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Heart valve bioprothesis; effect of different acellularizations methods on the biomechanical and morphological properties of porcine aortic and pulmonary valve

P. WILCZEK*

Foundation for Cardiac Surgery Development, 345a Wolności St., 41-800 Zabrze, Poland

Abstract. Tissue engineering is a promising tool for the creation of a new type of the heart valve bioprothesis. The biological scaffold composed of decellularized tissue has been successfully used for the constructions of the valve prosthesis. An analysis of the efficiency of the valve leaflet acellularization methods and the influence of those methods on the morphology and the biomechanical properties of the ECM (extra cellular matrix) was performed. Fresh porcine hearts obtained from a slaughterhouse were used in the experiments. The efficiency of the acellularization methods was dependent on the tissue type and the acellularoization methods used. The more effective were the enzymatic methods, both because of the cell removal efficiency and the effect on the biomechanical properties of the heart valve. The differences in the biomechanical and morphological properties of the porcine aortic and the pulmonary valve after different types of the acellularization process could influence the hemodynamic conditions of the heart after the valve replacement, which limited the range of the tissue types used for the creations of the tissue engineered heart valve.

Key words: tissue engineering, heart valve, bioprothesis, acellularization.

1. Introduction

A heart valve failure remains one of the most challenging problems in cardiac surgery. A diseased heart valve can by replaced by the use of a biological or mechanical bioprosthesis. Both of them have many disadvantages. Tissue engineering is a promising tool for the creation of a new type of the heart valve bioprothesis [1]. The biological scaffold composed of decellularized tissue has been successfully used for the constructions of the valve prosthesis. The calcification and structural changes are the most difficult problems concerning the use of the biological heart valve prosthesis [2]. The potential immune response can by another risk factor witch can limit the use of the biological valve prosthesis [3]. The need for the long-life anticoagulation therapy can affect the use of the mechanical prosthesis. Consequently, all the disadvantages can strongly influence the long term durability of the valve prosthesis.

Currently, we are looking for a new concept to obtain a biological heart valve prosthesis which can avoid the disadvantages concerning the use of the commercially available heart valves. A progress in tissue engineering can give a promising tool in the creation of a new type of the valve prosthesis. Consisting of autologous cells seeded on the scaffold, the tissue engineered heart valve has a potential of growth, repair and remodeling, similarly to the native tissue. Both the acellular tissue and the biodegradable material can be used as the source for the scaffold construction. Because of the instability of the polymeric biodegradable scaffold and the potential toxicity of the degradation products, the most promising concept seems to be the use of the acellular tissue. The preparation of the acellular valve leaflets is important for a complete removal of the cells from the extracellular matrix, which maintains the intact native structure of the matrix. The methods suitable for the cell removing from the donor tissue which differ in the decellularization efficiency; cyclic freezing/defreezing, incubation in Trypsin/EDTA, incubation in a detergent solution are used [4]. The methods vary in the cell removal efficiency, and in the effect on the ECM structure and the biomechanical properties.

The aim of the study was to analyze the efficiency of the heart valve acellularization methods and their influence on the morphology and biomechanical properties of the ECM.

2. Materials and methods of examination

The fresh porcine hearts obtained from a slaughterhouse were transported to the laboratory in a Medium 199. The pulmonary and aortic valves were dissected from the heart aseptically. After the dissection, the valves were stored in an antibiotic solution containing PBS, 1% Difflucan(Pfizer), 1% Cyiprobay (Bayer) for 24 h at 4°C. After the antibiotic bathe, the valves were rinsed in phosphate buffer saline (PBS) and treated with the use of one of the following acellelarization methods: a) 48h Trypsin/EDTA (1X) incubation (0.5 g/l porcine trypsin and 0.2 g/l EDTA in. 4Na in Hank's Balanced Salt Solution – Sigma – kat.nr. T3924, combined with SDS incubation, b) detergent method – 48 h incubations in Deoxyholan combined with Tritin X-100 incubation.

