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Evaluation of decellularization of porcine pericardium: *Decellularization of porcine pericardium*

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Abstract: The porcine pericardium has been used for its great potential as a biological scaffold, produced from the extracellular matrix (ECM) and used mainly in surgeries reconstructive, tissue repair and surgical procedures for corneal reconstruction. The adequate preservation and biocompatibility of the pericardial ECM structure during the decellularization process is fundamental, the biggest challenge being the total removal of cellular material without damage to the structure. All agents used in decellularization change the composition and cause some damage to the ultrastructure. Sodium Dodecil Sulfate (SDS) is the most effective for removing cell residue from tissue compared to other detergents, which is also the most used for the decellularization process. This work aimed to test 3 different concentrations of SDS, in order to assess the concentration (0.1, 0.5 and 1%) that best preserves the structure of the ECM pericardial. In addition, we listed the type of daily wash to make the process more effective (only distilled water or PBS 1x), in order to assess the concentration capable of decellularizing the tissue and better preserving the pericardial ECM. The concentration of SDS at 1%, when compared to the lowest concentrations of 0.1 and 0.5%, was more effective in the decellularization process, however it did not obtain good results in the preservation of the ECM. Regarding daily washing, there was no difference in the frequency assessed in the experimental groups.

Keywords: Decellularization.Tissue engineering. Pericardium. Scaffold.

Introduction

The decellularized pericardial membrane is a promising source of biomaterial [1]. Its shape, function and biological signaling to cells are used in tissue bioengineering and regenerative medicine, for the construction of heart valves, vascular grafts, and tissue repair [2,3]. However, the biomaterial's antigenicity represents the first barrier to expand the use of xenogenic tissues in practice, and the use of scaffold as an option to remove its antigenic properties [4]. The advantage of using this tissue is precisely in its high collagen content [5].

The decellularization process consists of the complete removal of cells from tissues, keeping the components of the extracellular matrix (ECM) in a structural support [6]. The complex and three-dimensional composition of the structure of this matrix, present in a decellularized organ, must have better preservation and good biocompatibility, the biggest challenge being the total removal of cellular material without damage to the ECM. In addition, the residual cellular material attenuates the constructive advantages of tissue remodeling [7,8].

Various chemical, physical and enzymatic methods have been developed to produce a scaffold [9]. The most effective agents for each tissue will depend on many factors, including cellularity, density, lipid content and thickness. Every cell removal agent alters the composition of the ECM and causes some degree of disturbance to the ultrastructure. The protocol to be adopted must take into account the minimization of these effects [7].

The purpose of this study is to test different concentrations of SDS. A smooth concentration (0.1%), an average (0.5%) and higher (1%), in order to assess the concentration that best removes cells from the tissue and preserves the extracellular matrix, with susceptibility to potential recellularization. The samples were also subjected to two types of washings with distilled water and PBS 1x, in order to compare which way is more efficient in the aid of decellularization.

Experimental

Porcine pericardium

The porcine pericardium was obtained from slaughterhouses, according to a project approved by the Ethics Committee on the Use of Animals (ECUA) 2288170519 of the Faculty of Veterinary Medicine and Zootechnics of the University of São Paulo. After collection, the material was placed in a sterile plastic bag and frozen at −20 C.

Decellularization process of the porcine pericardium

After thawing, the porcine pericardium was dissected into pieces of approximately 1.5 cm, placed in containers with the visceral part facing downwards and the serous part upwards. The samples were separated into two experimental groups with different types of washings and SDS concentrations and a control group (Fig. 01). In the first group, the samples were washed with distilled water, placed on the shaker for 1 hour, followed by a second wash and placed on the shaker for another 30 minutes. The second group performed the same procedure, but washes were done with 1x PBS. The control group was also submitted to the same procedure, but the PFA 4% was used. Right after the washing procedures, the group of both distilled water and that of PBS 1x, were separated into 3 subgroups of different concentrations of SDS using gram per liter of distilled water (0.1% – 1g/L, 0.5% 0 5g/L and 1% – 10g/L) for 24 hours in a shaker with 100 revolutions per minute, SDS concentrations changed, this same procedure was performed two more times. At the end of this period, all samples were fi-

