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P. WILCZEK*#

BIOCOMPATIBILITY ISSUE OF TISSUE ENGINEERED HEART VALVES

PROBLEM BIOZGODNOŚCI W INŻYNIERII TKANKOWEJ ZASTAWEK SERCA

Tissue engineering is a new field of knowledge which creates the possibilities for producing bioactive cardiac prostheses that will characterize by biomechanical and morphological properties similar to native tissue. It is expected that it will be characterized by high durability, which is very important from the social and clinical point of view. The aim of the study was to compare the cytotoxic effect of enzymatic and detergent acellularization methods commonly used for the biological scaffold preparation. It seems that the use of enzymatic methods, allows efficient donor cells removal while maintaining the ability to autologous cell seeding. Heart valves bioprosthesis created using these techniques, may be a good alternative to the currently used prostheses.

Keywords: tissue engineering, heart valve, cytotoxicity, scaffold.

Inżynieria tkankowa jest nową dziedziną wiedzy, która stwarza możliwości wytwarzania protez serca bioaktywnych charakteryzujących się właściwościami biomechanicznymi i morfologicznymi zbliżonymi do tkanki rodzimej. Oczekuje się, że proteza serca będzie charakteryzować się wysoką trwałością, co jest bardzo ważne z punktu widzenia społecznego i klinicznego. Celem badania było porównanie efektu cytotoksycznego enzymatycznych i detergentowych metod usuwania komórek, powszechnie stosowanych do wytwarzania biologicznych rusztowań. Wydaje się, że stosowanie metod enzymatycznych, umożliwia wydajne usunięcie komórek dawcy przy zachowaniu zdolności do autologicznego posiewu komórek. Bioprotezy zastawek serca tworzone za pomocą tej techniki mogą być dobrą alternatywą dla obecnie stosowanych mechanicznych i biologicznych protez zastawek serca.

1. Introduction

Currently, heart and cardiovascular-associated conditions are one of the main causes of death worldwide with a constantly growing number of people affected [1, 2]. According to the World Health Organization (WHO) in 2005, 17.5 million deaths were due to cardiovascular conditions, which accounts for 30% of the world's mortality. A large proportion of cardiovascular conditions are due to heart valve failure [1]. The demand for heart valve replacement is estimated to grow from 290,000 a year in 2003 to more than 850,000 a year in 2050 [3]. The surgical procedure for the exchange of diseased heart valve is commonly used method of treatment in a situation in which conventional pharmacological treatments are exhausted. A significant limitation of this method is a lack of heart valve substitutes with sufficient durability, preserving functional characteristics of the native valve. At present in the treatment of cardiac valve disease, mechanical or biological prosthesis, are successfully used for many years. However, both of them have limitations. Mechanical valves are characterized by high durability, however, the need for the long-term anticoagulation is their major limitation of this type of prosthesis [4,5,6]. Potential immunogenicity, a risk of calcification, are the major limitations of biological heart valves. This may lead to progressive tissues degeneration, loss of functionality and thus reducing the durability [7,8]. This causes the search for alternative methods of bioprosthesis preparation and one of them is the tissue engineering techniques [9,10,11]. The concept of heart valve prostheses preparation, based on the tissue engineering technique assumes that using enzymatic or chemical methods the donor's cells can be removed, while leaving the intact structure of the extracellular matrix, such scaffold can be seeded with autologous cells. Chemical or enzymatic factors used for acellularization procedure can indicate potential cytotoxicity and reduce the cell seeding ability. The aim of the study was to

* HEART PROSTHESIS INSTITUTE, BIOENGINEERING LABORATORY, 345A WOLNOSCI STR., ZABRZE 41-800 POLAND
# Corresponding author: mildes@post.pl

compare the cytotoxic effect of the enzymatic agents and detergent, as the compounds commonly used in the acellularization procedure, and thus to determine their role in the development of heart valves prosthesis based on tissue engineering techniques.

2. Material and Methods

2.1. Acellularization procedure

Enzymatic acellularization: For the acellularization procedure, the valves were rinsed in phosphate buffer saline (PBS; GIBCO) and placed in trypsin/EDTA (190.5 g l-1porcine trypsin and 0.2 g l-1EDTA in Hank's Balanced Salt Solution; GIB-CO) for 48 h and then placed in 0.5% sodium dodecylsulfate (SDS; Sigma) for 15 min. To remove residual enzymes and detergent, the heart valves were washed in PBS for 1 h at room temperature under shaking.