The uniaxial tensile test was used to estimate the biomechanical properties of the aortic and pulmonary valves. The length/width ratio of the tested samples was more than 10,

*e-mail: mildes@post.home.pl

to minimize the influence of the Poisson effect on the strain measured in the longitudinal direction of the sample. A universal testing machine Tytron 250 MTS was used for the analysis of the mechanical properties. The machine control and the measurements were performed with use of the TestWorks®software (MTS Systems Corporation, Eden Prairie, MN, U.S.A.). The test speed was 20 mm/min. The morphology of the tested valves was analyzed using light microscopy.

Light microscopy. The specimens for the microscopic examinations were fixed in 99% ethyl alcohol. They were embedded in paraffin blocks and cut in 5 μm thick sections with the use of a microtome. The valve tissues were stained with hematoxylin and eosin (H-E) and visualized in the Reihert Polyvar 2 microscope. The degree of cell-removing and the degree of the morphological changes in the extracellular matrix which are distinguished in the extracellular matrix in the significant or minor manner as well as without morphological changes were estimated.

Tensile test. The uninaxial tensile test was used to estimate the biomechanical properties of the aortic and pulmonary valve. The length/width ratio of the tested samples was more than 10, to minimize the influence of the Poisson effect on the strain measured in the longitudinal direction of the sample An universal testing machine Tytron 250 MTS was used for the analysis of the mechanical properties. The machine control and the measurements were performed with use of the TestWorks®software (MTS Systems Corporation, Eden Prairie, MN, U.S.A.), and the test rate was 20 mm/min.

The elastic modulus was obtained from the slope of the initial linear section of the stress-strain curve ($tg\varphi$). The secant modulus, defined as the slope ($tg\varphi_1$) of the line drawn from the graph origin and intersecting with the selected point was calculated at 5% strain point on the stress-strain curve (Fig. 1).

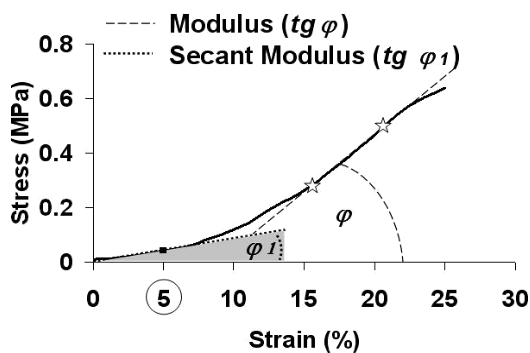

Fig. 1. Description of moduli determination from the stress-strain curve. Modulus is calculated as tangent φ, and Secant modulus is a $tg\varphi_1$ measured in this case at 5% strain point

The analysis was performed up to 30% strain in the mechanical tests. At this value, the corresponding stress is equal to or higher than the maximal stress in valve the leaflets in situ. Above this value, there was an increased possibility of sample slippage and crack formation on the edges.

Cell culture and seeding procedure. A fibroblast cell line clone L929 (ATTC line CCL-1) was used for the cell seeding procedure. The cells were cultured according to the standard procedure; briefly: they were cultured on a 75 cm^2 culture flask in a Medium 199 supplemented with 20% FBS (Gibco kat.nr. 10106169), 0,01mg/ml bFGF (basic Fibroblast Growth Factor – Sigma F0291). The culture flask was incubated at 37°C with 5% CO_2. The culture medium was changed every second day. After the cells had grown to confluence, they were detached by trypsynization. The cells were resuspended in the culture medium. Before seeding, the valves were washed in PBS and than incubated for 72 h in the culture Medium 199, supplemented as above. The acellularized valve leaflets were inserted in culture dishes and the cell suspension was given onto the leaflets' surface and cultured for four days at 37°C with 5% CO_2 in the complete medium. The cell seeding ability was confirmed using the fluorescent microscopic technique.

3. Results

3.1. Acellularization. The acellularization of the valve leaflets with the use of Trypsin/EDTA, followed by SDS washing, leaves the valves free of endothelial and interstitial cells, as well as cell debris in all the treated samples (Figs. 2–6). The morphology of the extracellular matrix appeared to be well preserved with a minor damage of the ECM. Additionally, the differences in the morphology of the native and the acellular valve were more visible for the pulmonary valve (Fig. 4) than for the aortic ones (Fig. 2).