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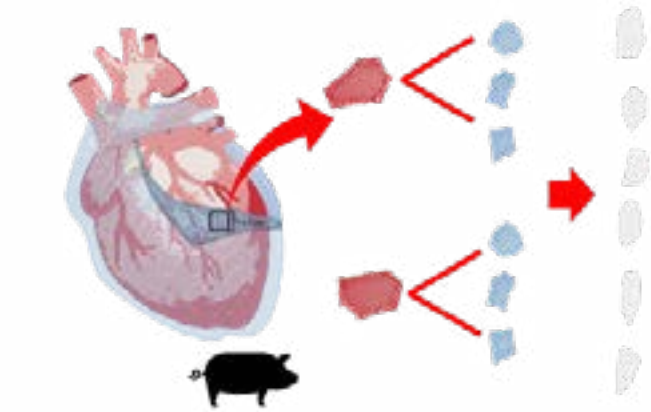
Figure 01– Schematic model of the porcine pericardium decellularization process.



xed with 4% buffered paraformaldehyde (PFA).

Microscopy – Histology and Scanning Electron Microscopy (SEM)

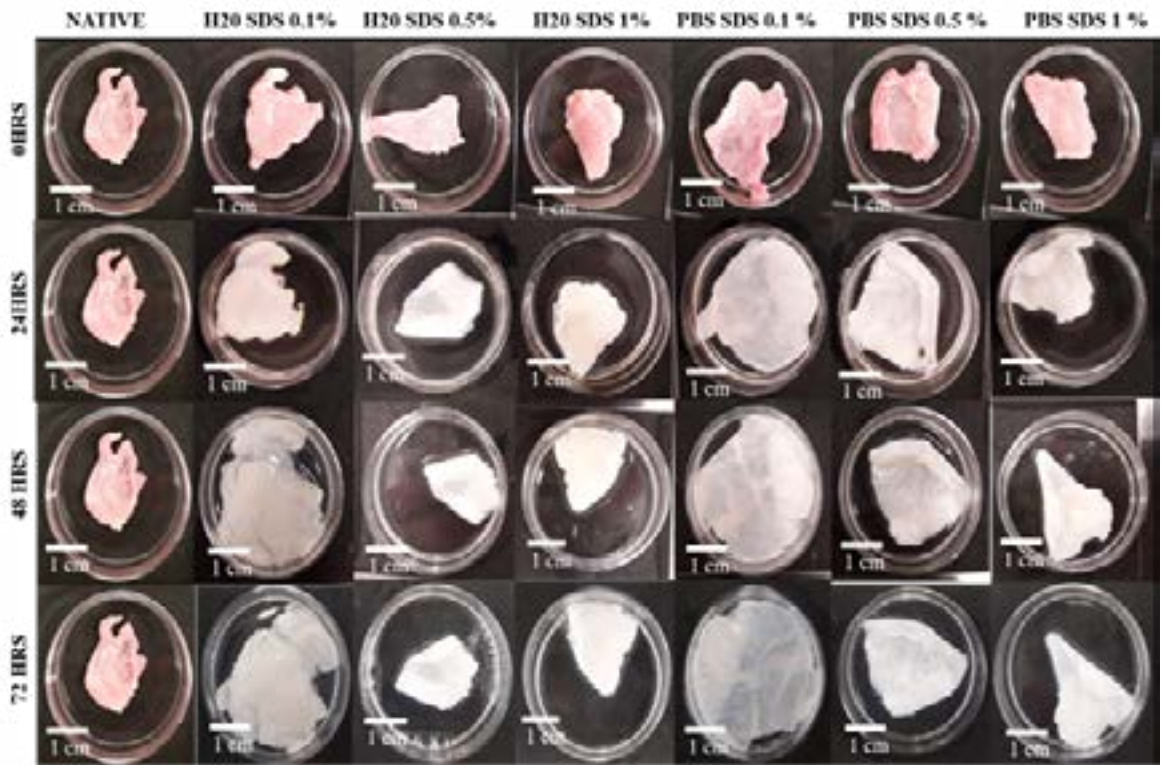
Pericaricardium porcine native (control) and decellularized samples were fixed in 4% buffered paraformaldehyde for 48 hours. Subsequently, dehydrated in ethanol, diaphanized in xylol and embedded in paraffin. Microscopes of 5 μm were produced in a microtome (# RM2265, Leica – Nussloch, GE) and transferred to glass slides. The slides were stained with hematoxylin and eosin (HE), colloidal iron and Picrossirius red, evaluating the cells before and after decellularization [10]. For the SEM, the native and decellularized samples were fixed in 4% buffered paraformaldehyde for 48 hours, washed in an ultrasound bath and post–fixed in 1% osmium tetroxide (SEM® – Hatfield, USA) for 90 minutes. The samples were also dehydrated in an increasing series of ethanols under vigorous agitation, then dried using an automated critical point dryer (EM CPD300, Leica), transferred to stubs and metallized with gold (# K550, Emitech – Ashford, United Kingdom). The samples were analyzed and photographed using a Leo 435 VP scanning electron microscope.