Detergent acellularization: The valves were rinsed in phosphate buffer saline (PBS; GIBCO) and placed in 0.5% sodium dodecylsulfate (SDS; Sigma) for 48 h. To remove residual detergent, the heart valves were washed in PBS for 1 h at room temperature under shaking.

2.2. Indirect contact cytotoxicity assay

Cytotoxicity assays were conducted in accordance with the recommendations of the ISO 10993-5 standard. For the assay fibroblasts cell lines clone L 929 from the American Type Culture Collection (ATCC) free of mycoplasma was used. The cytotoxicity of selected concentrations of trypsin / EDTA solution and the SDS solution, was performed using the indirect cytotoxicity test in which the cell cultures were supplemented with following concentrations of trypsin / EDTA and SDS solution: 0,2%; 0,4%; 0,6%; 0,8%; 1%; 2%; 5%; 10%. Cytotoxicity assays were performed in two following intervals: A – after a period of 4 hours, B – after 24 hours. For the determination of cell viability the fluorescent microscopy and flow cytometry technique was used with the help of AxioObserver inverted microscope (Zeiss) and FC 500 flow cytometer (Beckman Coulter).

2.3. Direct contact cytotoxicity assay

For the direct contact cytotoxicity assay tissue samples subjected to acellularization procedure were used. The acellular tissue discs were placed in culture dishes and incubated for 24 hours under standard conditions. After this period the samples were subjected to microscopic cell morphology and viability analysis. For the cell viability the cells were stained with Fluorescein diacetate (FDA) and propidium iodide (PI) Fluorescein diacetate has the ability to cross over the intact cell membrane. Intracellularly the FDA is converted by esterase to polar, bright green fluorescent monomer which labeling the viable cells. In turn, PI enter inside the cell through the damaged cell membranes, PI exhibit the red fluorescent, the PI positive cells were classified as necrotic. The cells nucleus was stain with DAPI, which allows for assessment of apoptotic changes. Phalloidyne and Vimentine staining was performed for the evaluation of cytoskeleton.

2.4. Cell seeding

For the cell seeding test the Mesenchymal Stem Cell line (MSC) from the ATCC collection was used. Before cell growth the acellular matrix was incubated for at least 48 h in culture medium in order to wash out the remaining detergent. Then the cells were seeded on the acellular scaffold and incubated at 37°C with 5% CO_2. The culture was carried out for 1 week. After this period The cell viability assay was performed using the inverted fluorescent microscope Axio Observer CarlZeiss, for the acquisition and image analysis the AxioVision 4.8 software Carl Zeiss were used.

2.5. Platelet activation assay

Especially for long-term use of cardiac bioprosthesis as is the case of tissue engineering techniques applications, the blood is exposed to non-physiological conditions. During the acellularization procedure the endothelial cell lining is removed and changes in ECM are observed. In such cases, the blood is contact with the modified surface which may result on platelet adhesion or activation of platelets and the formation of platelet-leukocyte complexes. For the test whole blood was used. Native or acellular tissue was incubated with whole blood at room temperature for 15 min. After the incubation, platelet activation was assessed.

3. Results

3.1. Indirect contact cytotoxicity assay

In all groups treated with increasing concentrations of trypsin / EDTA solution the necrotic cells has been observed. However, greater changes were observed after 24 hours. Decrease of the total number of cells was also observed (Fig. 1).

In the SDS treated group both after 4 and 24 hours, about 90% of the cells in the field of view was classified as necrotic. Also significant decrease in total number of cells and the changes in cell morphology was observed (Fig. 2).

However the flow cytometry studies indicate the greater percent of necrotic cells after 4h exposure to Trypsin/EDTA compared to 24 hours of exposure to proteolytic enzymes (Fig. 3).

The number of viable cells after 4h incubation in Trypsin. EDTA was about 70% at the same time an increase in the percent of viable cells after 24 was observed, with the and maximum value about 90% (Fig. 4).

