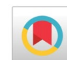

Decellularization Agents on Porosity and Pore Size in Bovine Pericardium Scaffolds for Pediatric Heart Surgery

Mario Hendri RW[1], Heroe Soebroto [1], Ito Puruhito [1]

Abstract

Background: Tissue engineering in pediatric and congenital heart surgery relies on developing biomaterials with extracellular matrix (ECM)-like properties. The acellular bovine pericardium membrane (BPM) is a promising scaffold due to its strength, low infection rates, and cost-effectiveness. Decellularization is essential to optimize BPM's properties for tissue regeneration. This study investigates the effects of different decellularization methods on BPM's porosity and pore size. Methods: A true experimental design was used to evaluate BPM scaffolds treated with sodium dodecyl sulfate (SDS), hydrogen peroxide (H2O2), or ASB-16, compared to a control group. Porosity and pore size were measured using image analysis software after 4 weeks of incubation. Statistical analysis was performed using the Kruskal-Wallis test, Mann-Whitney U test, and one-way ANOVA. Results: ASB-16 decellularization significantly increased BPM porosity (50.14 ± 3.71%) compared to the control (3.06 ± 0.99%) and other treatments (SDS: 6.23 ± 2.94%, H2O2: 4.47 ± 1.34%). Pore size was also significantly larger in the ASB-16 group (26.9 ± 5.93 μm) compared to SDS (8.99 ± 2.77 μm), H2O2 (3.13 ± 1.00 μm), and control (2.00 ± 0.29 μm). Conclusion: ASB-16 decellularization effectively enhances BPM porosity and pore size, making it a promising method for optimizing scaffolds in tissue engineering applications. Further research should focus on its impact on cell proliferation and tissue regeneration.

Keywords: Bovine pericardium membrane, Decellularization, Porosity, Pore size, Tissue engineering

Significance | This study showed optimal decellularization methods for enhancing porosity and pore size in bovine pericardium scaffolds, crucial for pediatric heart surgery.

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Introduction

In pediatric and congenital heart surgery, the need for effective substitute materials to replace removed body tissues is paramount (Shah et al., 2020). Tissue engineering offers a promising solution through the development of biomaterials that closely mimic the extracellular matrix (ECM) (Hinderer et al., 2016; O'Brien, 2011). One significant approach within tissue engineering is the use of acellular scaffolds, which are designed to replicate the structural and functional properties of native tissues (Badylak et al., 2015; Crapo et al., 2011). Among these scaffolds, acellular bovine pericardium membrane (BPM) stands out due to its favorable characteristics, including high tensile strength, low infection rates, and cost-effectiveness (Lin et al., 2022; Zheng et al., 2019). BPM can be used directly in clinical settings without extensive preparation, making it a valuable resource for regenerative therapies, especially in thoracic and cardiovascular surgeries (Feng et al., 2020; Rodrigues et al., 2013). For BPM to be effective as a scaffold, it must first undergo a process of decellularization (Liao et al., 2008; Gilbert et al., 2006). This process is crucial for removing cellular debris such as DNA, plasma membranes, and mitochondria, which could otherwise

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provoke inflammatory responses and impede the tissue reconstruction process (Crapo et al., 2011; Keane et al., 2015). The goal of decellularization is to retain the ECM's structural integrity while eliminating potential contaminants that could hinder cellular activities like proliferation and migration (Ott et al., 2008; Petersen et al., 2010). Effective decellularization ensures that the scaffold supports optimal tissue regeneration and function by maintaining a balanced ECM structure (Limpert et al., 2009; Badylak, 2002). Porosity and pore size are critical factors in the functionality of decellularized scaffolds (Grayson et al., 2008; Boccafoschi et al., 2016). These parameters influence cell adhesion, proliferation, and migration, all of which are essential for successful tissue regeneration (Takahashi et al., 2022; Wang et al., 2017). High porosity is particularly important as it facilitates the distribution of cells throughout the scaffold, ensuring adequate nutrient and oxygen supply, and supports the formation of new tissue (Liu et al., 2017; Zhang et al., 2020). Conversely, scaffolds with low porosity and small pore sizes can restrict cell infiltration and impede tissue growth (Bashur et al., 2009). This study explores the effects of different decellularization methods—specifically SDS 0.5%, H2O2 3%, and ASB-16 3%—on the porosity and pore size of BPM scaffolds (Keane et al., 2015; Dahl et al., 2003). By evaluating these parameters, the research aims to identify the most effective decellularization strategy for enhancing scaffold performance in regenerative medicine (O'Brien, 2011; Badylak et al., 2015). Understanding how these methods influence scaffold characteristics will help optimize their use in clinical applications, particularly in pediatric and congenital heart surgeries where tailored biomaterials are essential for successful outcomes (Alizadeh et al., 2021; Shah et al., 2020).

