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Balancing Cellular Removal and Extracellular Matrix Preservation for Cardiac Tissue Engineering

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Abstract : This study evaluates the balance between cellular removal and extracellular matrix (ECM) preservation in cardiac tissue engineering by comparing chemical and physical decellularization methods. Cardiac tissues were treated with chemical agents (sodium dodecyl sulfate and Triton X-100) and physical methods (freeze-thawing and ultrasound). These methods were assessed based on residual cellular content, DNA quantification, ECM structural integrity, and preservation of key ECM components like collagen and glycosaminoglycan (GAG). The results revealed that while chemical methods, particularly SDS, achieved more complete cell removal, they significantly compromised ECM integrity. In contrast, physical methods, such as freeze-thawing, preserved ECM structure more effectively, despite moderate cellular removal. The findings underscore the importance of tailoring decellularization techniques to specific cardiac tissue engineering needs, with chemical methods excelling in cell removal and physical methods offering superior ECM preservation. Future research should aim to optimize these methods to achieve a better balance between decellularization efficiency and ECM integrity.

Keywords : Decellularization, tissue engineering, extracellular matrix, cardiac tissue.

1. Introduction

Tissue engineering aims to create functional tissue replacements to address the limitations of current treatment options for damaged or diseased tissues [1]. A critical step in this process is the decellularization of native tissues, which involves removing cellular components while preserving the extracellular matrix (ECM) structure and composition [2,3]. The ECM provides a scaffold that supports cell attachment, proliferation, and differentiation, making its preservation crucial for the success of engineered tissues.

Various decellularization methods have been developed, broadly categorized into chemical and physical approaches [4]. Chemical methods typically involve the use of detergents and enzymes to lyse and remove cellular components [5,6,7]. Commonly used chemical agents include

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sodium dodecyl sulfate (SDS) and Triton X-100. These agents are effective in removing cells but often result in the disruption of the ECM, potentially compromising the structural and functional integrity of the scaffold [8]. On the other hand, physical methods such as freeze-thaw cycles and ultrasound apply mechanical forces to dislodge cells [9,10]. These methods tend to be gentler on the ECM but may not achieve complete cell removal.

Previous studies have provided valuable insights into the effectiveness of these methods, however, many have primarily focused on simpler tissues with less complex ECM structures, such as dermal or cartilage tissues [11]. The unique challenge presented by cardiac tissues, with their dense and intricate ECM composition, has been less explored, particularly in a comparative context. Furthermore, while some studies have examined individual decellularization methods, few have directly compared the impact of different chemical and physical methods on both cellular removal and ECM preservation within the same tissue type.

This study addresses these gaps by directly comparing the efficiency of chemical (SDS and Triton X-100) and physical (freeze-thawing and ultrasound) decellularization methods in cardiac tissues. Specifically, this research evaluates not only the residual cellular content and DNA quantification but also the structural integrity of the ECM and the preservation of key ECM components such as collagen and glycosaminoglycan (GAG) [12]. By focusing on cardiac tissues, this study provides novel insights into the trade-offs involved in different decellularization methods and offers guidance for optimizing protocols for tissue engineering applications involving complex organs.

2. Materials and Methods

2.1. Tissue preparation

Cardiac tissues were obtained from a commercial slaughterhouse (Daejeon, Republic of Korea), where the animals were slaughtered as part of the standard food production process. Importantly, the animals were not euthanized specifically for the purpose of this research, and no live animals were involved in the experimental procedures. The tissues used in this study were derived from the left ventricular myocardium of pigs. After procurement, the tissues were thoroughly washed with phosphate-buffered saline (PBS; 1X, pH 7.4, Welgene, Gyeongsangbuk-do, Republic of Korea) to remove any residual blood and debris. Uniform samples, approximately 1 cm^3 in size, were then prepared to ensure consistency in subsequent experimental treatments. This approach aligns with ethical guidelines, as the tissues were collected post-mortem and do not require additional animal ethical approval under the IACUC guidelines, given that the tissue collection did not involve any active intervention on living animals.

2.2. Tissue decellularization

2.2.1. SDS Treatment

Cardiac tissue samples were immersed in a 1% (w/v) SDS (Sigma-Aldrich, St. Louis, MO. USA) solution, prepared in deionized water. The samples were incubated at room temperature (22-25° C) with gentle agitation using a laboratory shaker (Shaker Tech, Seoul, Repulic of Korea) set at 50 rpm for 24 hours. After incubation, the samples were extensively washed with 500 mL of PBS for 15 mins per wash cycle, repeated three times, to remove residual SDS until no foam was observed, indicating complete removal of the detergent.