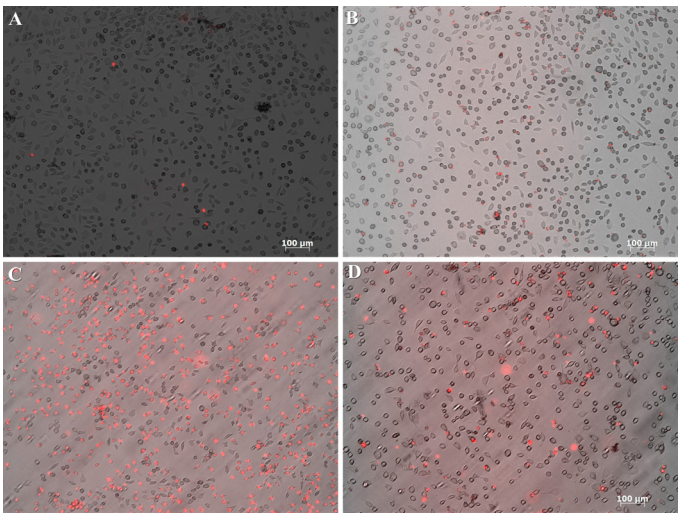

Fig. 1. The microscopic image of cells treated with 0, 2% and 10% Trypsin / EDTA after 4 hours (A, B) and 24 hours (C, D) incubation. Cells exhibiting red fluorescence were classified as necrotic

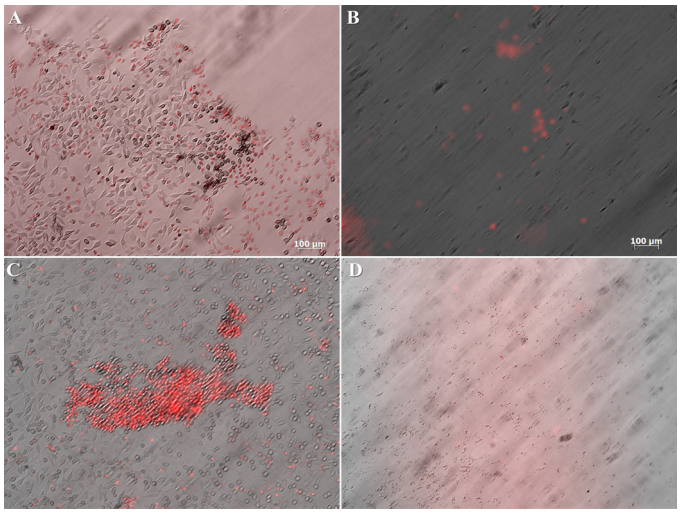

Fig. 2. The microscopic image of cells treated with 0, 2% and 10% SDS after 4 hours (A, B) and 24 hours (C, D) incubation. Cells exhibiting red fluorescence were classified as necrotic

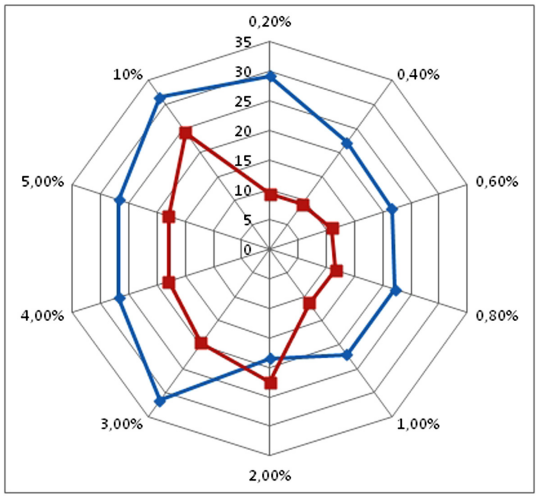

Fig. 3. Comparison of the percentage of necrotic cells in the culture after 4 hours (blue line) and 24 hours (red line) incubation with selected concentrations of Trypsin EDTA solutions

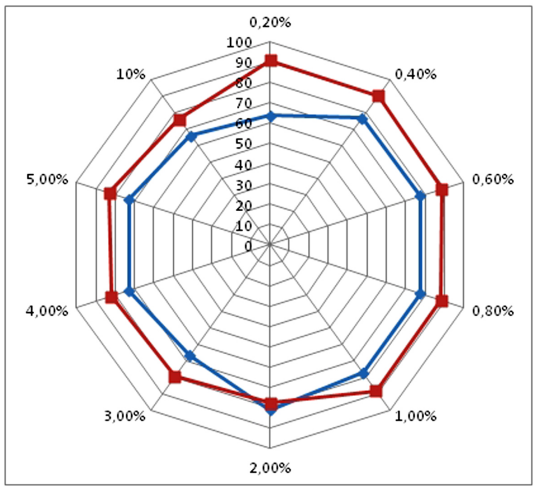

Fig. 4. Comparison of the percentage of viable cells in the culture after 4 hours (blue line) and 24 hours (red line) incubation with selected concentrations of Trypsin EDTA solutions

Since most of the SDS-treated cells were lysed it was not possible to perform flow cytometry analysis.