2. Materials and Methods

2.1 Study Design

This study utilized a True Experimental design with both treatment and control groups to assess the porosity and pore size of bovine pericardium membrane (BPM) scaffolds subjected to different decellularization methods. The decellularization methods compared were sodium dodecyl sulfate (SDS), hydrogen peroxide (H2O2), and ASB-16, with a control group consisting of untreated BPM scaffolds. The control group scaffolds were immersed solely in a 0.9% NaCl solution, while the treatment groups were exposed to SDS 0.5%, H2O2 3%, and ASB-16 3%.

2.2 Data Collection

Bovine pericardium membranes were selected based on specific inclusion and exclusion criteria. The inclusion criteria required that the pericardium come from slaughtered cattle certified healthy by a veterinarian. Exclusion criteria were applied to exclude pericardium that was either torn or damaged during preparation or infected with fungi.

Selected bovine pericardium samples meeting the inclusion criteria and free from exclusion factors were chosen using simple random sampling. These samples were then randomly assigned to one of the four groups: the control group, the SDS 0.5% treatment group, the H2O2 3% treatment group, and the ASB-16 3% treatment group. The decellularization process was conducted as per the study protocol, with samples incubated for 4 weeks post-treatment. After this incubation period, the microstructure of the BPM scaffolds in each group was evaluated, and porosity and pore size measurements were performed using ImageJ software.

2.3 Statistical Analysis

Data were organized and analyzed using spreadsheet software. Normality and variance homogeneity of the data were assessed using the Shapiro-Wilk normality test. For normally distributed data, parametric analysis was performed using an unpaired one-way ANOVA with a 95% confidence level to determine differences in mean values among the four groups. Significant results were followed by post hoc testing to identify specific group differences. Statistical significance was set at a p-value of less than 0.05, and the Bonferroni correction was applied to account for multiple comparisons and reduce the risk of Type I errors.

3. Results

3.1 Porosity of Bovine Pericardium

The porosity of bovine pericardium (BPM) was analyzed after decellularization with different methods: 0.9% NaCl (control), SDS 0.5%, H_2O_2 3%, and ASB 16 3%. The results showed a wide range of porosity values among the groups (Figure 1, Table 1). The control group exhibited the lowest porosity at 3.0600 ± 0.99%. The H_2O_2 3% group had a slightly higher porosity at 4.4675 ± 1.34%, while the SDS 0.5% group exhibited a porosity of 6.2275 ± 2.94%. The ASB 16 3% group showed a significant increase in porosity, with an average value of 50.1425 ± 3.71%, indicating that ASB 16 3% substantially enhances porosity compared to the other treatments.

Statistical analysis using the Kruskal-Wallis test revealed no significant differences in porosity among the groups overall ($p = 0.197$), but pairwise comparisons using the Mann-Whitney test indicated significant differences between the ASB 16 3% group and all other groups ($p < 0.05$). Specifically, significant differences were found between the ASB 16 3% group and the control group ($p = 0.000$), SDS 0.5% group ($p = 0.000$), and H_2O_2 3% group ($p = 0.000$). However, no significant differences were observed between the control group and the SDS 0.5% group ($p = 0.088$) or the H_2O_2 3% group ($p = 0.144$), nor between the SDS 0.5% and H_2O_2 3% groups ($p = 0.319$) (Table 2, Table 3).

