Cell and Organ Transplantology. 2020; 8(2):193-197.
doi: 10.22494/cot.v8i2.110

Biocompatibility analysis of the decellularized bovine pericardium

Sokol A.[1,2], Grekov D.[1,2], Yemets G.[1], Galkin O.[2], Shchotkina N.[1,2], Rudenko N.[1], Yemets I.[1]

[1]*Ukrainian Children's Cardiac Center, Kyiv, Ukraine*
[2]*Igor Sikorsky Kyiv Polytechnic Institute, National Technical University of Ukraine, Kyiv, Ukraine*

e-mail: cardiotissue@gmail.com

ABSTRACT

The decellularized bovine pericardium and its potential use as a natural scaffold is a promising approach in the field of tissue engineering and regenerative medicine. The reaction of the host toward decellularized scaffolds depends on their biocompatibility, which should be satisfied being before applied in clinical use.

***PURPOSE**: to evaluate the biocompatibility of the extracellular matrices, which were decellularized by trypsin enzyme and anionic sodium dodecyl sulfate (SDS) detergent.*

***MATERIAL AND METHODS.** Pericardial sacs were acquired from 12-18 months' age bulls. Tissue decellularization was performed by using 0.25 % Trypsin solution and 1 % ionic SDS for group I and 0.1 % SDS for group II samples. The implantation was performed on Wistar rats. The tissue samples were stained with hematoxylin & eosin, Congo red and Masson's Trichrome for histological analysis.*

***RESULTS.** In group 1 in 3 months after subcutaneous implantation in rats we noticed the inflammation in surrounding tissue and degradation of the implant. Under the same conditions in animals of group 2 implant replacement with growing immature connective tissue was noted. Bio-implant of this group did not degrade, moreover it's integrated to the tissues of experimental rats.*

***CONCLUSION.** Our results showed that decellularized bovine pericardium by 0.1 % SDS can become an alternative material for tissue engineering and has the potential for further use in human surgery.*

***KEY WORDS:** bovine pericardium; decellularization; implantation; regenerative medicine*

The decellularized bovine pericardium and its potential use as a natural scaffold is a promising approach in the field of tissue engineering and regenerative medicine. Bovine pericardium can be used in tissue engineering, as a biomaterial for the reconstruction of congenital heart defects and for the construction of heart valves, vascular grafts and patches [1, 2]. Worldwide, over 300,000 heart valve replacements and over 570,000 arterial bypasses are performed, therefore, there is a great need for developing bioprosthetic scaffolds [3]. Moreover, congenital heart disease occurs with a frequency of about 9 % [4]. Today, cross-linked bovine pericardium with glutaraldehyde is widely used in cardiovascular surgery for acquired and congenital heart defects (closure of atrial and ventricular septal defects) correction; manufacturing of tricuspid, mitral and aortic heart valves; prosthetics and plasty of vessels, heart valve leaflets, pericardial tissue; prevention of the mediastinum adhesions; bioprosthetics and main vessels plasty (ascending aortic plasty); aortic root plasty [5-10]. Despite a number of advantages of this biomaterial, the problem of calcification and degeneration *in vivo* has not been solved yet [11]. Therefore, the search for new protocols for pericardial decellularization continues.

Therefore, the **PURPOSE** of our study was to evaluate the biocompatibility of the extracellular matrices of bovine pericardium, which were decellularized by trypsin enzyme and anionic sodium dodecyl sulfate (SDS) detergent.

MATERIALS AND METHODS

Tissue isolation and processing. The research was performed in accordance with the General Ethical Principles of Animal Experiments (Strasbourg, France, 1985) and Law of Ukraine No. 3447-IV «On Protection of Animals from Cruelty» (2006, edited in 2009). Pericardial sacs were obtained from 12-18-month bulls and randomly selected from a local abattoir «Antonovsky Meat Factory» LLC. Pericardial sacs were dissected and non-fibrous components were removed. The tissue samples were cut to the 40×40 mm pieces. In this study, three groups (n = 5 each) were tested, including two pericardial processing conditions and native pericardial tissues.