2.2.2. Triton X-100 treatment

Cardiac tissue samples were immersed in a 1% (v/v) Triton X-100 (Sigma-Aldrich) solution, prepared in deionized water. The samples were incubated at room temperature with gentle agitation at 50 rpm for 24 hours. Following incubation, the samples were thoroughly washed with 500 mL of PBS for 15

minutes per wash cycle, repeated three times, to eliminate residual Triton X-100 until the solution was clear of detergent.

2.2.3. Freeze-Thawing

Cardiac tissue samples were subjected to freeze-thaw cycles by freezing at -80° C in a deep freezer (Duksan, Seoul, Republic of Korea) for 24 hours, followed by thawing at room temperature. This process was repeated for a total of three cycles. After the final thaw, the samples were rinsed with 500 mL of PBS to remove lysed cellular debris.

2.2.4. Ultrasound treatment

Cardiac tissue samples were placed in a 100 mL PBS solution and subjected to ultrasound treatment at a frequency of 40 kHz using an ultrasonic bath (Model 3210, Branson Ultrasonics, Danbury, CT, USA) for 30 minutes at room temperature. Post-treatment, the samples were washed with 500 mL of PBS for 15 minutes per wash cycle, repeated three times, to remove dislodged cellular material.

2.3. DNA quantification

Total DNA content was extracted from the decellularized tissue samples using the phenol-chloroform extraction method. In brief, approximately 25 mg of tissue was homogenized in 500 μL of lysis buffer (containing 10 mM Tris-HCl, 100 mM NaCl, 10 mM EDTA, 0.5% SDS, pH 8.0; Sigma-Aldrich) using a mechanical homogenizer (TissueRuptor II, Qiagen, Hilden, Germany). The homogenate was mixed with an equal volume of phenol: chloroform:alcohol (25:24:1, v/v, Sigma-Aldrich) and centrifuged at 12,000 g for 10 minutes at 4℃. The aqueous phase was carefully transferred to a new tube, and DNA was precipitated by adding 2 volumes of cold 100% ethanol and 0.1 volume of 3M sodium acetate (pH 5.2). The DNA pellet was washed with 70% ethanol, air-dried, and resuspended in 50 μL of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0; Sigma-Aldrich).

DNA concentration was determined by measuring the absorbance at 260 nm using a NanoDrop spectrophotometer (ND-1000, Thermo Scientific, Wilmington, DE, USA). The DNA concentration (ng/μL) was calculated automatically by the NanoDrop software using the formula (1):

$$DNA\,concentration = A_{260} \times 50 \times dilution\,factor \quad (1)$$

where A_{260} is the absorbance at 260 nm, which reflects the DNA concentrations, as DNA absorbs light most strongly at this wavelength. The factor of 50 is used because 1 OD at 260 nm equals approximately 50 ng/μL of double-stranded DNA. Since the DNA samples were measured without dilution, the diultion factor is 1.

2.4. Collagen contents

Collagen content in the tissue samples was quantified using a hydroxyproline assay. Tissues were hydrolyzed in 1 mL of 6N HCl (Sigma-Aldrich) at 120℃ for 3 hours in sealed glass vials. The hydrolyzate was neutralized with 1M NaOH and reacted with 0.056M chloramine-T (Sigma-Aldrich) at room temperature for 20 minutes, followed by addition of 1M Ehrlich's reagent (Sigma-Aldrich). Absorbance was measured at 558 nm using a spectrophotometer (UV-1800, Shimadzu, Kyoto, Japan).

2.5. GAG contents

GAG content was quantified using the dimethylmethylene blue (DMMB) assay. Tissue samples were digested with 1 mL of papain enzyme solution (1 mg/mL in 0.1M sodium phosphate buffer, pH 6.0, containing 5 mM EDTA and 5 mM cysteine hydrochloride, Sigma-Aldrich) at 60℃ for 24 hours. The digested samples were mixed with 1 mL of DMMB dye (16 μg/mL, Sigma-Aldrich), and absorbance was measured at 525 nm using a spectrophotometer (UV-1800, Shimadzu).

2.6. Biomechanical properties

Tensile strength and elasticity of the treated tissues were assessed using a universal testing machine (Instron 5943, Norwood, MA, USA). Samples were cut into standardized strips (10 mm width × 20 mm length × 1 mm thickness) and subjected to uniaxial tensile loading at a crosshead speed of 5 mm/min until failure. The tensile strength and elasticity (Young's modulus) were calculated from the stress-strain curves obtained using Bluehill software (Instron).

2.7. Statistical analysis

Each experiment was conducted a minimum of three times, and the resulting data were subjected to one-way ANOVA to identify statistically significant differences among group means. Pairwise comparisons of multiple groups were subsequently performed using Tukey's post-hoc test. Results are expressed as mean ± standard deviation, with "n" representing the number of samples from distinct experiments. Additionally, dot-and-whisker plots displaying averages and ranges were used for data presentation. Statistical significance was established at $p < 0.05$, with different significance levels indicated as follows: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and N.S. for not significant. The analyses were carried out using Excel, KyPlot 6.0 (Kyenslab, Tokyo, Japan), Origin 2018 (OriginLab, Northampton, MA, USA), and SigmaPlot V.12.0 (Systat Software Inc., San Jose, CA, USA).

