standard conditions of 37 °C and 5% of CO_2 [21]. For histological examination, DEM samples were fixed in 10% buffered formalin embedded in paraffin. Sections were made (5 μm). Hematoxylin / eosin samples were obtained (light microscopy) [22]. Images were taken using an Olympus BX 51 light microscope (Tokyo, Japan).

In vivo biocompatibility test

For the study, 10 Wistar rats were kept in a pathogen-free environment. Decellularized and pre-sterilized bovine pericardium was implanted subcutaneously in the interscapular space and explanted after 8 weeks. The animals were divided into 2 groups. The groups were as follows: Control group: native bovine pericardium (n = 5), group 1: bovine pericardium decellularized with 0.1% solution of SDS (n = 5). Wool was removed from the skin surface of rats in the area of the operating field and treated with a 70% solution of ethyl alcohol. The operation was performed under sterile conditions. Xylazine (Alfasan, Netherlands) at a dose of 1 mg/kg of body weight in combination with ketamine (Biolik, Ukraine) at a dose of 10 mg/kg was used for intramuscular anesthesia. An incision of 2 cm was made in the back of the animal, and the subcutaneous pockets were formed with a pointed spatula, separating the subcutaneous tissue from the muscle layer. The prepared 1×1 cm implants were placed in the lumen of the pocket, which were fixed at the corners to the muscle tissue with noose sutures "Polypropylene" ("Golnit", Ukraine). The skin was closed with a continuous suture, the thread, which did not delaminate, was treated with an antiseptic — a solution "Betadine" ("EGIS Pharmaceuticals, PLC", Hungry). The implants were removed for histological analysis 3 months after surgery.

Statistical analysis. Statistical significance was analyzed using variation analysis and t-test, if necessary. $P < 0.05$ value was regarded as statistically significant. Variation and statistical processing of the obtained results was performed using StataIC software.

Results and Discussion

It is known that cellular components, including nucleic acids of xenogeneic tissues, are strong antigens that trigger immunological reactions in the recipient, which provokes graft rejection. A decellularization protocol is considered to be effective, using which all components of the cell and nucleic residues are completely removed [23].

To assess the decellularization process, histological examination of samples stained with hemotoxylin-eosin and by the DAPI method was performed. Basophilically stained bovine pericardial cells and the bright glow of fluorescent dye were detected in the control samples, which confirms the presence of nucleic acids (Fig. 1). At the same time, the

absence of cells in hematoxylin-eosin staining and the absence of glow in DAPI were recorded for the bovine decellularized pyricard of the experimental group. The absence of nuclear elements was seen by staining in both ways after 21 days of decellularization. There was also no obvious difference in the structure and distribution between the collagen and elastin fibers of the decellularized extracellular matrix and the native bovine pericardium.

In the samples of the experimental group, the DNA concentration was recorded at the level of 5 ng / mg on the 35^{th} day of decellularization (Fig. 2). Thus, the degree of matrix purification of decellularized bovine pericardium tissue from nucleic acid residues was 99.8 % compared with native samples (Table 1). A statistically significant difference was found between the DNA concentrations of the experimental and control groups ($P < 0.05$).