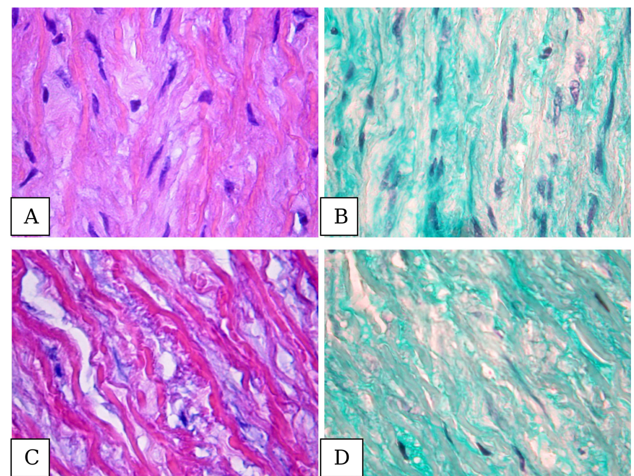

Fig. 2. Light microscopy patterns of section of porcine aortic heart valve conduit treated with SDS incubation combined with 48 h Trypsin/EDTA incubation: A,B – native tissue, C,D – acellular. (H&E staining – A,C, Masson staining B,D)

After the acellularization of the heart valve with the use of detergent methods, most of the cells were not removed from the tissue; the interstitial and fibroblast cells were observed in the tissue after the acellularization process. The morphology of the extracellular matrix appeared to be damaged with signs of layer detachment. The ECM was lost, and some inter-fiber spaces were observed.

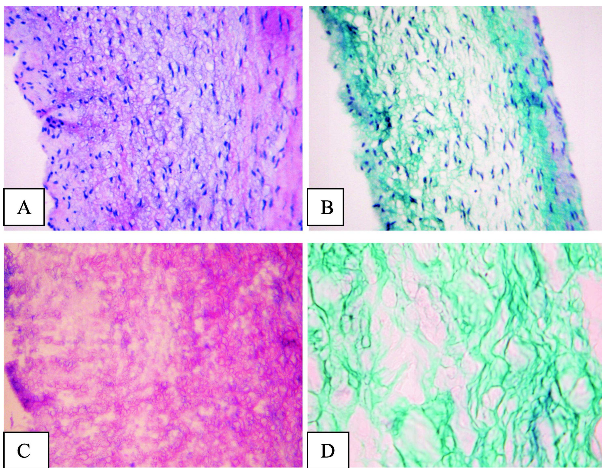

Fig. 3. Light microscopy patterns of section of porcine aortic heart valve leaflets treated with SDS incubation combined with 48 h Trypsin/EDTA incubation: A,B – native tissue, C,D – acellular. (H&E staining – A,C, Masson staining B,D)

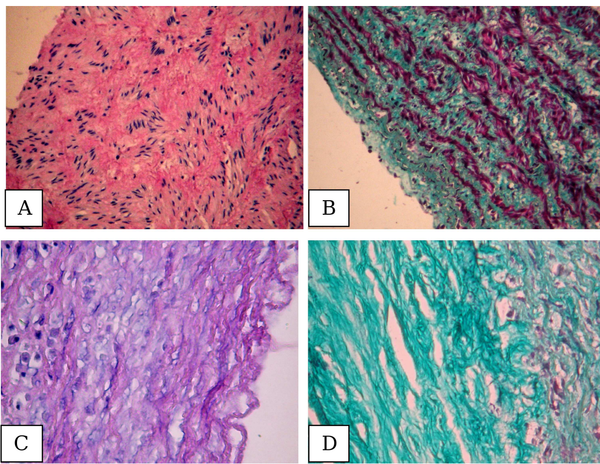

Fig. 4. Light microscopy patterns of section of porcine pulmonary heart valve conduit treated with SDS incubation combined with 48 h Trypsin/EDTA incubation: A,B – native tissue, C,D – acellular. (H&E staining – A,C, Masson staining B,D)