Results

Decellularization of the porcine pericardium

The porcine pericardium decellularization protocol was performed in 72 hours. After decellularization of the tissue, it was whitish in different concentrations of SDS,

Figure 02 – Process of decellularization of porcine pericardium. It is possible to observe that after 72 hours the tissues of all groups were whitish and with increased thickness.



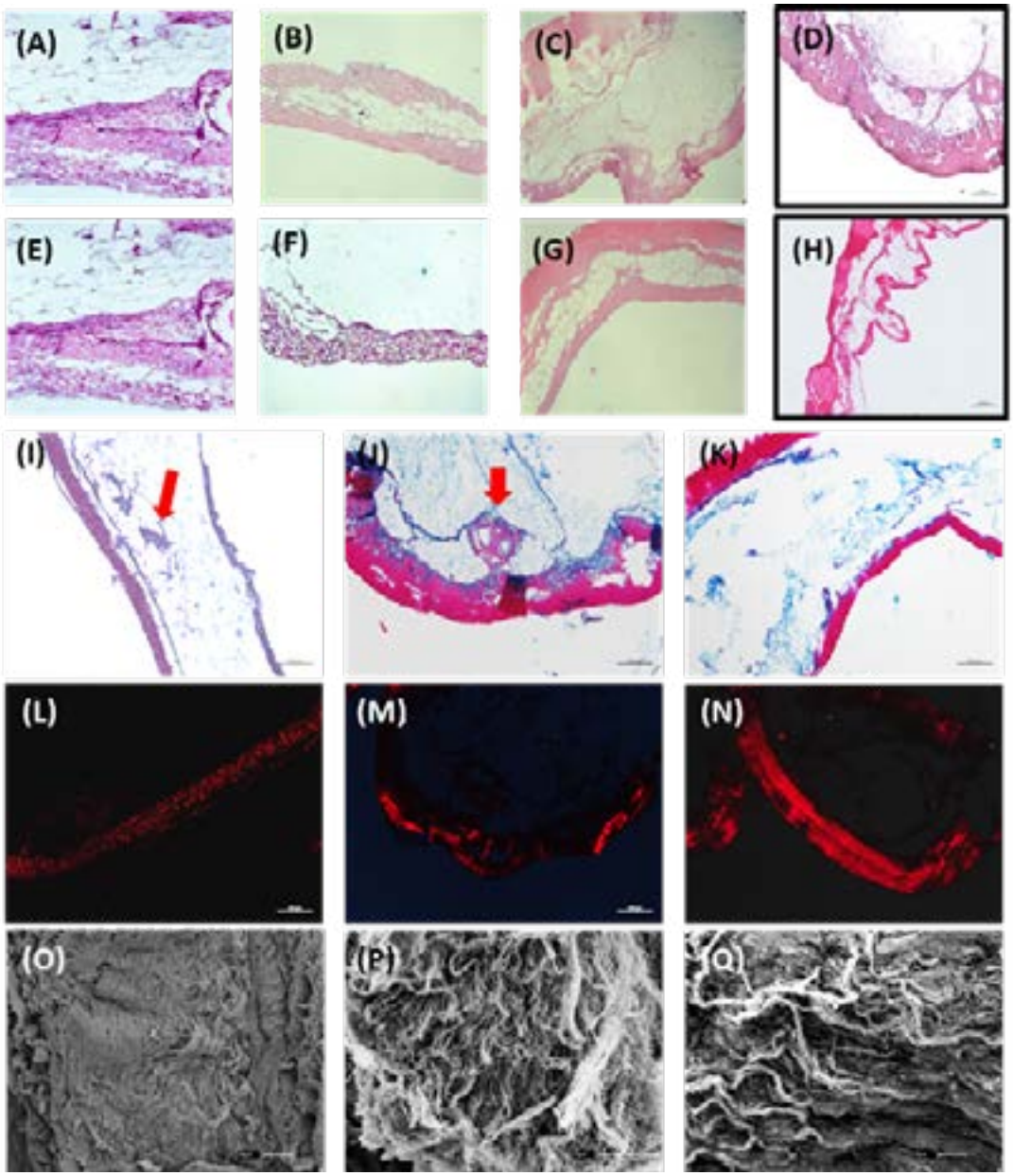
when compared to the native tissue in the washes with distilled water and PBS (Fig. 02).