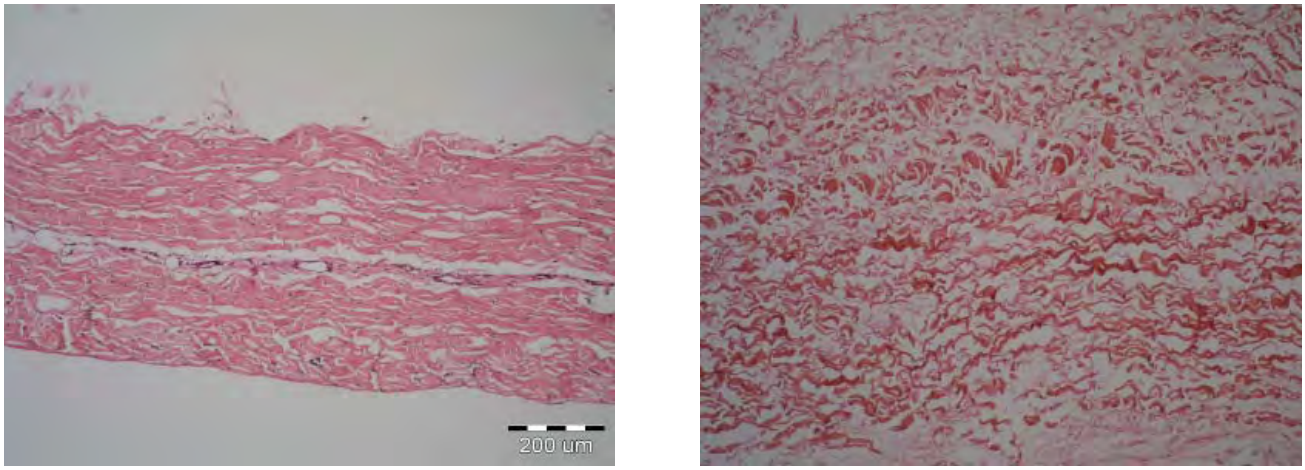
The study of biomechanical properties showed that the decellularized matrix not only did not change its natural properties, but also gained greater strength after the process of purification and cross-linking. This is evidenced by an increase almost 2 times of the maximal tensile strength value in the samples of the experimental group (Table 1).

The study of the cytotoxic effect of the decellularized extracellular matrix showed that after two months of cultivation the culture of human fibroblast cells were placed in a uniform layer on the surface of the sample. Some groups of fibroblasts penetrated to a depth of 350 μm, but only in places where the fiber bundles were less dense (Fig. 3). Collagen and elastin components of the matrix are well expressed, fiber bundles are strong, ordered.

The ultimate goal of implantation is bioimplant integration into the host tissue, with its subsequent regeneration [24]. Fig. 5 presents a histological examination of the explanted pericardium in control rats implanted with untreated/native bovine pericardium. As expected, the pericardium is completely degraded and eliminated, there is only connective and muscle tissue of the operated animal. The tissue is infiltrated with leukocytes, which indicates inflammatory processes in this area.

At the same time, in comparison with the control, the histology data indicate the successful biointegration of the implant in the rats of the experimental group (Fig. 6). In the tissues, implant replacement with growing immature connective tissue was noted. In the area of implant, the increased vascularization of connective tissue is also observed, capillaries are formed, which are filled with erythrocytes. There was a lower level of macrophages and monocytes filtration in decellularized tissues of the pericardium compared to non-decellularized tissues of the control group.

This study made it possible to obtain a scaffold made of xenotissue, which in its biomechanical and biological characteristics is similar to the native bovine pericardium. Ionic detergent SDS was used in the decellularization process. The scientific literature confirms the effective use of this detergent, the action of which is aimed at cleaning the matrix by solubilizing the cytoplasmic and nuclear



A *B*

Fig. 1. **Imaging of histological sections of bovine cellular and decellulorized pericardium. Hematoxylin and eosin staining (light microscopy, ×200) and DAPI (fluorescence microscopy, ×200):**
A — control group (native pericardium); *B* — experimental group (0.1% solution of SDS)

Table 1. **Comparative evaluation of native and decellularized bovine pericardium**

Research / indicators	Control group	Experimental group
Histology	Presence of basophilically stained cells	Absence of cells
DAPI	The presence of the glow of cells nuclear material	No glow of cells nuclear material
Average DNA content (ng/mg), $n = 5$	1436 ± 116.8	0.5 ± 0.448*
Fmax, maximal tensile strength (kgf)	6.84 ± 0.69	9.548 ± 0.65*
Cytotoxicity	-	None after 2 months
Biocompatibility, inflammatory reaction**	++++	+

* statistically significant compared with the control group ($P < 0.05$).

