Original Paper

Optimization Techniques of Single-Detergent Based Protocols for Heart Tissue Decellularization

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ABSTRACT: The extracellular matrix (ECM) scaffolds are considered a gold standard for the engineering of appropriate grafts used in regenerative medicine for tissue repair, and decellularization of myocardial tissue is one of the most studied processes for obtaining natural ECM to date. Decellularization methods, agents used, or treatment durations can be varied to optimize cardiac tissue decellularization parameters. In this work we performed a morphological and morphometric analysis of cardiac tissue subjected to decellularization protocols based on Sodium Deoxycholate (SD) or Sodium Lauryl Sulfate (SLS) to identify factors that allow optimization of single-detergent based protocols for cardiac ECM manufacturing. For this, Wistar rat hearts (n=10) were subjected to 5 different decellularization protocols (n=2) and then histologically processed to achieve H&E or Azan trichrome stained sections for the morphological and morphometric analysis of the obtained ECM. The results of this study showed that SLS alters the spatial distribution of cardiac ECM collagen fibers, and SD can be successfully used in tailoring single-based detergent decellularization protocols by appropriately adjusting the application times of hypo/hyperosmotic shocks, which increases the lytic action of the detergent, and the washing times for the efficient elimination of cellular residues.

KEYWORDS: *Cardiac ECM, collagen fibers, decellularization, sodium deoxycholate, sodium lauryl sulfate.*

Introduction

The decellularization process represents the process of removing all cellular elements from a tissue, leaving behind the histological skeleton formed exclusively from the extracellular matrix [1].

The first attempt at decellularization was made by Poel in 1948 [2].

After that, a few studies documented the decellularization process in the 1970s, mostly performed in the case of less complex structures (arteries, veins, tendons, skin) [3].

In 1995, Badylak's team succeeded in decellularizing the submucosa of the small intestine, which was then used to repair the Achilles tendon [4].

Since then, various 3D biological constructs derived from decellularized tissues or whole organs have been harvested and applied in a wide range of procedures, such as dermal tissue repair, heart valve replacement, and vascular tissue regeneration.

An important milestone in the history of decellularized organs was reported by Ott in 2008, where an extracellular matrix derived from a rat heart was fabricated using perfusion decellularization [5].

Since then, many research groups have demonstrated the feasibility of obtaining acellular structures obtained from a variety of organs.

In 2010, Ott and Petersen, obtained bioartificial lungs using the decellularization process and verified their utility by demonstrating that the acellular lung could participate in gas exchange [6,7].

There is a wide range of methods by which this process can be achieved, be they chemical, physical, enzymatic or a combination of those listed.

The most used chemicals agents are non-ionic, ionic and zwitterionic detergents.

Triton X-100 is representative of the class of nonionic detergents, its properties to denature lipid-lipid and lipid-protein interactions while leaving protein-protein interactions intact make it often used, but results are mixed, depending largely on tissue part.

Non-ionic detergents are represented by sodium dodecyl sulfate (sodium lauryl sulfate) and sodium deoxycholate, which have the property of solubilizing cell membranes with a tendency to denature proteins, remove glycosaminoglycans, but due to their proteolytic property, they can destroy native tissue.

Zwitterionic detergents have the combined properties of non-ionic and ionic detergents, the most commonly used representative of the group is CHAPS (3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate) and has been successfully used to study the decellularization of arteries [8].

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The effects of these substances on the myocardial extracellular matrix are intensely debated [9].

From a structural point of view, it is known that the extracellular matrix is formed almost entirely of connective tissue and extracellular vesicles [10].

Electrophoretic analysis of the extracellular matrix demonstrated that rat [5] and pig [11] myocardium contains type I and type III collagen, type I predominating with a percentage of 85%, type III collagen representing only 11%, the rest being represented of collagen specific to the basement membrane type IV and collagen type V [12], together with fibronectin, laminin and elastic fibers form the myocardial connective tissue.

Extracellular vesicles are synthesized by the cells of a tissue and released into the extracellular matrix.