Decellularization. Following preparation, pericardial tissue was then randomly subjected to one of two processing conditions. The first stage

Fig. 1. Micrographs of histological sections of decellularized bovine pericardium before implantation; hematoxylin/eosin staining, light microscopy, x200. **A** – native pericardium; **B** – group 1 (trypsin + 1 % SDS); **C** – group 2 (0.1 % SDS)

Fig. 2. Micrographs of histological sections of decellularized bovine pericardium before implantation; Congo red staining, luminescent microscopy x200. **A** – native pericardium; **B** – group 1 (trypsin enzyme + 1 % SDS); **C** – group 2 (0.1 % SDS)

of processing is common for both groups: samples were exposed to osmotic shock placing to the sterile distilled water (5 pieces per 500 ml solution) at 4 °C for 72 hours (200 rpm). Water was changed every 6-8 hours.

Group 1 samples were prepared using 0.25 % Trypsin solution at 24 °C constantly shaking for 3-hours at 50 rpm. Then, the samples of this group were placed into the 1 % ionic Sodium dodecyl sulfate (SDS) detergent. Group 2 samples were prepared using a low concentration 0.1 % SDS. Detergent solution was replaced every 24 hours. In general, the decellularization process lasted 30 days. Samples detoxification was carried out by washing in sterile distilled H_2O for 7 days at 4 °C constantly stirring at 200 rpm. H_2O replacement was performed every 12 hours. The next step was to stabilize and fix obtained samples. We provided stabilization and fixation of all obtained samples in a 70 % ethanol solution during 24 h at 4 °C with constant stirring at 200 rpm. Then all fragments of the decellularized pericardium were washed in sterile saline for 24 h at 4 °C with constant stirring at 200 rpm. Extra stage for the chemical decellularization was achieved using the cross-linking method using EDC/NHS solution: 10 mM 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC), 10 mM N-Hydroxysuccinimide (NHS) and 0.05 M 2-morpholinoethane sulfonic acid (MES) at pH 5.6 [12]. Non decellularized pericardial tissues served as controls.

Surgical procedure. Fifteen male Wistar rats 4-5-month Wistar rats weighing 190-230 g were kept in a pathogen free environment. Decellularized and sterilized bovine pericardium were implanted subcutaneously in a rat back and explanted after 3 months. The animals were divided into 3 groups. The groups were as follows: control group – native bovine pericardium (n = 5), group 1 – trypsin enzyme + 1 % SDS treated bovine pericardium (n = 5), group 2 – 0.1 % SDS treated bovine pericardium (n = 5).

The surgical field was shaved and cleansed with 70 % solution of ethyl alcohol. The operation was performed in sterile conditions. Intramuscular anesthesia was used with xylazine (*Alfasan,* Netherlands) at a dose of 1 mg/kg body weight in combination with ketamine (*Biolik*, Ukraine) at a dose of 10 mg/kg. A 2 cm incision was performed on the animal's back and subcutaneous pockets were formed using a pointed spatula, separating the subcutaneous tissue from the muscular layer. Prepared 1 × 1 cm implants were placed in the pocket, which were fixed at the corners to the muscle tissue using «Catgut Chrome» (*Golnit*, Ukraine). The skin was sutured and treated with an antiseptic solution of 1 % «Diamond Green» (*Halychpharm*, Ukraine). The implants were isolated for histological analysis after 3 months post-operation. Euthanasia was performed using diethyl ether.

Histological analysis. Segments of non decellularized (n = 5) and decellularized (n = 10) pericardial tissues were fixed in 10 % neutral buffered formalin (*Sigma-Aldrich,* USA) for 1 h, embedded in paraffin, cut into 5-μm sections, and stained with hematoxylin & eosin (*Sigma-Aldrich*, USA), Congo Red Kit (*Sigma-Aldrich*, USA) and Masson's Trichrome stain (*Sigma-Aldrich*, USA).The stained samples were examined with BX 51 light microscope (Olympus, Japan) and BX 40 luminescence microscope (*Olympus,* Japan).