** To obtain more objective data, a semi-quantitative assessment of inflammatory reaction severity was performed: 0 — no signs; + — single cells (neutrophils, lymphocytes, macrophages, eosinophils); ++ — small foci; +++ — separate and massive foci; ++++ — large infiltrates.

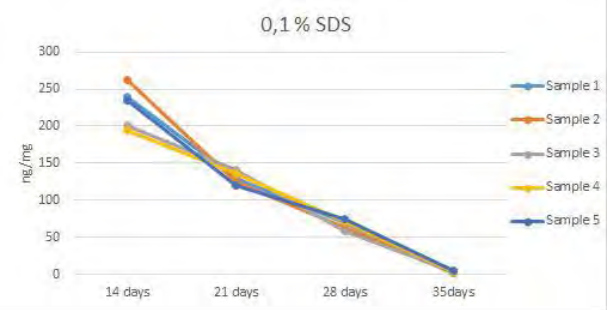


Fig. 2. **DNA concentration (ng/mg) in pericardium samples decellularized with 0.1% solution of SDS**

membranes, denaturation of proteins and removal of nuclear residues [25–27].

At the same time, it is known that SDS can bind to and deform collagen fibers. Due to hydrogen bonds of collagen fibers rupture, tissue edema may develop [23, 28]. SDS is reported to interact very strongly with extracellular matrix proteins, making it difficult to remove SDS completely [29]. Ning Lia and colleagues also noted that residual SDS in decellularized tissues can lead to insufficient settling of host cells after implantation, causing less implant durability and inhibiting growth. This was clearly correlated in rat implantation studies, where peri-implant necrosis was found around about 1% of SDS-treated implants [22]. Based on these facts, it was hypothesized that the use of a low concentration of 0.1% solution of SDS will allow for effective decellularization, while maintaining the architectonics of the matrix and not affect the biocompatible properties of scaffold. This study confirms the hypothesis and demonstrates the effective use of low-concentration SDS detergent for decellularization of the bovine pericardium. Tran Ha Le Bao et al. also showed that 0.1% solution of SDS is better fit for porcine pericardial decellularization than 0.3% or 0.5% solution of SDS, because the biological properties of scaffold were better preserved [31].

In our study, the absence of cells and their components was confirmed by histological and molecular genetic studies. Examination of hematoxylin-eosin-stained samples showed the absence of nuclear elements and the preservation of scaffold matrix structure. Complete removal of all cellular components is virtually impossible with any method of decellularization [32]. Quantitative evaluation of residual DNA can be used as an additional marker in determining the effectiveness of the decellularization process. DNA analysis showed that decellularization was able to remove 99.8% of nucleic acids from the extracellular matrix.

The absence of scaffold toxic effects on donor cells is an important part of selection for further transplantation. In this study, the cytotoxicity of scaffold was determined by comparing different microscopy samples to identify the number and location of human fibroblasts cultured on the obtained tissue sample. Histological analysis made it possible to establish the levels of structural changes in the fibers together with the position of fibroblast cells after prolonged cultivation. In our study, we did not observe any *in vitro* cytotoxic effect for a decellularized matrix