They contain a variety of regulatory proteins, lipids, and nucleic acids used in intercellular signaling [13].

Maintaining an architectural stability and preserving extracellular vesicles represented a challenge for the decellularization process, the lack of a universal protocol being the main impediment [14].

Chemical detergents used for cardiac tissue decellularization have different capacities for removing cellular components and damaging the ECM ultrastructure [15], and the time of exposure to the action of the detergent plays a key role in maintaining structural and functional proteins [8].

Therefore, an optimization of the complexity of the procedures and the duration of the decellularization treatments is still necessary to obtain an adequate cardiac ECM [16].

Because lauryl and deoxycholate are some of the most used cardiac tissue decellularization agents [17], in this study we performed a morphological and morphometric analysis of the effect of different decellularization protocols based on these detergents on heart ECM collagens.

Since lauryl and deoxycholate are some of the most widely used cardiac tissue decellularization agents [17], in this study we performed a morphological and morphometric analysis of cardiac tissue subjected to 5 different decellularization protocols based on these detergents to identify factors that allow optimization of single-based detergent protocols for cardiac ECM manufacturing.

Materials and Methods

Animals

The protocols used in this study were approved by the Scientific Ethics and Deontology Commission of the University of Medicine and Pharmacy in Craiova.

Hearts harvested from 8-week-old Wistar rats (n=10) were used.

They were anesthetized with Ketamine (100mg/kg) and Xylazine (10mg/kg), after which they received i.v. 1000IU heparin 10 minutes before harvesting the cardio-pulmonary complexes.

After a longitudinal thoraco-laparotomy, the anterior chest wall was removed.

The retrosternal fat was dissected to identify the aorta and pulmonary artery.

The left lung was retracted, and the parietal pleura was dissected to expose the descending thoracic aorta.

The aorta was cannulated using a 16G venous cannula placed through the descending thoracic aorta into the aortic arch.

The innominate artery, the carotid artery, the subclavian artery, and the superior vena cava were sectioned after ligation with 4-0 nylon suture, to prevent retrograde leakage of infused solutes.

The heart and both lungs were harvested as a single block by sectioning the trachea and inferior vena cava.

Washing Protocols

The hearts were subjected to different washing protocols marked as P1, P2, P3, P4.

These were conducted at room temperature, and the used solutions were perfused retrogradely using a cannula inserted in the aorta and maintaining a pressure of 100cm H2O.

P1 protocol: hearts were perfused with distilled water for 24 hours.

P2 protocol: hearts were perfused with distilled water for 24 hours, and then with 2% SD (Sigma-Aldrich®) for another 24 hours.

P3 protocol: hearts were perfused 2 hours with PBS, 24 hours with distilled water, 4 hours with PBS, 18 hours with 2% SD, 3 hours with PBS, 3 hours with distilled water, and finally 3 hours with PBS.

P4 protocol: the same as P3 protocol, but the SD was replaced with 2% SLS (Sigma-Aldrich®).

P5 protocol: hearts were perfused 24 hours with ultrapure water (18.2 MΩ/cm), 4 hours with 4% SD, 30 minutes with PBS, 3 hours with 15% NaCl mixed with 2000 KU deoxyribonuclease

I from bovine pancreas (Sigma-Aldrich®), and finally 30 minutes with PBS.

After applying the 5 washing protocols the hearts were fixed 48 hours in 4% formaldehyde buffered to a neutral pH for later histological analysis.

Histological Analysis

The harvested and fixed hearts were processed by classical histology techniques by embedding in paraffin, sectioning with the Leica RM2255 rotary microtome at a thickness of 7 microns and collected on slides covered with poly-L-lysine.

The analysis of the morphological changes induced by the washing protocols was carried out with the light microscope on sections stained with HE and Azan trichrome.

The relative area of the ECM (RaECM) was used to compare the degree of decellularization after the application of protocols P3, P4 and P5, being calculated according to the formula RaECM=ECM area/measured area.

To achieve this, 20 fields were obtained for each studied heart with the ×20 objective, which were processed using Image Pro Plus® software.