3.2. Direct contact cytotoxicity assay

In the direct contact cytotoxicity test, both for the native and acellular tissues the cells exhibited normal morphology and growth. Staining of nuclei with DAPI showed no apoptotic cells in the field of view (Fig. 5, 6).

However, for the tissue subjected to acellularization procedure with the use of SDS almost 90% of the cells in the field of view were identified as necrotic (Fig. 7).

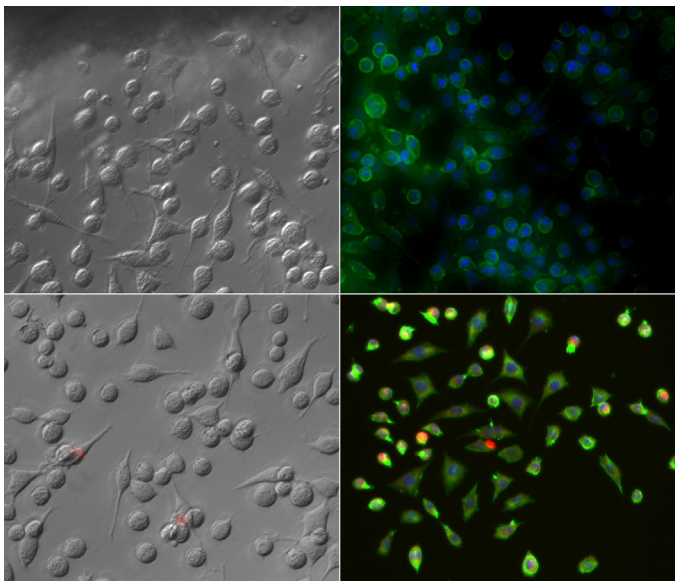

Fig. 5. The microscopic image of the control cells in the direct contact cytotoxicity assay; A – cells observed under phase contrast Plas DIC, B – cells stained with FDA showing green fluorescence classified as live, blue stained nuclei, C – cells stained with PI, showing red fluorescence classified as necrotic, D – cells stained with a phalloidyne – green fluorescence and vimentin (red fluorescence) for labeling the cytoskeleton

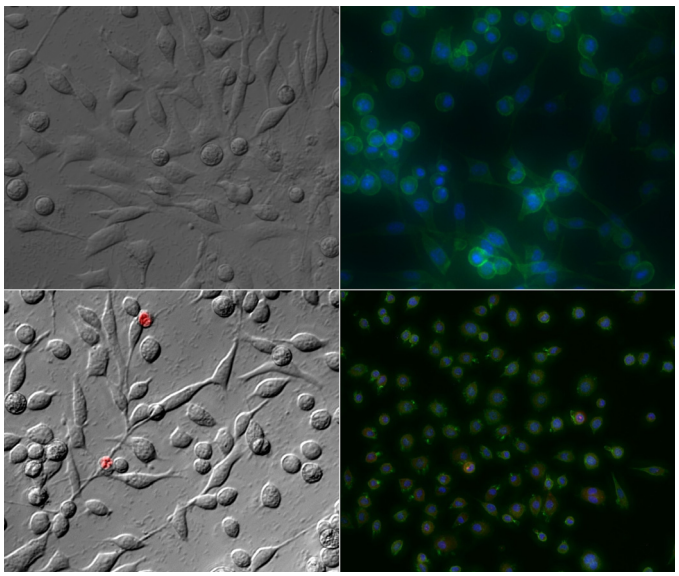

Fig. 6. The microscopic image of the cells exposed to Trypsin/EDTA treated tissue in the direct contact cytotoxicity assay; A – cells observed under phase contrast Plas DIC, B – cells stained with FDA showing green fluorescence classified as live, blue stained nuclei, C – cells stained with PI, showing red fluorescence classified as necrotic, D – cells stained with a phalloidyne – green fluorescence and vimentin (red fluorescence) for labeling the cytoskeleton