3. Results and Discussion

3.1. Residual cellular content and DNA quantification

The decellularization efficiency was evaluated by measuring residual cellular content and DNA quantification (Figure 1). SDS-treated tissues exhibited the lowest residual DNA content (average 12 ng/mg of tissue), followed by Triton X-100-treated tissues (average 25 ng/mg of tissue). Physical methods, freeze-thawing, and ultrasound, showed higher residual DNA content (average 60 ng/mg and 75 ng/mg of tissue, respectively). These results indicate that chemical methods, particularly SDS, are highly effective in removing cellular components from cardiac tissues. However, the higher residual DNA in physically treated tissues suggests incomplete cellular removal, highlighting the need for method optimization to improve efficacy.

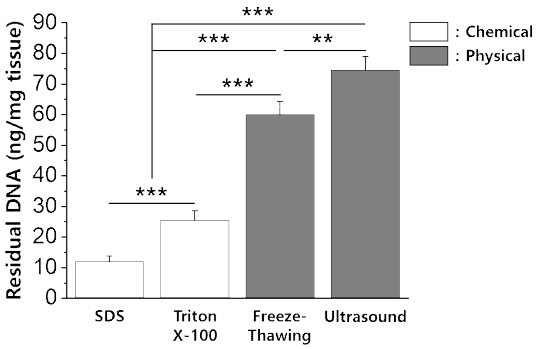

Fig. 1. Comparison of residual DNA amounts after various tissue decellularization. Statistical and p-value analyses are carried out through one-way ANOVA with Tukey's post hoc test. Data = mean ± standard deviation (n = 3). **$p < 0.01$; ***$p < 0.001$ between the lined groups.

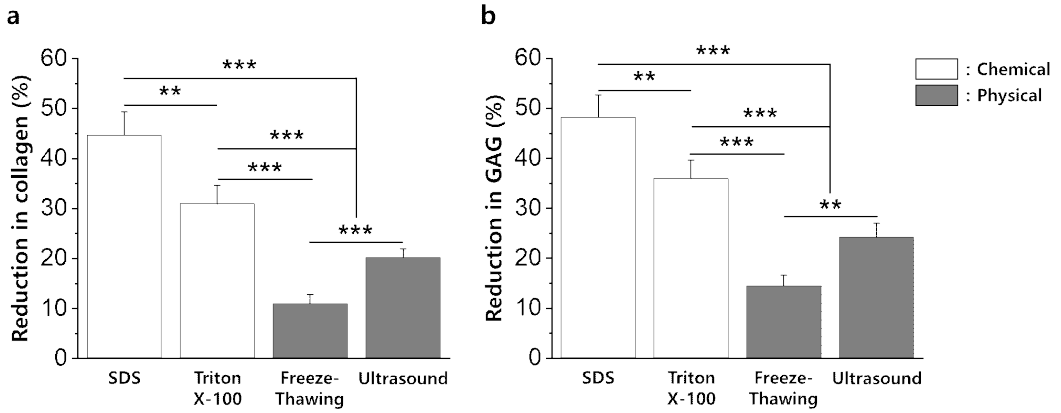

Fig. 2. Comparison of (a) collagen and (b) GAG reduction (%) following different decellularization treatments. Statistical and p-value analyses are carried out through one-way ANOVA with Tukey's post hoc test. Data = mean ± standard deviation (n = 3). $^{**}p < 0.01$; $^{***}p < 0.001$ between the lined groups.

3.2. Preservation of ECM Components

Quantification of key ECM components including collagen (Figure 2a) and GAG (Figure 2b), revealed that SDS-treated tissues had a 45% reduction in collagen content compared to untreated controls, while Triton X-100 caused a 30% reduction. Freeze-thawing preserved collagen more effectively, with only a 10% reduction, and ultrasound treatment resulted in a 20% reduction. GAG content showed a similar trend, with SDS causing a 50% reduction, Triton X-100 a 35% reduction, freeze-thawing a 15% reduction, and ultrasound a 25% reduction. These results underscore the importance of selecting a decellularization method that balances cell removal with the preservation of critical ECM components essential for maintaining the biomechanical properties and functionality of the tissue scaffold.

3.3. Biomechanical Properties

Biomechanical testing revealed that SDS-treated tissues had significantly reduced tensile strength (Figure 3a) and elasticity (Figure 3b), correlating with observed ECM damage. Triton X-100-treated tissues showed moderate reductions in these properties. Freeze-thawing preserved biomechanical properties more effectively, with only slight decreases in tensile strength and elasticity. Ultrasound-treated tissues maintained intermediate biomechanical properties, better than chemical methods but not as well as freeze-thawing. These results suggest that physical decellularization methods, particularly freeze-thawing, are more suitable for applications requiring the retention of the tissue's mechanical integrity.