Statistical Analysis

The results were presented as mean±standard deviation, and were compared with ANOVA post-hoc Tukey test, having set the level of significance at 0.05, using SPSS v.16 software.

Results

The microscopical analysis of the heart sections revealed specific morphological features to each washing protocol.

To analyze the degree of heart tissue decellularization, we evaluated at the level of the ventricular walls, the lysis of the cells and the integrity of the nucleus.

The histological evaluation of the P1-decellularized heart tissue

In the case of the P1 protocol, a complete emptying of blood of the large caliber vessels from the subepicardic region of the ventricles was observed (Figure 1 a,b).

The blood's cellular components were pushed towards the subendocardial arteriolo-capillary network, blocking it in some places by cell-based plugs (Figure 1, c,d).

Washing with distilled water caused vacuolization of the myocardial cells near large caliber blood vessels highlighted by H&E stain (Figure 1 b).

In addition to cellular edema, this protocol can also induce the muscle fibers dissociation, with the stretching of collagen fibers which in certain regions can fracture (Figure 1 e,f).

After applying the P1 protocol, no changes were observed in the nuclei of the myocardial cells, on H&E staining.

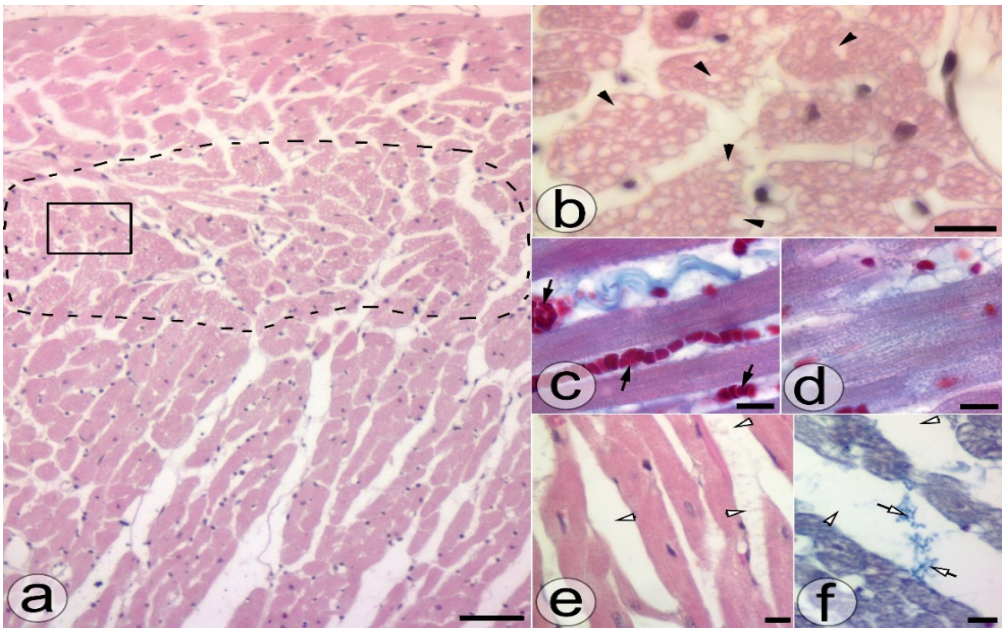

Figure 1. Histological aspects of P1 washed heart sections. a: interstitial edema and patches with early cell lysis (broken line) located around large vessels in the subendocardial region. b: detailed image of the rectangular area marked in the previous image, highlighting the vacuoles (black head arrows) formed in the muscle fibers. c: subendocardial area with interstitial edema and capillaries blocked by erythrocyte plugs (black arrows). d: subendocardial area with unblocked capillaries and incipient muscle fiber lysis injuries. e: interstitial edema with the dissociation of muscle fibers and tension of the ECM collagen fibers (white head arrows). f: areas with dissociated muscle fibers and fragmentation of the ECM collagen fibers (white arrows). H&E (a,b,e) and Azan trichromic (c,d,f) stains. Bar=50µm (a), 10µm (b-f).