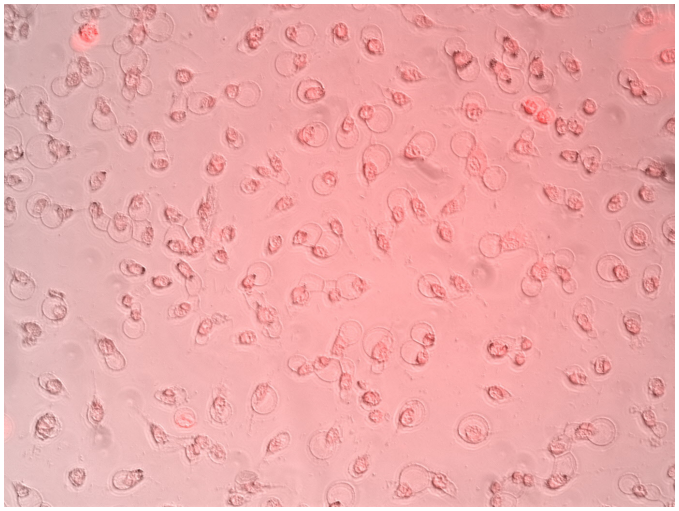

Fig. 7. The microscopic image of the cells exposed to SDS treated tissue in the direct contact cytotoxicity assay

3.3. Cell seeding

After 1 week culture the cell seeded on the Trypsin/EDTA treated acellular scaffold indicate suitable morphology, high viability, more than 90% of cells was FDA positive – green fluorescent (Fig. 8).

After 1 week culture on the SDS treated acellular scaffold, the cells are not adhere to the tissue, only a few necrotic cells on the surface was observed (Fig. 9).

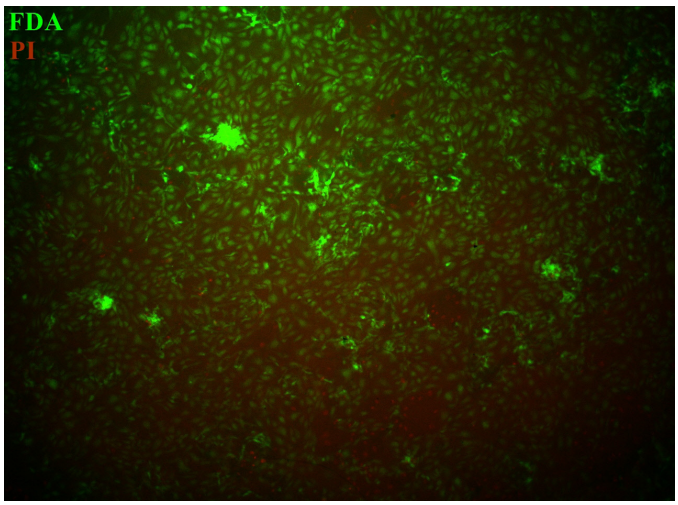

Fig. 8. The microscopic image of cells seeded on Trypsin/EDTA treated acellular tissue after 1 week culture

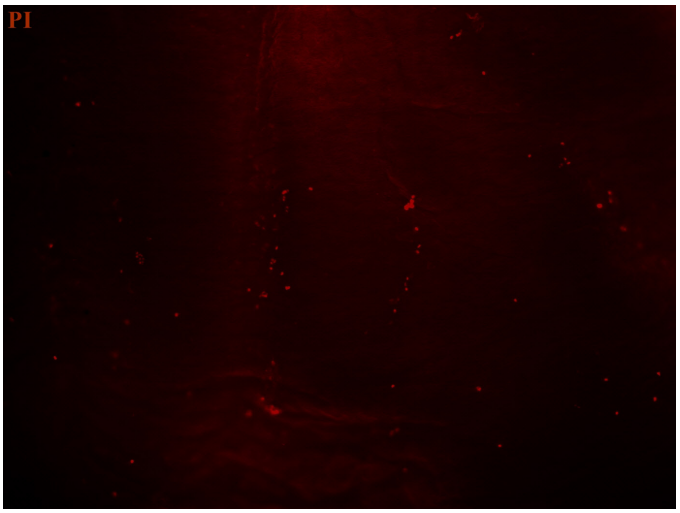

Fig. 9. The microscopic image of cells seeded on SDS treated acellular tissue after 1 week culture

3.4. Platelet activation

Comparison of average values, of P-selectin (CD62P), CD-45 positive cells and the number of platelet-leukocyte aggregates cells between native and acellular tissue indicative of a higher activation of the platelet on the native tissue compared with the acellular one (Fig. 10).