3.2 Pore Size of Bovine Pericardium

The average pore size of BPM also varied significantly among the groups (Figure 3, Table 4). The control group had the smallest average pore size of 2.00 ± 0.29 µm. The H_2O_2 3% group showed a

slight increase in pore size to 3.13 ± 1.00 µm, while the SDS 0.5% group exhibited a more substantial increase to 8.99 ± 2.77 µm. The ASB 16 3% group demonstrated the largest pore size, with an average of 26.9 ± 5.93 µm.

A One-Way ANOVA test confirmed significant differences in pore size among the groups (p = 0.000). Post Hoc Tukey test comparisons revealed that significant differences in pore size were found between the ASB 16 3% group and the control group (p = 0.000), SDS 0.5% group (p = 0.000), and H_2O_2 3% group (p = 0.000). However, there were no significant differences between the control group and the SDS 0.5% group (p = 0.49) or the H_2O_2 3% group (p = 0.963), nor between the SDS 0.5% and H_2O_2 3% groups (p = 0.110) (Table 5, Table 6).

3.3 Electron Microscopy Observations

Electron microscopy provided a visual confirmation of the porosity and pore size differences among the decellularization methods (Figures 1, 2, and 3). The control group, treated with 0.9% NaCl, showed minimal porosity and very small pore sizes. The SDS 0.5% and H_2O_2 3% groups displayed moderate increases in porosity and pore sizes, with the SDS 0.5% group exhibiting slightly larger pores than the H_2O_2 3% group. The ASB 16 3% group, however, showed a dramatic increase in both porosity and pore size, indicating that ASB 16 3% is the most effective among the tested methods for enhancing these properties in bovine pericardium scaffolds.

In summary, the decellularization of bovine pericardium using ASB 16 3% resulted in significantly higher porosity and larger pore sizes compared to other methods, making it a potentially superior approach for scaffold preparation in tissue engineering applications.

4. Discussion

Porosity in scaffolds is a critical factor in tissue formation and function. Adequate porosity ensures uniform cell distribution and interconnection throughout the artificial tissue, which is crucial for the diffusion of nutrients and oxygen in environments lacking functional vascular systems. This porosity mimics the body's extracellular matrix (ECM), interacting effectively with cells and playing a vital role in tissue engineering (Annabi et al., 2010).

Porosity also supports local angiogenesis after implantation, a key requirement for the development of vascularized tissue. Additionally, it significantly impacts the mechanical properties of scaffolds; as porosity increases, stiffness decreases due to fluid flow during mechanical deformation. Specific pore architectures, including their interconnections, are critical in various cellular processes within hydrogels, such as enhancing cell viability, promoting proliferation, facilitating migration, and encouraging ECM component secretion (Khademhosseini & Langer, 2007).

In an experiment, the average porosity of the SDS 0.5% group was found to be 6.2275 ± 2.94, while the ASB 16 group had an average porosity of 50.1425 ± 3.71. These results indicate that washing with ASB-16 increases scaffold porosity by 8-10 times. Pairwise comparisons using the Mann-Whitney test revealed no significant differences between the control group and Treatment Group I (SDS 0.5%) (p = 0.088) or Treatment Group II (H2O2 3%) (p = 0.144), nor between Treatment Group I (SDS 0.5%) and Treatment Group II (H2O2 3%) (p = 0.319). However, significant differences were observed between Treatment Group III (ASB 16 3%) and the control group (p = 0.000), Treatment Group I (SDS 0.5%) (p = 0.000), and Treatment Group II (H2O2 3%) (p = 0.000).