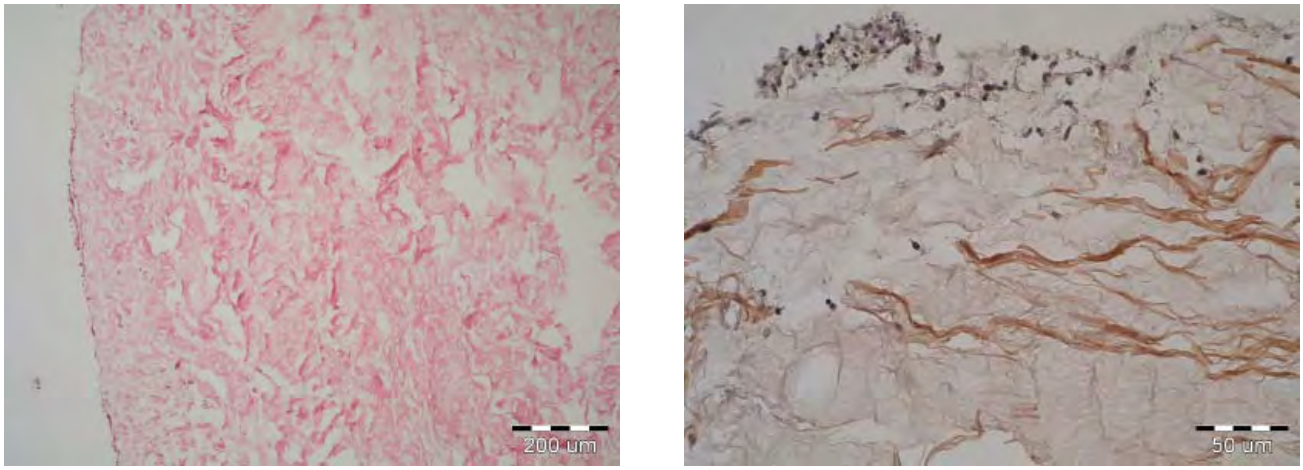


Fig. 3. **Histological examination for cytotoxicity in samples of the experimental group (pericardium decellularized with 0.1% solution of SDS) after 2 months of cultivation** (Congo staining and H/E, light microscopy, × 200, ×50)

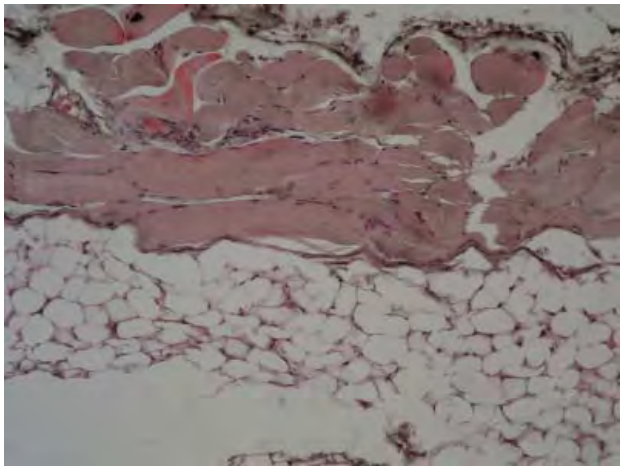


Fig. 4. **Histological examination of the explanted pericardium of control rats** (tricolor Masson staining method, light microscopy, ×200)

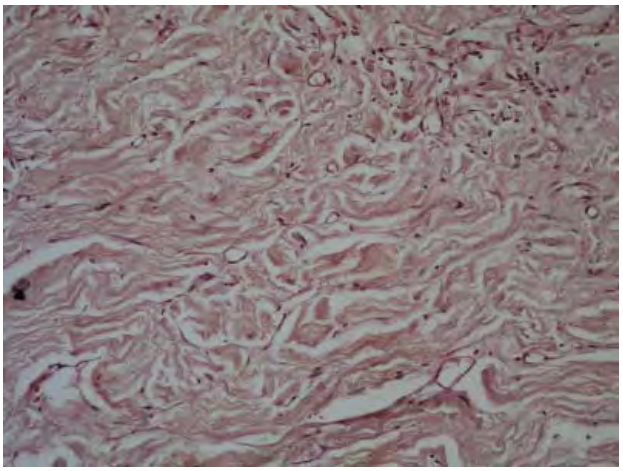


Fig. 5. **Histological analysis of decellularized pericardial implants and evaluation of rat tissue response after subcutaneous implantation** (tricolor Masson staining method, light microscopy, ×200)

with a law concentration of 0.1% solution of SDS for 2 months of cultivation. Although in most experiments the cytotoxic effect for a short period of time (24 to 48 hours) was studied, long-term studies are more effective for the transplant stage [25–27, 31].