A microscopic examination of tissues incubated with whole blood cells was observed only a few CD62P positive. The greater the number of receptor positive cells was observed CD 62 P for acellular tissues (Fig. 11).

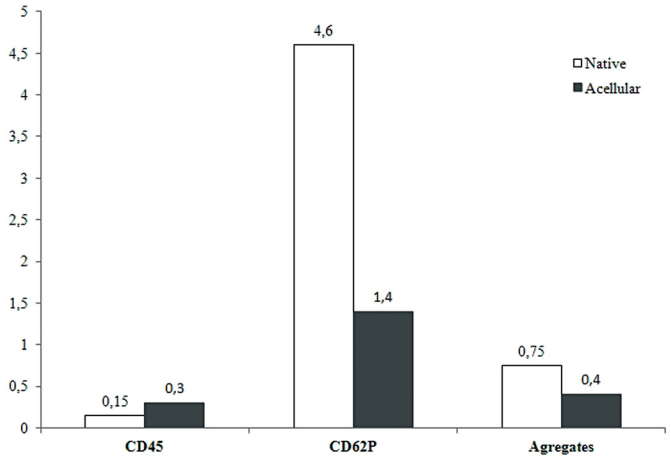

Fig. 10. Mean values of the activated CD62P platelets, CD45 positive cells and platelet-leukocyte aggregates in contact with the surface of the native and acellular tissue

4. Discussion

An increasing number of patients with heart valve diseases cause that it is still ongoing search for optimal cardiac bioprosthesis which shown a high durability and a corresponding high biocompatibility. Due to the availability and morphological similarity the porcine tissue are commonly used for the creation of bioprosthesis. The valve prosthesis can be constructed on the basis of bovine pericardium. These include stended, Ionescu-Shiley valve or Carpentier-Edwards pericardial valves, but both of them do not have sufficient durability. In the case of xenogeneic tissues the major problem is their immunogenicity, due to the presence of porcine cell epitope 1, 3 α-GAL, which is not present on human cells, and that can be an indicator of immune response leading to the bioprosthesis degradation [12]. Although there is the possibility of the use of cross-linking agents such

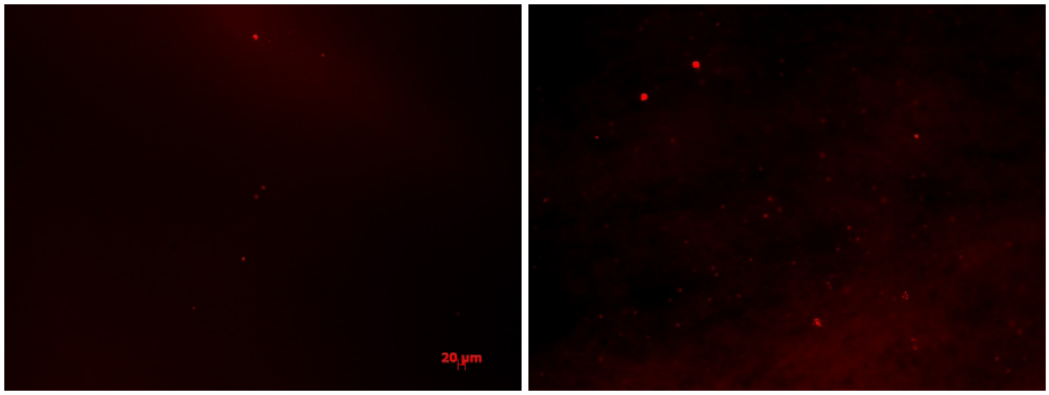

Fig. 11. The microscopic image of native and acellular tissue incubated with whole blood. Visible CD62P positive cells (red fluorescence) on the tissue surface