Small pore sizes can hinder cell interactions, including adhesion, proliferation, and migration, particularly in stem cell applications on scaffolds. Ensuring adequate pore size in scaffold design is therefore crucial for facilitating tissue regeneration processes. Techniques such as applying a vacuum to bovine pericardium scaffolds can enlarge pore sizes to a sufficient dimension, such as 75 µm. Generally, larger pore sizes support cell adhesion, proliferation, and migration more effectively than smaller ones, as they provide more space for cells to penetrate, adhere to the pore walls, and proliferate on the scaffold's surface (Alizadeh et al., 2019).

Research indicates that bovine pericardium scaffolds decellularized using ASB-16 exhibit significantly larger pore sizes compared to those treated with the SDS 0.5% method. This demonstrates that the choice of decellularization method directly affects pore size and porosity, which are critical factors for the effectiveness of scaffolds in regenerative applications. Studies have shown that pores smaller than 100 µm can restrict cell movement, impeding efficient migration and proliferation. Conversely, pores larger than 100 µm enhance cell infiltration, which is essential for tissue formation and regeneration (Alizadeh et al., 2019).

In this study, the largest pore size was observed in scaffolds decellularized with ASB 16, followed by SDS 0.5% and H2O2 3%. The smallest pore size was noted in scaffolds decellularized with H2O2 3%, which was nearly identical to the control group. Specifically, the pore sizes were as follows: control group - 0.65 (± 0.27) µm, SDS 0.5% group - 8.33 (± 1.70) µm, H2O2 3% group - 9.08 (± 4.80) µm, and ASB-16 3% group - 33.27 (± 12.17) µm. These results indicate that washing with ASB-16 increased pore size by 4-5 times. There was a notable increase in pore size with SDS 0.5%, but this method has cytotoxic effects on cells and requires more extensive washing (Alizadeh et al., 2019). ASB-16 offers a viable alternative, as it does not present significant differences in comparison to SDS 0.5% and demonstrates good tolerance to adaptive and innate immunity due to the reduction of xenogenic material (Dalgliesh et al., 2018).

The decellularization methods ASB-16 and SDS 0.5% showed significant differences in pore size compared to other treatments, while SDS 0.5%, H2O2, and the control did not exhibit significant differences. In another study involving bovine pericardium

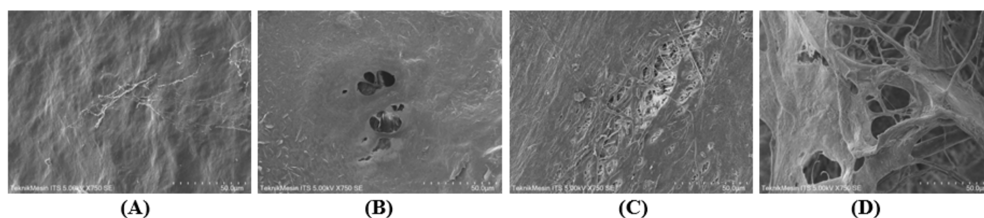

Figure 1. Bovine pericardium that was decellularized with (A) 0.9% NaCl (B) SDS 0.5% (C) H202 3% (D) ASB 16 3% observed using Electron Microscope

Table 1. Average Porosity of Bovine Pericardium Decellularized with Various Materials

Decellularization Method	Porosity (%)/Field of view
ASB 16	50.1425 (± 3.71)
H2O2	4.4675 (± 1.34)
SDS 0,5%	6.2275 (± 2.94)
Control	3.0600 (± 0.99)