The ideal scaffold should not only be free of any cells, have sufficient stability, be able to withstand mechanical loads for a long time, while maintaining the structure of the extracellular matrix, but also when implanted to integrate into the donor tissue [23]. That is, the created matrix is the basis for its settling by donor cells. One of the most important requirements for the safety of the scaffold is the biocompatible properties of the biomaterial determination [33, 34]. Our study has demonstrated a high level of scaffold biocompatibility with tissues of Wistar rats. The bioimplant was not only destroyed, but also became a full-fledged part of the tissues of experimental animals, as evidenced by its replacement by immature connective tissue.

Thus, the scaffold from bovine pericardium was obtained by decellularization using a low concentration of 0.1% solution of SDS which can then be used in cardiac surgery, subject to a series of preclinical and clinical tests.

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ПЕРСПЕКТИВА ВИКОРИСТАННЯ СКАФФОЛДУ З ПЕРИКАРДА ВЕЛИКОЇ РОГАТОЇ ХУДОБИ ДЛЯ ПОТРЕБ КАРДІОХІРУРГІЇ

А. А. Сокол
Д. А. Греков
Г. І. Ємець
Н. В. Щоткіна
А. А. Довгалюк
Н. М. Руденко
І. М. Ємець

ДУ «Науково-практичний медичний центр дитячої кардіології та кардіохірургії» МОЗ України, Київ

E-mail: cardiotissue@gmail.com

Метою дослідження було оцінити властивості скафолду, отриманого після децелюляризації перикарду великої рогатої худоби 0.1% розчином додецилсульфату натрію. Експеримент включав стандартні гістологічні, мікроскопічні, молекулярно-генетичні, біомеханічні методи. Скаффолд протестований на цитотоксичність *in vitro* та біосумісність *in vivo*. Показано високу ступінь очистки позаклітинного децелюляризованого матриксу від клітин та їх компонентів. Біомеханічні характеристики були такими самими, як і нативного перикарда. При довготривалому контакті не спостерігали цитотоксичного впливу на клітини людини. Відмічено біоінтеграцію скаффолду в тканини лабораторних тварин, що підтверджує потенційну можливість використання імпланту в кардіохірургічний практиці.

Ключові слова: перикард великої рогатої худоби, скафолд, децелюляризація, кардіоімплант, тканинна інженерія.

ПЕРСПЕКТИВА ИСПОЛЬЗОВАНИЯ СКАФФОЛДА ИЗ ПЕРЕКАРДА КРУПНОГО РОГАТОГО СКОТА ДЛЯ КАРДИОХИРУРГИИ

А. А. Сокол
Д. А. Греков
Г. И. Емец
Н. В. Щоткина
А. А. Довгалюк
Н. Н. Руденко
И. Н. Емец

ГУ «Научно-практический медицинский центр детской кардиологии и кардиохирургии МОЗ Украины», Киев

E-mail: cardiotissue@gmail.com

Целью исследования было оценить свойства скаффолда, полученного при децелюляризации перикарда крупного рогатого скота 0.1% раствором додецилсульфата натрия. Эксперимент включал стандартные гистологические, микроскопические, молекулярно-генетические, биомеханические методы. Скаффолд протестирован на цитотоксичность *in vitro* и биосовместимость *in vivo*. Показана высокая степень очистки внеклеточного децелюляризированого матрикса от клеток и их компонентов. Биомеханические характеристики были такими же, как и нативного перикарда. При длительном контакте не наблюдали цитотоксического воздействия на клетки человека. Отмечено биоинтеграцию скаффолда в ткани лабораторных животных, что подтверждает потенциальную возможность использования импланта в кардиохирургической практике.

Ключевые слова: дперикард крупного рогатого скота, скаффолд, децеллюляризация, кардиоимплант, тканевая инженерия.