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The histological evaluation of the P2-decellularized heart tissue

The microscopical analysis showed areas with a patchy disposition around the large subepicardial vessels with advanced phases of decellularization of the myocardial tissue (Figure 2 a,b,c).

These zones of advanced decellularization presented myocardial cell lysis which highlighting the ECM network that remained in places attached to the remains of the cytoskeleton of some myocardial cells (Figure 2 f).

Towards the periphery the decellularization process is heterogenous.

The decellularization process is gradual, presenting areas with myocardial cells that have kept all the cytoskeleton anchored to the ECM (Figure 2 e) or areas with only partially lysed myocardial cells (Figure 2 d).

The nuclear integrity was affected by the P2 protocol, but it failed to eliminate the nuclear debris.

In the areas with advanced decellularization H&E stain showed, in addition to the persistence of nuclear shadows, an accumulation of basophilic material among the collagen fibers of the ECM, which acts as a filter for them.

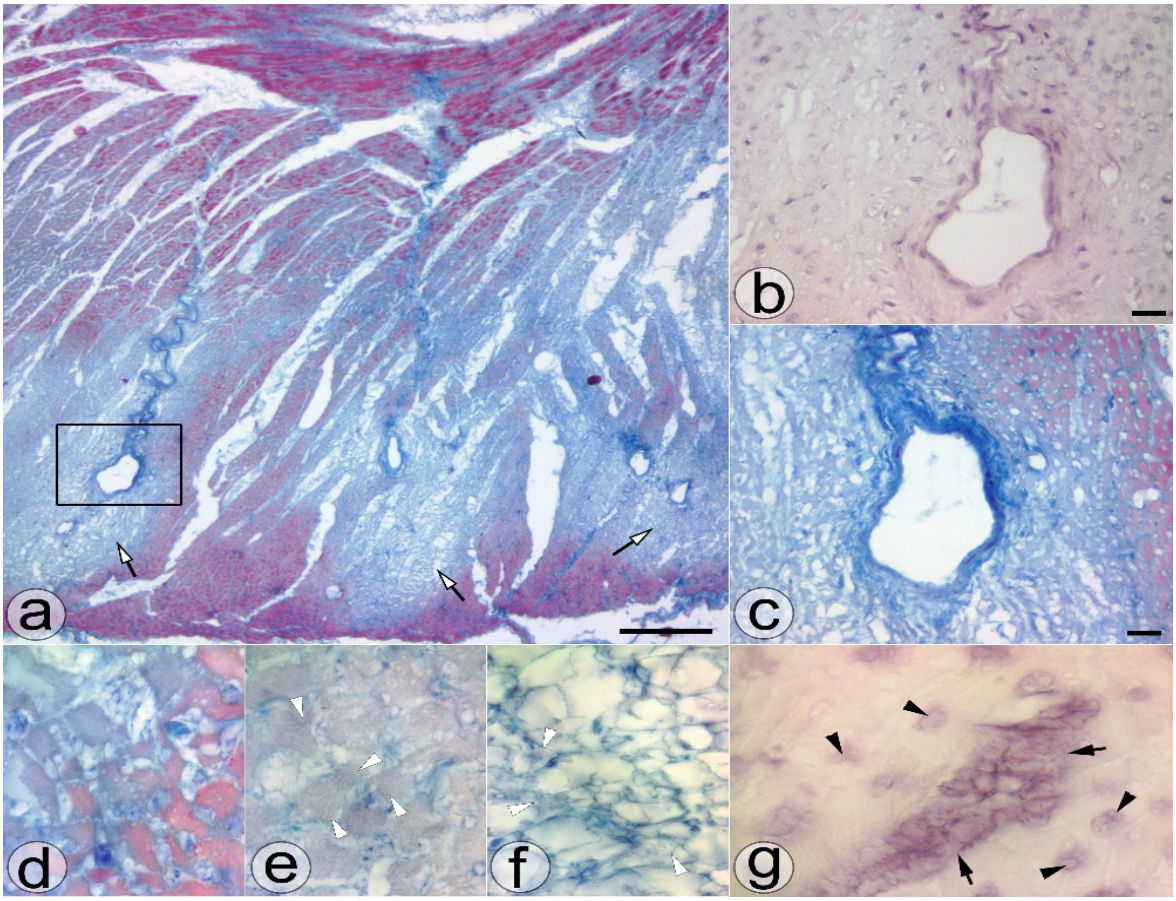

Figure 2. Histological aspects of P2 washed heart sections. a: gradual progression of cell lysis lesions, with advanced lesions located around large vessels (white arrows). b, c: detailed images of the rectangular area marked in the previous image. d: lysed muscle fibers with partial removal of cellular content. e: areas with the persistence of the muscle fiber cytoskeleton (white arrowhead) anchored to the ECM. f: areas with advanced decellularization of the ECM. g: nuclear shadows (black arrowheads) and basophilic material (black arrows) among the ECM collagen fibers in areas with advanced decellularization. H&E (b, g) and Azan trichromic (a, c, d-f) stains. Bar=500µm (a), 10µm (b-g).

The histological evaluation of the P3-decellularized heart tissue

The histological analysis of the sections through the ventricles showed advanced subepicardial and subendocardial decellularization process (Figure 3 a,b).

Most myocardial cells kept a part of the cytoskeletal structures attached to the ECM.

Also, ECM collagen fibers have maintained their three-dimensional arrangement.

The nuclear components are removed, but the basophilic debris were noticed by H&E stains in some zones of the ECM (Figure 3 a).