as glutaraldehyde, to mask the antigen and reduce the immune response, however, cytotoxicity is the disadvantage of this type of chemical agent [13]. This type of valve has proper mechanical properties, but it is impossible to growth the cells "in vitro" or "in vivo" and thus does not have the ability to grow and remodeling. Using enzymatic or chemical methods the cells, which are responsible for immunological reaction, can be removed from the recipient tissue leaving the intact structure of the extracellular matrix. Next the autologous cells can be seeded on the biological scaffold, this minimizes the immune response, thereby reducing the risk of degenerative processes, and consequently bioprosthesis durability can be increased. However, it should be noted that the factors used in the acellularization process may adversely affect the extracellular matrix. In the case of enzymatic acellularization methods the damage of collagen, laminin, fibronectin, elastin, glicosaminoglicnas and changes in the ECM architecture can be observed [14]. The use of ionic and non-ionic detergents may in turn cause damage to DNA – proteins interactions, lipids, lipoproteins, protein denaturation. It can also change the ultrastructure of the ECM [15]. Furthermore, acellularization factors may exhibit cytotoxic activity. Prolonged exposure to Trypsin treatment may lead to irreversible cell damage. Most of the surface proteins will be destroyed by Trypsin detachment during the procedure. Trypsin needs to be actively inactivated to stop the reaction [16]. Similarly, the cytotoxic effect may also be present by the use of detergents [17,18]. Despite repeated rinsing is difficult to completely remove the rest of the surfactant from the tissue which may enhance the cytotoxic effect [19,20]. Acellularization process can also affect the efficiency of cell culture on the scaffold. In the study of Iop [21] human and porcine heart valves were acellularized, then HBM-MSC (Human Bone Morrow-Mesenchymal Stem Cells) seeded statically on the ventricularis fibrosa layers and grown for 30 days. It was observed that the characteristics of the cell growth on both layers (fibrosa and ventricularis) was different suggesting that cell growth may be determined by changes in the microstructure of the ECM. In the study of Xu the endothelial cell response to surface of the acellular scaffold prepared on the basis of bone marrow mesenchymal stem cells was assessed. To remove the cells from the tissue the Triton X-100 was used. It was observed that HUVEC properly adhere to the tissue surface and for a regular layer. Also induction of migration and proliferation processes was observed [22]. Cytotoxic effect of different concentrations of SDS and its removal ability from pericardial tissue was exanimate. In the

study studies ovine vascular cells were used, it was observed that SDS at a concentration above 10 μM significantly reduces the total amount of cells while concentrations above 100 μM causes a significant increase in necrotic cells. It was also observed that SDS can be removed depending on the concentration used. It can be concluded that the cytotoxic effect should be taken into account in the design of bioprosthesis with the use of tissue engineering techniques [19]. The Gratzer in his work applied two methods of SDS leaching from acellular tissues, using a buffer at pH 9 and 75% ethanol. It was observed that the SDS concentration after intense washing was about 8 and 23 times lower than the reported cytotoxic concentration. At the same time it was reported that the SDS causes a significant reduction of GAG. This may suggest that the reduced ability to cell repopulation observed by SDS treated tissue, may result from the changes in biochemistry and architecture of the ECM and not by the SDS cytotoxic effects [23]. These findings are consistent with studies in which the distribution of structural components and water content (hydration) in the pulmonary and aortic valves both native and acellular was estimated. There has been a high water and collagen contents, different distribution of elastin and of glycosaminoglycan along the conduits and pulmonary scaffold. There was no such relationship for the aortic valve. Similar results were recorded for native and acellular tissue [24]. In our studies the significantly higher cytotoxicity of SDS compared to Trypsin / EDTA was observed. The use of the detergent resulted not only in an increase in the amount of necrotic cells but also significantly reduced the total number of cells in the cultures. It was also found that in the case of tissue SDS treated tissue, by despite the long-term washing procedure, no cell repopulation was observed. In contrast, the use of Trypsin/EDTA for acellularization allow the proper cell growth. The seeded cell indicates high viability.

5. Concluding remarks

In the case of acellular scaffolds used in the creation of bio-prosthetic heart valve using tissue engineering techniques, the proper cell repopulation is essential. The cell growth on the bioprosthesis may depend on the acellularization method. Commonly, there are two methods that can be utilize: enzymatic and chemical with detergents. Our study presents that the enzymatic method has a lower cytotoxicity in comparison with the detergent. Therefore, it can be seeded by autologous cells, which allow the induction of tissue repair and remodeling process. In contrast, the use of detergent methods utilizing SDS significantly reduces the ability of cell growth on the scaffold. This may be due to SDS cytotoxicity, by the limited ability to remove the detergent from the tissue or as a result of ECM degradation. Therefore, it seems that by using enzymatic acellularization methods it is possible to create heart valve prosthesis with properties mimicking native tissue.

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