RESULTS AND DISCUSSION

Morphological analysis of decellularized pericardial tissue. Histological analysis showed that decellularized pericardial tissues retained the ECM components without cells or nuclei (**Fig. 1**). Stained with hematoxylin and eosin showing the presence of visible cell nuclei in native pericardium. There was no obvious difference in the structure of the ECM, including the distribution of collagen and elastin fibers, between native and decellularized bovine pericardium in both groups.

Staining of pericardial tissues with Congo red showed that the collagenous and elastic components of the extracellular matrix were well preserved (**Fig. 2**). In sections of decellularized bovine pericardium group 1, the distribution of collagen fibers appeared less dense than in the group 2.

In vivo durability of decellularized pericardial tissue. The replacement efficiency and biocompatibility of decellularization in pericardial tissues

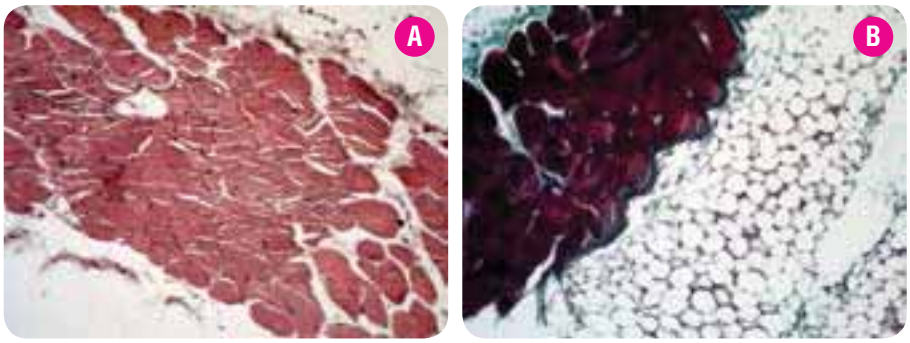

Fig. 3. Micrographs of histological sections of native bovine pericardium implants of control group in 3 months after subcutaneous implantation in rats; hematoxylin/eosin and Masson's Trichrome staining, light microscopy, x200. **A** – no visible implanted native pericardium, leukocyte infiltration (blue arrows); **B** – no visible implanted native pericardium, swelling of muscle tissue (blue arrow).

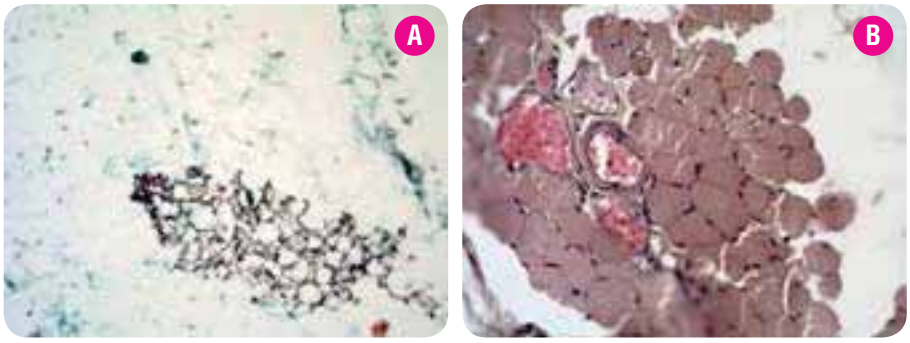

Fig. 4. Micrographs of histological sections of decellularized bovine pericardium implants of group 1 in 3 months after subcutaneous implantation in rats; light microscopy, x200. **A** – striated muscle tissue of a rat around the implant with formation of blood clots and swelling (blue arrow); hematoxylin/eosin staining; **B** – degraded part of the implant (blue arrow); Masson's Trichrome staining.