Microscopy analysis

Histological analysis showed that the pericardial tissues decellularized with 1% SDS maintained the ECM components without any evidence of cellular and nuclear material . In hematoxylin and eosin staining, after decellularization the tissue retained only the eosin dye, without cell debris (Fig. 03–d/h). Staining with colloidal iron showed preservation of hyaluronic acid and glycosaminoglycan(Fig. 3–j/k), while staining with Picrossirius red in polarized light showed preservation of collagen fibers(Fig. 03–m/n). In the scanning electron microscopy, the tissue presented a loose network composed of randomly oriented fibers (Fig. 03–p/q).

The SDS concentrations in 0.1 and 0.5% were insufficient for the decellularization process (Fig. 03–b/c/f/g), remaining cells remaining to histological analysis (HE). This information can also be evidenced according to the ultra–structural analysis of ECM, through SEM. Although they appear to be decellularized, these results demonstrate that the remaining ECM was not

Figure 03 – Decellularization of porcine pericardium. A/B/C– hematoxylin and eosin. A– native tissue; B– PP descel. PBS/ 0.1% SDS, 10x objective; C– PP descel. PBS/ 0.5% SDS, 10x objective; D– PP descel. PBS/ 1% SDS, 10x objective; E– PP descel. H20/ 0.1% SDS, 10x objective. F– PP descel. H20/ 0.5% SDS, 10x objective; G– PP descel. H20/ 1 % SDS, 10x objective, without any evidence of cellular and nuclear material. I/J/K– Colloidal Iron Coloration. I– native tissue, 10x objective; J– PP decell. H20/ 1% SDS, 10x objective; K– PP descel. PBS/ 1% SDS, 10x objective. It is possible to notice in the arrow, the preservation of vascularization, even after the decellularization process. L/M/N– Colouring of Picrossirius red . L– native tissue, 10x objective; M– PP descel. H20/ 1% SDS, 10x objective; N– PP descel. PBS/ 1% SDS, 10x objective. O/P/Q – SEM. O– native tissue; P–PP decell. H20/ 1% SDS, 10 μm; Q– PP descel. PBS/ 1% SDS, 10 μm. The tissue presented a loose network composed of randomly oriented fibers.



properly preserved.