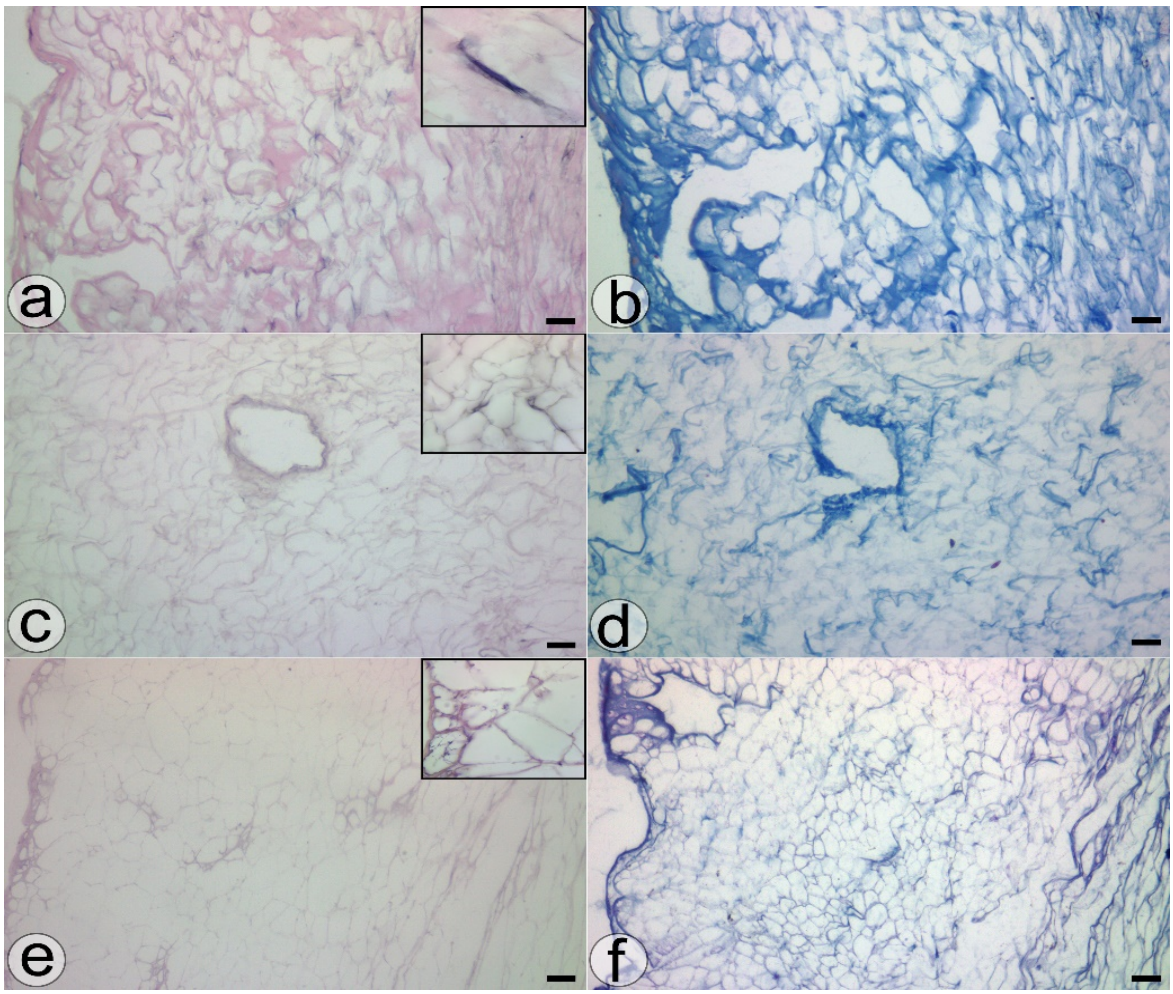

Figure 3. Histological aspects of P3 (a, b), P4 (c,d), and P5 (e,f) washed heart sections. The boxes show detailed images of the basophilic material on the H&E stain retained by the ECM collagen fibers. H&E (a,c,e) and Azan trichromic (b,d,f) stains. Bar=20µm.

The Histological Evaluation of the P4-Decellularized Heart Tissue

P4 protocol caused a complete lysis of the myocardial cells with the complete removal of cellular content including cytoskeletal structures (Figure 3 c, d).

Basophilic material was still identified attached to the collagen fibers in H&E stains (Figure 3 c).

After applying this protocol, the spatial arrangement of the ECM was modified through collagen fibers fracture (Figure 3 d).

The Histological Evaluation of the P5-Decellularized Heart Tissue

The microscopical analysis of the histological sections through heart washed with P5 protocol showed a complete lysis of the myocardial cells with the removal of cellular debris without the alteration of the spatial arrangement of the collagen fibers of the ECM (Figure 3 e, f).