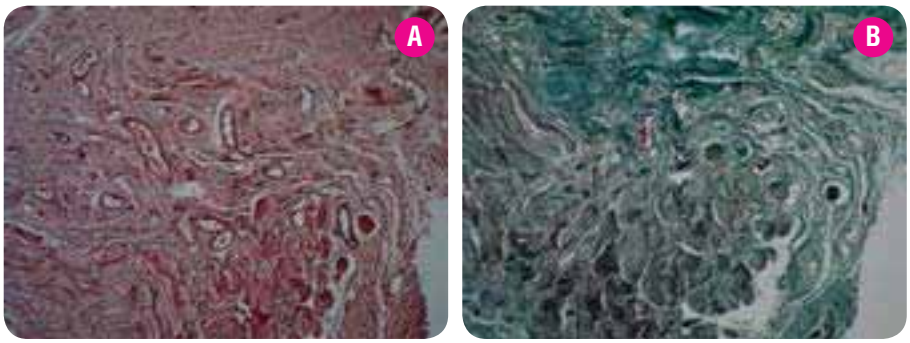

Fig. 5. Micrographs of histological sections of decellularized bovine pericardium implants of group 2 in 3 months after subcutaneous implantation in rats; light microscopy, x200. **A** – the formation of immature connective tissue over the entire surface of the graft; hematoxylin/eosin staining; **B** – the formation of immature tissue over the entire surface of the graft and newly formed capillaries with erythrocytes (blue arrow); Masson's Trichrome staining.

was evaluated by implantation in the interscapular area of Wistar Rats. All rats that were implanted with the non decellularized and decellularized grafts survived and successfully recovered after the surgery. The grafts remained within the Wistar Rats for 3 months.

Twelve weeks following the surgery, non decellularized grafts of control groups were resorbed and tissue were infiltrated by macrophages mostly as shown by H&E and Masson's Trichrome staining. In **Fig. 3** presents the results of histological examination of the explanted pericardium of rats of the Control group, which was implanted untreated/native bovine pericardium. As expected, the pericardium is completely degraded and eliminated, there is only connective and muscle tissue of the animal. The tissue is infiltrated with leukocytes, which indicates inflammatory processes in this area.

In group 1 visual evaluation showed a lower leucocyte infiltration in decellularized pericardial tissues, compared to non-decellularized tissues of the control group (**Fig. 4**). The presence of new vessels and immature tissue over the entire surface of the graft of group 2 suggested the potential of graft remodeling over a long-term implantation. In group 1 animals, where implanted pericardium was treated with Trypsin and 1 % ionic SDS detergent, in the striated muscles surrounding to the implant there was swelling of muscle fibers and slight lymphocytic infiltration, indicating the development of inflammation. The collapsing area of the implant is clearly visible. In the striated muscle tissue, there is swelling of muscle fibers and stasis, which indicates a stop or slowing of blood flow in the microcirculatory vessels (mainly in the capillaries). Also in some areas recorded sludge phenomenon, characterized by adhesion of erythrocytes and leukocytes resulting in complications of blood perfusion through the vessels of the microcirculatory tract and the formation of blood clots.

In the tissues of group 2, implant replacement with growing immature connective tissue over the entire surface of the graft was noted. Also observed in the area of the implant enhanced vascularization of connective tissue, newly formed capillaries are filled with erythrocytes (**Fig. 5**). In a part of the capillaries erythrocyte stasis was observed. Bio-implant of group 2 did not degrade, moreover, it integrated to the surrounding tissues of experimental rats.

The main objective of this study was to use modern approaches of tissue engineering to produce xenogeneic tissue grafts suitable for transplantation. Since the decellularization provides the biomaterial mechanically stable, functionally reliable, non-immunogenic and able to support cell growth, the next stage of the study was to test xenogeneic tissue for biocompatibility *in vivo*. Therefore, two samples of bovine pericardial tissue decellularized by two different protocols against native pericardial tissues were compared, followed by an assessment of the effect of treatment on degradation. Thus, the ideal graft must be free of any cells, as these are potential antigens that can provoke host immune response, have sufficient stability and be able to withstand mechanical stress for a long time, while maintaining the structure of the extracellular matrix [13, 14]. One of the most important requirements for the safety of the scaffolds is biocompatibility that is a property of a biomaterial and its environment [15, 16]. A rat subcutaneous implantation model was used

to evaluate local tissue reactions following implantation of the two types of decellularized pericardium membranes intended for the use in cardiac surgery. After 3 months from implantation, no signs of rejection were observed for 2 types of extracellular matrices. Nevertheless, different responses of rat tissue to the two decellularized bovine pericardia can be displayed in the histological sections of Fig 4, 5.