Discussion

The present study demonstrated three protocols with different concentrations of SDS, in order to assess the concentration capable of maintaining the structures of the pericardial ECM preserved, to evaluate the effectiveness of a simpler protocol, *e.g*. easy to reproduce and without association of numerous chemical agents to make decellularization on a large scale viable. In this context, we use two types of daily washing of our material, with distilled water and PBS 1x. The ineffective removal results in less durability of the porcine pericardium, calcification and an unfavorable immune-mediated response on the part of the host. Although SDS has a toxicity rate for ECM after the decellularization process, it is still the most effective agent for removing cell residues from tissue compared to other agents [7,11], therefore, protocols with different concentrations of SDS was tested in our study.

Heuschke [1], evaluated the structural integrity and biocompatibility of the bovine pericardium after a smooth decellularization process with 0.1% SDS solution. This protocol was able to preserve the ECM, as well as its biomechanical properties, allowing the adherence of human adipose tissue stem cells and a suitable scaffold for cell repopulation. Another study was carried out using the same concentration with 0.1% SDS, using human pericardium. There was a reduction in the visible nuclei present in the pericardial tissue after decellularization and retention of collagen and elastin bundles [12].

In the present study, the histological analysis of the porcine pericardium decellularized with 0.1% SDS was insufficient to remove all cells, both in the daily wash with distilled water and with PBS; proving ineffective at this SDS concentration. Therefore, the effect of the decellularization method on the properties of animal tissues must be analyzed, since they present differences in compositional and structural characteristics. In other studies, the process of decellularization of heart valves with SDS in porcine and bovine also achieved different results. While the process was effective for the porcine pericardium, the bovine pericardium failed to preserve the content of GAGs, altering their biomechanical properties [13,14].

In the literature, the concentration with the 0.5% SDS, demonstrated histological analysis, complete removal of cells, interrupted ECM and decrease in the mechanical properties of the decellularized porcine pericardium [15]. Mallis and collaborators [11] used 0.05% SDS followed by several washes in PBS and obtained favorable results regarding the preservation of ECM and potential use as a scaffold in humans. In the present study, the 0.5% SDS protocol, with PBS and distilled water, was not sufficient to guarantee the complete removal of the cells. The differences between the studies are due to the association of other agents with the protocol along with SDS.

The complete removal of cells was described with the 1% SDS protocol in PBS. However, in scanning electron microscopy, it was detected significant changes in the extracellular matrix [16]. Lopera and Griffiths [17], also obtained good results regarding the removal of cells in the pericardial tissue, however the same result was not obtained with regard to the preservation of ultraestructural ECM, compromising the scaffold function and leading to consider that this concentration has a certain degree of toxicity for recellularization. In yet another study, the use of a detergent-based protocol using Triton X-100 followed by SDS in concentrations of 0.3%, 0.5% and 1%, demonstrated extensive loss of elastin and glycosaminoglycans when treated with SDS a 0.5% or 1% SDS, only the concentration of 0.3% SDS combined with Triton X-100 has shown promise [18].

In this study, only the decellularized group with 1% SDS did not reveal any remaining cells in the histological evaluation. Regarding the daily washes, there was no significant data regarding the type used during the protocol days, showing that the detergent concentration is strictly related to the final result. Scanning electron microscopy revealed changes in the architecture of the ECM, when compared to the control group. It was observed that the fabric had a loose network, small pores and randomly oriented fibers. It is known that structural changes in tissue lead to changes in mechanical properties and can contribute to tissue degeneration due to presenting greater rigidity [19]. It is important to note that greater rigidity of the decellularized pericardial tissue was observed, appearing to be brittle.

Conclusion

In conclusion, the protocol with SDS at 1% (10g/L) is superior, when compared to the processes of decellularization with SDS at 0.1 (1g/L) and 0.5% (5g/L), since it obtained positive results regarding the process of decellularization. However, it has not proved to be efficient in terms of preserving the ECM. The protocols with lower concentrations, according to the literature, need to be associated with other agents, to guarantee a complete removal of the cells. It was also possible to observe that the same protocol used in pericardiums of different species, does not have the same result, making the necessary analysis necessary, considering the differences in the compositional and structural characteristics.

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