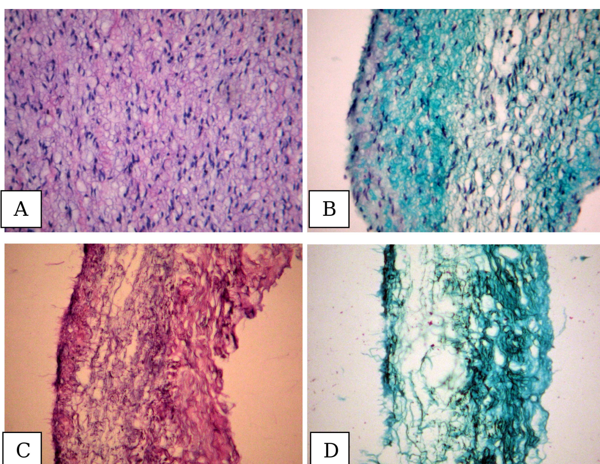

Fig. 5. Light microscopy patterns of section of porcine pulmonary heart valve leaflets treated with SDS incubation combined with 48 h Trypsin/EDTA incubation: A,B – native tissue, C,D – acellular. (H&E staining – A,C, Masson staining B,D)

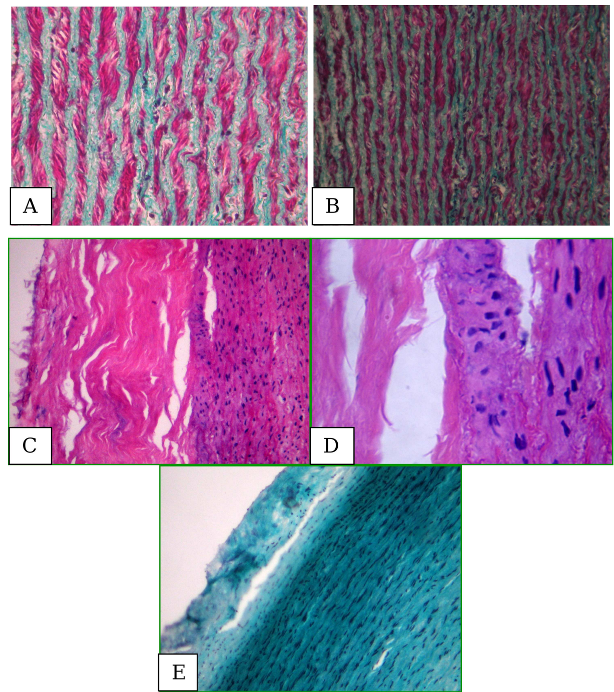

Fig. 6. Light microscopy patterns of section of porcine pulmonary heart valve leaflets treated with detergent methods: A,B, – native, C,D,E – acellular. (H&E staining – A,CD, Masson staining B,E)

3.2. Cell seeding. The valve leaflets were washed in PBS and subsequently incubated for 72 h in the complete medium. In the shaking conditions, they were completely covered with the cells showing a confluent cell monolayer. More than 90% of the cells, seeded on the tissue surface, were viable. The cells formed a monolayer on the tissue surface, but in most cases, they did not migrate inside the tissue. In some cases of the pulmonary valve examination, the tissue was not seeded by the cells. These results were confirmed in a fluorescent microscopy and with the use of a light microscopy, in the section with a H-E staining (Figs. 7–8).

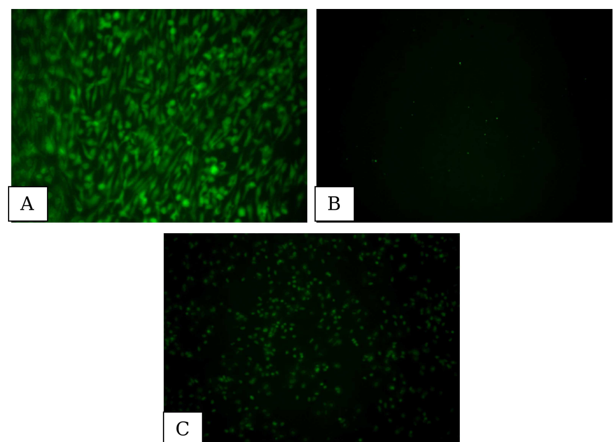

Fig. 7. Fluorescent microscopy pattern of section of porcine pulmonary and aortic heart valve leaflets seeded with the cells: A – aortic valve after Trypsin/EDTA incubations following SDS treatment, B – pulmonary valve after Trypsin/EDTA incubations following SDS treatment, C – aortic valve after detergent incubations