Thus, testing of group 1 material did not provide the expected positive result *in vivo*. The implant degraded and caused inflammatory reactions in the surrounding recipient's tissue. Such results exclude this graft of its further use in cardiac surgery [17-21].

It is well known that SDS is an ionic detergent, capable of binding to collagen fibers, thus destroying them and promoting swelling of the tissue, caused by a potential break in hydrogen bonds of the collagen fibers [14, 16]. SDS has been reported to interact strongly with the ECM proteins, making its complete removal challenging [22]. Based on this fact, Courtman et al. proposed that using SDS as decellularization reagent could possibly alter the thermal stability of the collagen and glycosaminoglycans (GAGs) content [17]. Ning Lia et al. also noted that residual SDS in decellularized tissues could result in an insufficient repopulation with the host cells after implantation, causing a lower durability of the implant and inhibiting growth. This was clearly correlated in rat implantation studies, where peri-implant necrosis was found in around 1 % SDS-treated implants [23].

At the same time, histological data indicate successful integration of the implant in rats of group 2. In this case, we used 0.1 % SDS concentration, which is ten times lower than in the previous testing of group 1. Our results coincided with previous data, also showing that 0.1 % SDS is better for the pericardial decellularization than 3 % or 0.5 % SDS, as far as the biological properties were better preserved [24]. Based on these observations, it may be assumed that the acellular pericardium scaffold manufactured using 0.1 % SDS may be potentially eligible for clinical implantation in humans.

CONCLUSION

To summarize, bovine pericardium was decellularized successfully with 0.1 % SDS with NHS/EDC cross-linked additional and maintained its original biological properties after subcutaneous implantation in rats. Overall, decellularized bovine pericardium tissue holds promise for its application in the manufacturing of tissue-engineered materials for cardiac surgery.