Using H&E stain, basophilic material was noticed attached to some collagen fibers of the ECM (Figure 3 e).

Morphometric Evaluation of the P3, P4 and P5-Decellularized Heart Tissue

To estimate the amount of ECM obtained after applying the washing protocols P3, P4 and P5, the RaECM were measured on the Azan trichrome stained sections through ventricles (Figure 4).

The morphometric analysis of the RaECM showed that there is a statistical difference between their values, being greater after the application of the P3 (0.39±0.05) protocol than after the application of the P4 (0.25±0.05) or P5 (0.16±0.03) protocols ($p<0.01$, ANOVA with post-hoc Tukey test).

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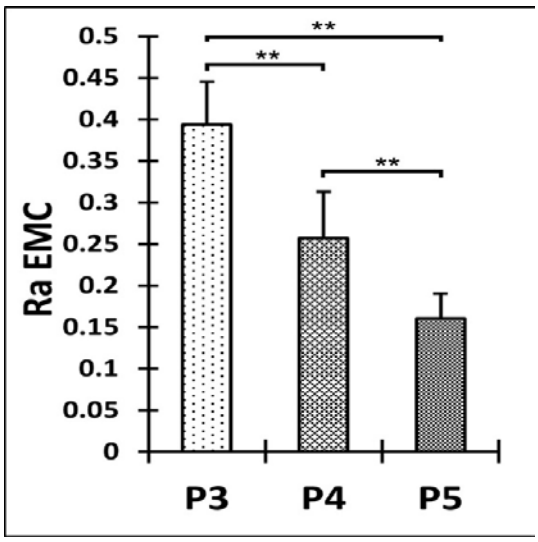

Figure 4. Relative area of ECM (RaECM) collagen in sections through hearts washed with protocols P3, P4 and P5 (**p<0.05).