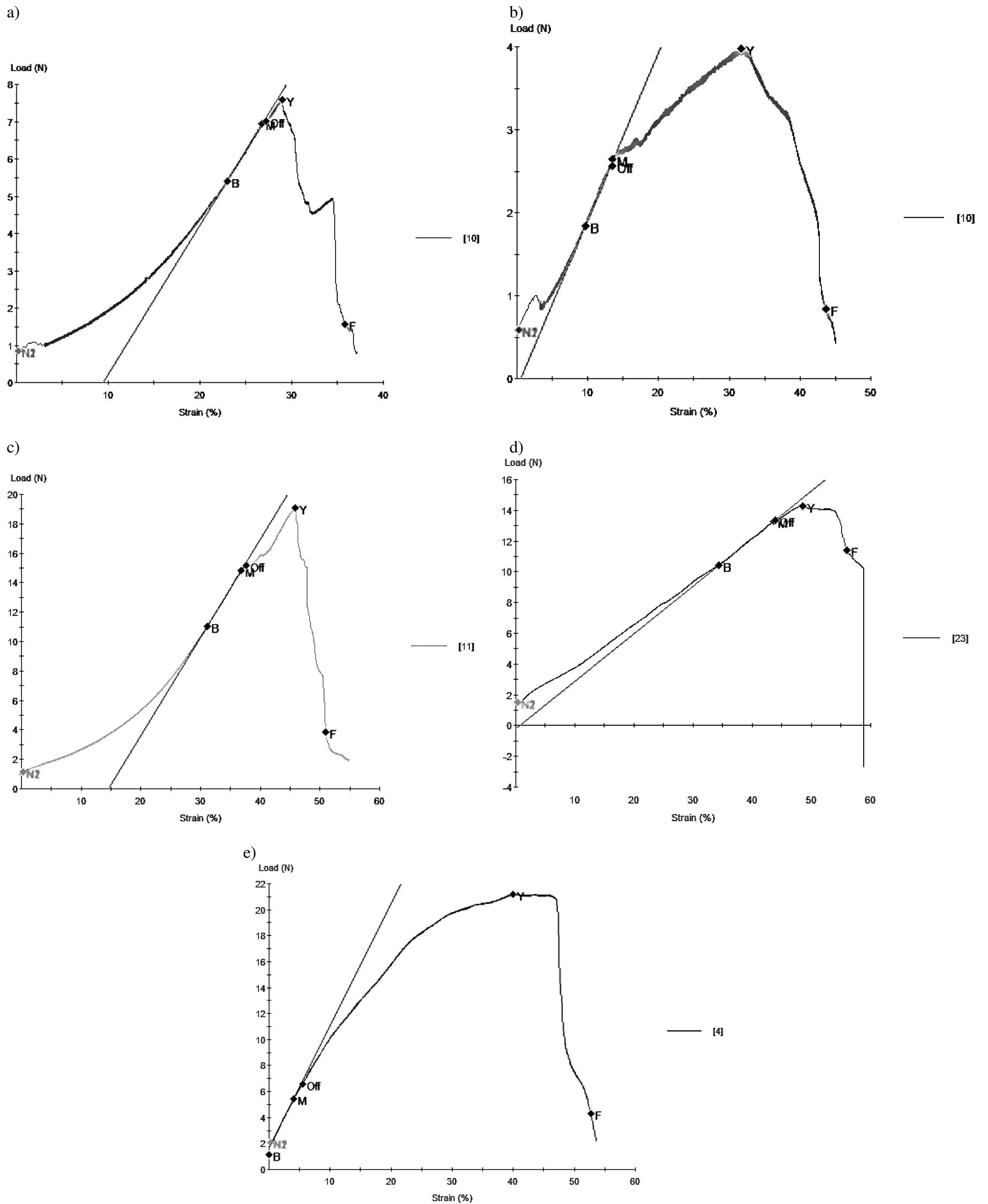

Fig. 8. Stress-Strain curves obtained in uniaxial tensile test, showing mechanical characteristics of: A – native pulmonary valve, B – acellular pulmonary valve treated with Trypsin/EDTA incubation combined with SDS, C – native aortic valve, D – acellular aortic valve treated with Trypsin/EDTA incubation combined with SDS, E – acellular heart valve treated with the detergent method

3.3. Tensile test. The uniaxial tensile test demonstrated significant differences in the elastic modulus and the biomechanical properties of the pulmonary and the aortic valve, both native and acellular. The most important differences were observed for the conduits. The experimental results showed that the modulus of elasticity for the pulmonary valve decreased two times, parallel to the significant decrease in the energy to break and % of strain. Small differences were observed between the native and the acellular aortic valve.

4. Discussion

The heart valves replacement is a commonly used method in the heart valve disease treatment. Although many types of valve prosthesis are developed, in many cases, the most important problem, which strongly determines the valve function and durability, is the body reaction to a foreign material. The commonly used heart valve prostheses, both biological and mechanical, have many limitations, such as: an infection risk, thromboembolism, the long-life anti-coagulation therapy [1, 2]. A possible cause of the valve prosthesis degeneration can also be an immune response to the viable cells in a cryopreserved allograft [3]. The tissue engineered heart valves (TEHV), which utilize a biological or biodegradable scaffold seeded with autologous cells, can be a promising tool for the creation of new types of valve prosthesis [4–6], which can eliminate most of the disadvantages concerning the use of other types of valves prosthesis.