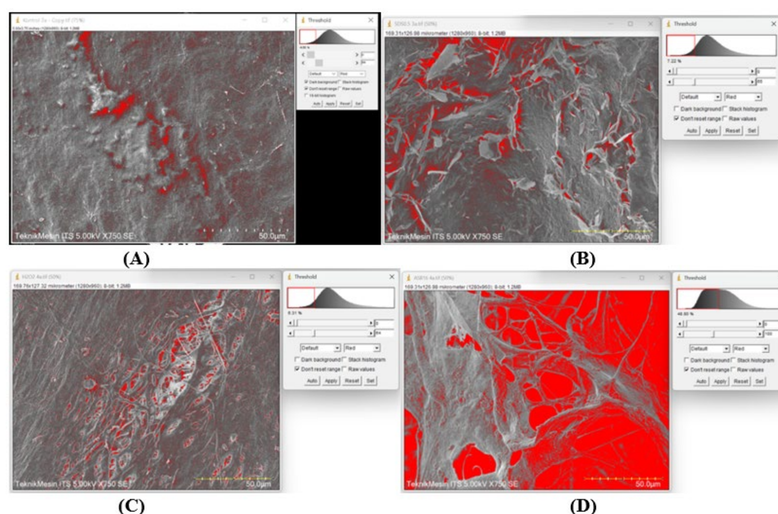

Figure 2. Porosity of Bovine pericardium that was decellularized with (A) 0.9% NaCl (B) SDS 0.5% (C) H202 3% (D) ASB 16 3% observed using Electron Microscope

Table 2. Saphiro-Wilk Test of Porosity of Bovine Pericardium Decellularized with Various Materials

Subject	Porosity (%)	Normality Test (Saphiro-Wilk)
Control	3.0600 (± 0.99)	.009
SDS 0,5%	6.2275 (± 2.94)	.479
H2O2 3%	4.4675 (± 1.34)	.705
ASB-16 3%	50.1425 (± 3.71)	.254

Table 3. Kruskal-Wallis Test of Porosity of Bovine Pericardium Decellularized with Various Materials

Subject	Porosity (%)	Kruskal-Wallis
Control	3.0600 (± 0.99)	P = 0.197
SDS 0,5%	6.2275 (± 2.94)	
H2O2 3%	4.4675 (± 1.34)	
ASB-16 3%	50.1425 (± 3.71)	

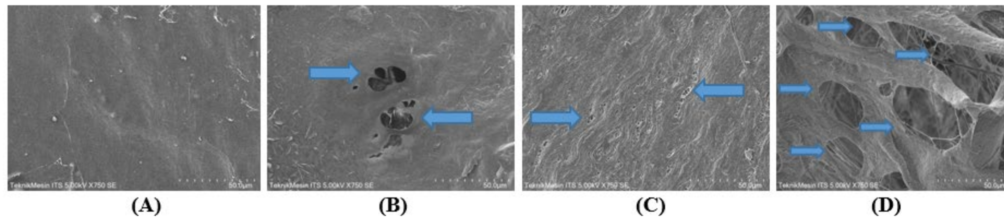

Figure 3. Pore Size of Bovine pericardium that was decellularized with (A) 0.9% NaCl (very small pore size) (B) SDS 0.5% (C) H202 3% (D) ASB 16 3% observed using Electron Microscope wth magnification 750x. Pore size shown with blue arrow.

Table 4. Average Porosity of Bovine Pericardium Decellularized with Various Materials

Decellularization Method	Pore Size (µm)
ASB 16	26.9 (± 5.93)
H2O2	3.13 (± 1.00)
SDS 0,5%	8.99 (± 2.77)
Control	2.00 (± 0.29)

Table 5. Saphiro-Wilk Test of Porosity of Bovine Pericardium Decellularized with Various Materials

Subject	Porosity (%)	Normality Test (Saphiro-Wilk)
Control	2.00 (± 0.29)	.977
SDS 0,5%	8.99 (± 2.77)	.586
H2O2 3%	3.13 (± 1.00)	.532
ASB-16 3%	26.9 (± 5.93)	.847

Table 6. One-Way ANOVA Test of Porosity of Bovine Pericardium Decellularized with Various Materials

Subject	Porosity (%)	One-Way ANOVA
Control	2.00 (± 0.29)	P = 0.000
SDS 0,5%	8.99 (± 2.77)	
H2O2 3%	3.13 (± 1.00)	
ASB-16 3%	26.9 (± 5.93)	

decellularized with SDS, an increase in pore size from 67 µm to 132 µm and porosity from 3% to 15% was observed (Alizadeh et al., 2021). This supports the current study's findings, where all decellularization methods resulted in varied increases in pore size compared to the control. Undecellularized bovine pericardium initially displayed no visible pores, but decellularization revealed increased pore size. The porous structure of the scaffold arises from voids left by removed cell layers and the breakdown of collagen fibers, without compromising the scaffold's mechanical integrity.