REFERENCES

1. *Nordmeyer S, Murin P, Schulz A, et al:* Results of aortic valve repair using decellularized bovine pericardium in congenital surgery. Eur J Cardiothorac Surg. 2018; **54**:986-992. DOI: 10.1093/ejcts/ezy181.
2. *Bell D, Prabhu S, Betts K, et al.* Durability of tissue-engineered bovine pericardium (CardioCel®) for a minimum of 24 months when used for the repair of congenital heart defects. Interact Cardiovasc Thorac Surg. 2019; **28(2)**:284-290. DOI: 10.1093/icvts/ivy246.
3. *Biasi GM, Sternjakob S, Mingazzini PM, Ferrari SA*. Nine-year experience of bovine pericardium patch angio-plasty during carotid endarterectomy. J Vasc Surg. 2002; **36**:271-277. DOI: 10.1067/mva.2002.123685.
4. *Hoffman J I E, Kaplan S*. The incidence of congenital heart disease. J Am Coll Cardiol. 2002; **39(12)**:1890-900. DOI: 10.1016/s0735-1097(02)01886-7.
5. *Cunanan CM, Cubbling CM, Dinh TT, et al*. Rutledge 3rd and M.C. Fishbein, Tissue characterization and calcification potential of commercial bioprosthetic heart valve. Ann Thora. Surg. 2001; **71**:417-421. DOI: 10.1016/S0003-4975(01)02493-6.
6. *Karpenko AA, Kuzhuget RA, Starodubtsev VB, et al.* Immediate and long-term results of various methods of carotid bifurcation reconstruction. Circulatory pathology and cardiac surgery. 2013; **1**:21-24.
7. *Mueller C, Dave H, Prêtre R*. Surgical repair of aorto-ventricular tunnel. Multimed Man Cardiothorac Surg. 2012: 1093-7.
8. *Chesnov UM*. Biocompatibility of xenopericardium fixed with epoxy compounds in in vitro and in vivo experiments. Current issues of cardiology: Sat. scientific tr. Manak NA, editor. Minsk: Encyclopedia. 2002. **2**:188-90.
9. *Nonaka M, Iwakura A, Yamanaka K*. Technique to treat extensive abscesses in double valve replacement for prosthetic valve endocarditis. J Heart Valve Dis. 2013; **22 (4)**:575-7.
10. *Akhmedov SD, Afanasyev SA, Dyakova ML, et al*. Use of a cell-free matrix for the formation of new blood vessels and heart by tissue engineering. Cell transplantology and tissue engineering. 2009; **4 (2)**:32-9.
11. *Salameh A, Greimann W, Vondrys D, Kostelka M*. Calcification or Not. This Is the Question. A 1-Year Study of Bovine Pericardial Vascular Patches (CardioCel) in Minipigs Semin Thorac Cardiovasc Surg. 2018; **30(1)**:54-59. DOI: 10.1053/j.semtcvs.2017.09.013.
12. *Park S, Kim SH, Lim HG, et al*. The Anti-calcification Effect of Dithiobispropionimidate, Carbodiimide and Ultraviolet Irradiation Cross-linking Compared to Glutaraldehyde in Rabbit Implantation Models. Korean J Thorac Cardiovasc Surg. 2013; **46(1)**:1-13. DOI: 10.5090/kjtcs.2013.46.1.1.
13. *Simões IN, Vale P, Soker S, et al*. Acellular Urethra Bioscaffold: Decellularization of Whole Urethras for Tissue Engineering Applications. Sci Rep. 2017; **7**:41934. DOI:10.1038/srep41934.
14. *Gilbert WT, Sellaro LT, Badylak FS*. Decellularization of tissues and organs. Biomaterials. 2006; **27**:3675-3683. DOI: 10.1016/j.biomaterials.2006.02.014.
15. *Aamodt JM, Grainger DW*. Extracellular matrix-based biomaterial scaffolds and the host response. Biomaterials 2016; **86**:68-82. DOI: 10.1016/j.biomaterials.2016.02.003.
16. *Oswal D, Korossis S, Mirsadraee S, et al*. Biomechanical characterization of decellularized and cross-liked bovine pericardium. J Heart Valve Dis. 2007; **16**:165-174.
17. *Courtman D, Pereira C, Kashef V, McComb D*. Development of a pericardial acellular matrix biomaterial: Biomechanical and mechanical effects of cell extraction. J Biomed Mater Res. 1994; **28**: 655-666. DOI:10.1002/jbm.820280602.
18. *Luo J, Korossis S, Wilshaw SP, et al*. Development and characterization of acellular porcine pulmonary valve scaffolds for tissue engineering. Tissue Eng. A. 2014; **20**:2963-2974. DOI: 10.1089/ten.tea.2013.0573.
19. *Korossis S, Wilcox H, Watterson K., et al*. In-vitro assessment of the functional perfor-mance of the decellularized intact porcine aortic root. J Heart Valve Dis. 2005; **14**:408-422.
20. *Yeh HS, Keller JT, Brackett KA, et al*. Human umbilical artery for microvascular grafting.Experimental study in the rat. J Neurosurg. 1984; **61**:737.

21. *Schmidt CE, Baier JM.* Acellular vascular tissues: Natural biomaterials for tissue repair and tissue engineering. Biomaterials. 2000; **21**:2215. DOI: 10.1016/S0142-9612(00)00148.
22. *Andre´e B, Bela K, Horvath T, et al*. Successful re-endothelialization of a perfusable biological vascularized matrix (BioVaM) for the generation of 3D artificial cardiac tissue. Basic Res Cardiol. 2014; **109**:441.
23. *Ning Lia, Yang Lia, Dejun Gong, et al:* Efficient decellularization for bovine pericardium with extracellular matrix preservation and good biocompatibility. ICVTS. 2018; **26**:68-776. DOI: 10.1093/icvts/ivx416.
24. *Tran HLB, Dihn TH, Nguyen TN, et al.* Preparation and characterization of acellular porcine pericardium for cardiovascular surgery. Turk J Biol. 2016; **40**:1243-1250. DOI: 10.3906/biy-1510-44.

ARTICLE ON THE SITE
TRANSPLANTOLOGY.ORG

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.