The proper function of the TEHV and the long-time durability depends strongly on the type of the acellularization method. The efficiency of the acellularization methods can affect the biomechanical and structural properties of the ECM. Several methods have been proposed for the accellularization of the heart valves. The simplest decellularization method is the cyclic freeze-thawing treatment [7, 8]. However, as it has been described in the literature [13], after the freeze-thawing treatment, several cells could be presented in the matrix. The leaflets showed a wave-like collagen structure with loosely widened interfibrillar spaces. After the endothelial cell seeding, the valve leaflets treated with the freeze-thawing method were not covered with the cells [9]. Another tissue acellularization method is a NaCl treatment combined with SDS incubation [9, 10]. As described by Kim [9], the valve leaflets treated with NaCl – SDS showed a completely-free cell structure, as well as revealed better characteristics than the endothelial cell seeding. The incubation in a Trypsin/EDTA solution is one of the most commonly used acellularization methods. Incubation in this solution allows to effectively remove the cells without visible changes in the extracellular matrix structure [11–13]. In our experience, the cell removal from the matrix is not completely effective after a 48 h incubation in a Trypsin/EDTA solution, and the rest of the cells or cell debris can cause degenerative changes within the tissue after a potential valve implantation. On the other hand, the prolonged time – to 72 h – can result in a structural alteration of the matrix fibers [14].