Additional research has identified differences in pore size following decellularization with SDS versus Triton X-100. SEM analysis showed that SDS-treated scaffolds retained dense collagen tissue with smaller pores, whereas Trypsin and Triton X-100 resulted in looser collagen tissue with larger pores. Electron microscopy further confirmed that SDS-treated tissue had denser collagen and smaller pores compared to Trypsin and Triton X-100 (Liao et al., 2008).

Research on biodegradable glass scaffolds coated with two different porogens, albumen and H2O2, revealed distinct differences in pore characteristics. Scaffolds coated with albumen displayed a monomodal pore size distribution around 150 µm and 82% porosity. In contrast, H2O2-treated scaffolds had lower porosity (37%), larger elongated pores, and a multimodal pore size distribution. After 2 weeks, the H2O2-treated glass scaffolds showed significantly reduced porosity and pore interconnectivity compared to the albumen-coated scaffolds, impeding colonization by newly formed tissue. Furthermore, the anisotropic porosity of the H2O2 samples hindered centripetal bone formation, affecting new tissue growth (Sanzana et al., 2014). Similar small pore sizes were observed in bovine pericardium decellularized with H2O2.

Large pore sizes are beneficial for the formation of intra-scaffold vessels post-implantation. However, pores smaller than 400 µm can restrict blood vessel growth. Research on aortic valves decellularized with SDS showed that while the extracellular matrix (ECM) of SDS-treated scaffolds closely resembled the original aortic valve, the dense collagen and small pore sizes could limit the repopulation of Aortic Valve Interstitial Cells (AVIC). If AVIC repopulation is inadequate, the ECM may degrade over time, leading to scaffold failure (Liao et al., 2008).

Another study comparing acellular bovine pericardium (ABP) with synthetic scaffolds found that ABP, with its original ECM, had a lower average pore size (±25 µm) and porosity (60%) compared to synthetic scaffolds (250 µm and 90%). This limited its suitability for cell growth. No significant improvement was observed in cell growth compared to synthetic scaffolds (Dong et al., 2009).

Acellular tissue treated with AcOH had an average pore size of 162.2 ± 24.3 µm and porosity of 94.7 ± 1.8%. AcOH treatment significantly increased pore size, with an average final size of 160 µm. Porosity also varied significantly among cellular tissue (58.1%), acellular tissue (67.3%), and AcOH-treated tissue (94.7%). Larger pore sizes enable the seeding of more stem cells for scaffold formation. Given that mesenchymal stem cells from sources such as bone marrow, placenta, and adipose tissue range from 17-18 µm, pores must be larger than the cells to facilitate effective seeding (Lam & Wu, 2012).

5. Conclusion

This study demonstrated that decellularization methods significantly impact the porosity and pore size of bovine pericardium membranes (BPM), with varying effects across different treatments. Among the methods tested, ASB-16 3% proved to be the most effective, resulting in the highest porosity and largest pore sizes compared to SDS 0.5% and H_2O_2 3%. These findings highlight the importance of selecting appropriate decellularization techniques to optimize scaffold properties for tissue engineering applications. The increased porosity and larger pore sizes achieved with ASB-16 3% enhance the scaffold's ability to support cell infiltration, nutrient exchange, and tissue regeneration, which are crucial for effective integration and function in regenerative therapies. In contrast, SDS 0.5% and H_2O_2 3% showed less pronounced improvements in scaffold characteristics. These results provide valuable insights for refining scaffold preparation methods and advancing their application in pediatric and congenital heart surgeries, where scaffold performance directly influences clinical outcomes.

Author contributions

M.H.R.W. prepared the original draft of the manuscript. H.S. and I.P. contributed to the review and editing of the manuscript.

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Competing financial interests

The authors have no conflict of interest.

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