Discussions

The scaffolds obtained from EMC are considered a gold standard for the engineering of appropriate grafts used in regenerative medicine for tissue repair, and decellularization of myocardial tissue is one of the most studied processes for obtaining natural ECM to date [17].

The main components of cardiac ECM confer strength, durability, and flexibility to the heart, being mainly represented by collagens, fibronectin, and elastin [16].

After cardiac tissue decellularization the degree of ECM preservation can influence scaffold properties such as mechanical strength, bioactivity, degradation process and tissue regeneration capacity in vivo [18].

To optimize cardiac tissue decellularization parameters, decellularization methods, agents used, or treatment durations can be varied [19].

Perfusion through the coronary arteries facilitates the homogeneous exposure of cardiac tissue to the action of the decellularization agent but presents the risk of ECM alteration by the perfusion pressure [20].

Based on these considerations, retrograde perfusion of decellularization agents with a pressure of 100cm H_2O (lower than the systolic pressure) was used in this study.

The histological analysis of the sections through the hearts washed with distilled water (protocol P1) showed that the cellular component of the blood that persisted in the arterial system was pushed towards the subendocardial capillaries with their partial blockage.

Because these blockages prevent the homogeneous perfusion of the washing agent, a complete emptying of blood from the arterial bed is necessary before applying any decellularization protocol.

Also, long-term washing with hypoosmotic solution caused the induction of an interstitial edema with partial fracturing of the collagen fibers of the ECM.

As a result, the perfusion time with the hypoosmotic solution must be reduced if the spatial arrangement of the ECM collagens is to be preserved.

Deoxycholate perfusion of cardiac tissue with interstitial edema (P2 protocol) showed a reduced decellularization capacity of the detergent, both in terms of cell lysis and the removal of nuclear material.

These observations showed the need to introduce an intermediate step to remove the interstitial edema by perfusing an isoosmotic or hypertonic solution to increase the efficiency of the detergent.

By using short cycles of distilled water/PBS before and after perfusion with a 2% deoxycholate solution (P3 protocol), the ability of the detergent to lyse cells and remove nuclear material increased preserving the morphological features of the ECM collagen fibers.

Cytoskeleton remnants attached to ECM components visible after the application of this protocol could be removed by increasing the detergent application time.

After applying the P4 protocol in which deoxycholate was replaced with lauryl, a much more effective decellularization agent [Moffat], it was observed that the distribution of basophilic remains marked by H&E stain was like that in the P3 protocol.

By correlating these results, we can state that both detergents are effective in removing the nuclei.

To remove the remnants of nuclear material that remain filtered by the collagen fibers of the ECM, which is generally associated with nuclear debris, the times of the last washing cycle of the protocols should be increased.

Cardiac ECM obtained after applying the P4 protocol no longer shows cytoskeleton remains but is characterized by fragmentation and alteration of the spatial distribution of collagen fibers.

These findings suggested that lauryl-based protocols are not recommended when maintaining the structural integrity of cardiac ECM collagens is desired, and are consistent with the observations published by other studies [15].

Hypo/hyperosmotic shocks in association with deoxyribonuclease I from bovine pancreas used in the P5 protocol showed a better removal of the cytoskeleton attached to the collagen fibers than the P3 protocol (P5 ArECM lower than P3 ArECM), without affecting the spatial arrangement of the collagen fibers.

The persistence of basophilic residues in H&E stain showed that DNase did not provide notable benefits and supports the finding above, about the role of the final washing cycles in the removal of nuclear material residues.

Conclusions

The results of this study showed that SLS fragmented collagen fibers and altered the spatial distribution of cardiac ECM.

SD can be successfully used in tailoring single-detergent based protocols for decellularization by appropriately adjusting the application times of hypo/hyperosmotic shocks, which increases the lytic action of the detergent, and the washing times for the efficient elimination of cellular residues.

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Conflict of interests

None to declare

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