In our study of an optimization of the acellularization procedure, a combination of a 48 h incubation in a trypsin/EDTA solution with the short SDS incubation was applied. It was observed that the Trypsin/EDTA incubations combined with short incubations with SDS resulted in a removal of all the cells from the valve leaflets. Almost all the cells were removed from the valve conduit. Only minor changes were stated in the ECM structure after the treatment of the tissue with the enzyme methods. Because of the risk of damage of the ECM structure, the detergent methods are extensively studied as the ones use for acellularizations. Triton X-100, SDS or a Deoxyholan are the most widely used detergents. As described by Kasimir [14], the treatment with Triton – X 100 leads to a complete acellularization and a preservation of the matrix structure. Opposite to this result, the investigation of Kim [9] showed that using Triton – X 100 it is difficult to achieve consistent results of an effective decellularization. Furthermore, it seems that, due to the chemical structure of Triton – X 100, it is more difficult to remove the rest of Triton – X 100 from the matrix than SDS. The use of SDS allows to remove the cells from the matrix [6]. The SDS treatment produces a more extensible tissue with equal strength, compared to the fresh valve [15]. In contrast, the other study demonstrates that a prolonged treatment with SDS leads to a denaturation of the collagen fibers, and their destabilization [16]. The SDS treatment can lead to a fragmentation and swelling of the collagen with a significant loss of the hydrothermal stability [17]. The detergent method is less effective in the cell removal, both for the valve leaflets and the valve conduit. With the use of the Deoxyholan methods combined with Tritin X-100, the incubations have been observed in the normal structure of the ECM fibers, but it is important to point out that a layer detachment in the valve conduit was observed. Even if the tissue after the acellularizations with the use of the detergent methods can be effectively seeded with the cells, the detachment of the layer can cause the risk of the aneurysm formation. On the other hand, the use of the enzymatic method can damage the ultra-fine structure of the collagen and the elastin fibers, but after the cell seeding the seeded cells can synthesize the ECM proteins, initiating the remodeling process, and restore the damaged structure of the ECM.

Concerning the biomechanical properties of the heart valve after the acellularizations process, we can observe a higher decrease of the estimated parameters in the detergent group compared to the valve treated with the enzymatic methods. The pulmonary valve can also be more sensitive to the enzymatic treatment, in comparison with the aortic valve.

From our study we can conclude that the efficiency of the acellularization methods are dependent on the tissue type and on their own type used. The more effective are the enzymatic methods because of the cell removal efficiency and the positive effect on the biomechanical properties of the heart valves. The differences in the biomechanical and morphological properties of the porcine aortic and pulmonary valves after different types of the acellularization process can influence the hemodynamic conditions of the heart after the valve

replacement. This limits the tissue type used for the creations of the tissue engineered heart valves.

5. Summary and conclusions

The uniaxial tensile test demonstrated significant differences in the elastic modulus and the biomechanical properties of the pulmonary and aortic valves, both native and acellular. The most important differences were observed for the conduits. The experimental results showed that the modulus of elasticity for the pulmonary valve decreased two times, and simulaneously, a significant decrease in the energy to break and % strain was observed. Small defferences between the native and the acellular aortic valve were observed. The morphological analysis indicated that 48 hours of the Trypsin/EDTA treatment led to a removal of the majority of the cells from the valve leaflets. The detergent acellularization procedure resulted in a not complete removal of the cells. After the trypsin/EDTA treatment, the extracellular matrix was lost and the inter-fiber spaces were observed. In the Masson staining, a degradation of the extracellular matrix was observed. The stated changes were more evident for the valve treated with the detergent methods, in which a detachment of the tissue layer in the valve conduit was observed. Additionally, the differences in the morphology of the native and the acellular valves were more visible for the pulmonary valve than for the aortic one.

6. Conclusions

- The efficiency of the acellularization methods is dependent on the tissue type and the method used,
- The enzymatic methods are more effective, both because of the cell removal efficiency and the effect on the biomechanical properties of the heart valve,
- The differences in the biomechanical and the morphological properties of the porcine aortic and the pulmonary valve after different types of the acellularization process can influence the hemodynamic conditions of the heart after the valve replacement, thus limiting the range of the tissue types used for the creations of the tissue engineered heart valves.

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REFERENCES

[1] S.A. North, R. Sadler, A.W. Stewart, L. McCowan, A.R. Kerr, and H.D White, "Long-term survival and valve-related complications in young women with cardiac valve replacements", *Circulation* 99, 2669–2676 (1999).

[2] S.S. Khan, A. Trento, M. DeRobertis, R.M. Kass, M. Sandhu, L.S.C. Czer, C. Blanche, S. Raissi, G.P. Fontana, and W. Cheng, "Twenty-year comparison of tissue and mechanical valve replacement", *J. Thorac. Cardiovasc. Surg.* 122, 257–269(2001).

[3] P. Hogan, L. Duplock, and M.F. O'Brien, "Human aortic valve allograft elicit a donor-specific immune response", *J. Thorac. Cardiovasc. Surg.* 112, 1260–1266 (1996).

[4] S.P. Hoerstrup, A. Kadner, S. Meltnichouk, A. Trojan, K. Eid, J. Tracy, R. Sodian, J.F. Visjager, S.A. Kolb, J. Grunenfelder, G. Zund, and M.I. Turina, "Tissue engineering of functional trileaflet heart valves from human marrow stromal cells", *Circulation* 106, I-143-I-150 (2002).

[5] S. Cebotari, H. Mertsching, K. Kallenbach, S. Kostin, O. Repin, A. Batrinac, C. Kleczka, A. Ciubotaru, and A. Haverich, "Construction of autologous human heart valves based on acellular allograft matrix", *Circulation* 106, I-63-I-689 (2002).

[6] C. Booth, S.A. Karossis, H.E. Wilcox, K.G. Watterson, J.N. Kearney, J. Fisher, and E. Ingham, "Tissue engineering of cardiac valve protheases I: development and histological characterization of an acellular porcine scaffold", *J. Heart Valve Dis.* 11 (4), 457–62 (2002).

[7] H.C. Grillo and C.F. McKhann, "The acceptance and evolution of dermal homografts freed of viable cells", *Transplantation* 2, 48–59 (1964).

[8] C.H. Fang, E.C. Robb, G.S. Yu, J.W. Alexander, and G.D. Wadden, "Observation on stability and contraction of composite skin grafts: xenodermis or allodermis with an isograft onlay", *J. Burn Care Rehab.* 11, 538–42 (1990).

[9] W.G. Kim, J.K. Park, and W.Y. Lee, "Tissue engineered heart valve leaflets: an effective method of obtaining acellularized valve xenografts", *Int. J. Artif. Organs* 25, 791–7 (2002).

[10] R.J. Walter, T. Matsuda, H.M. Reyes, J.M. Walter, and M. Hanumadass, "Characterization of acellular dermal matrices (ADMs) prepared by two different methods", *Burns* 24, 104–13 (1998).

[11] O.E. Teebken, A. Bader, G. Steinhoff, and A. Haverich, "Tissue engineering of vascular grafts: Human cell seeding of decellularized porcine matrix", *Eur. J. Vasc. Endovasc. Surg.* 19, 381–386 (2000).

[12] G. Steinhoff , U. Stock, N. Karim, H. Mertsching, A. Timke, R. Meliss, K. Pethig, A. Haverich, and A. Bader, "Tissue engineering of pulmonary hearts valves on allogenic acellular matrix conduits", *Circulation* 102, S III 50–55 (2000).

[13] A. Bader, G. Steinhoff, K. Strobl, T. Schilling, G. Brandes, H. Mertsching, D. Tsikas, J. Froelich, and A. Haverich, "Engineering of human vascular aortic tissue based on xenogenic starter matrix", *Transplantation* 70, 4–14 (2000).

[14] M-T. Kasimir, E. Rieder, G. Seebacher, G. Silberhumer, E. Wolner, and P. Simon, "Comparison of different decellularization procedures of porcine heart valves", *Int. J. Artif. Organs* 26, 421–427 (2003).

[15] S.A. Korossis, C. Booth, H.E. Wilcox, K.G. Watterson, J.N. Kearney, J. Fisher, and E. Ingham, "Tissue engineering of cardiac valve prostheses II: biomechanical characterization of decellularized aortic heart valve", *J. Heart Valve Dis.* 11 (4), 463–471 (2002).

[16] V.Samouillan, J.Dandurand-Lods, A.Lamure, E. Maurel, C. Lacabanne, G. Gerosa, A. Venturini, D. Casarotto, L. Gherardini, and M. Spina, "Thermal analysis characterization of aortic tissue for cardiac valve bioprostheses", *J. Biomed. Mater. Res.* 46, 531–8 (1999).

[17] E. Bodnar, E.G.J. Olsen, R. Florio, and J. Dobrin, "Damage of porcine aortic valve tissue by the surfactant sodium dodecylsulfate", *Thorac. Cardiovasc. Surg.* 34, 82–85 (1986).