3.4. Discussion

The comparative analysis of chemical and physical decellularization methods underscores the inherent trade-offs associated with each approach [13]. Chemical methods, particularly SDS, were found to be highly effective in removing cellular components from cardiac tissues [14]. However, this efficiency in decellularization came at a significant cost: the integrity of the ECM was markedly compromised. The structural and compositional damage observed in SDS-treated tissues included collagen fiber fragmentation and substantial loss of GAG, which are critical for maintaining the biomechanical properties and

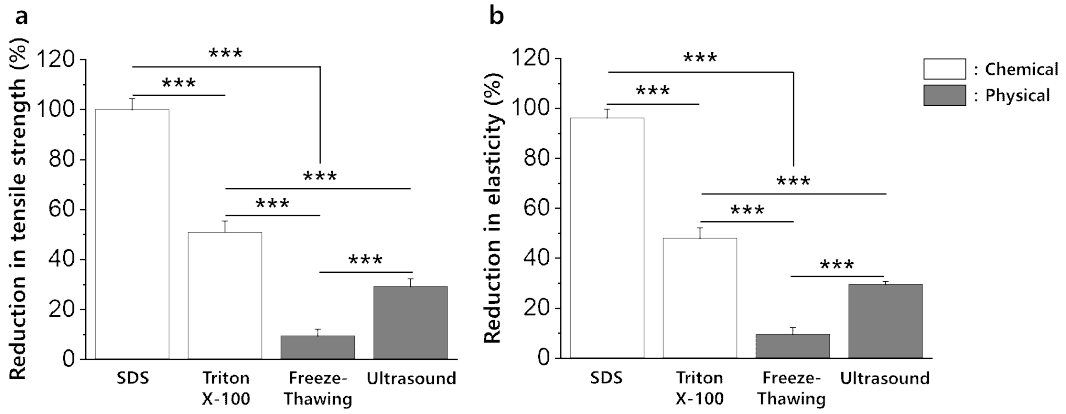

Fig. 3. Reduction in (a) tensile strength and (b) elasticity (%) following different decellularization treatments. Statistical and p-value analyses are carried out through one-way ANOVA with Tukey's post hoc test. Data = mean ± standard deviation (n = 3). ***$p < 0.001$ between the lined groups.

functionality of the tissue scaffold [15]. Triton X-100, another chemical agent, caused less ECM disruption compared to SDS but still resulted in considerable damage [16], indicating that while chemical methods can achieve high levels of cellular removal, they pose substantial risks to ECM integrity.

In contrast, physical decellularization methods demonstrated a more balanced approach. Freeze-thawing, in particular, achieved moderate cellular removal while preserving the ECM structure and composition to a greater extent [17]. This method maintained collagen alignment and the overall architecture of the ECM, with only slight reductions in collagen and GAG content. Ultrasound treatment, though less effective in cell removal compared to freeze-thawing, also preserved ECM integrity better than chemical methods [18], suggesting that physical approaches are more suitable when ECM preservation is a priority.

One of the significant challenges in tissue engineering, especially in the context of tissue remodeling, is the need to maintain the biomechanical and biochemical properties of the ECM while effectively removing cellular debris. This study addresses this challenge by providing a comparative analysis that can guide the selection of decellularization methods based on the specific needs of the tissue remodeling process. For instance, in applications where mechanical strength and ECM composition are critical for the scaffold's functionality, the findings suggest that physical methods like freeze-thawing might be preferable despite the moderate efficacy in cellular removal.

Moreover, the preservation of ECM components such as collagen and GAG is essential for successful tissue remodeling and regeneration, as these elements play a crucial role in cell signaling, migration, and integration within the host tissue [19]. Previous studies have shown that inadequate ECM preservation can lead to poor remodeling outcomes, such as scaffold failure or inadequate tissue integration [20]. By highlighting the strengths and limitations of various decellularization techniques, this study provides a foundation for optimizing scaffolds that can better support tissue remodeling, potentially improving outcomes in clinical applications such as heart valve replacement or myocardial repair.

Future research should focus on evaluating these decellularized scaffolds *in vivo* to

determine their long-term performance in tissue remodeling contexts. For example, it will be essential to investigate how these scaffolds support cellular infiltration, angiogenesis, and functional integration in animal models, as these factors are critical for successful remodeling. Additionally, exploring combined decellularization approaches or sequential treatments could offer new strategies to enhance both ECM preservation and cellular removal, further advancing the field of tissue engineering.

Despite the valuable insights gained, this study has certain limitations that should be acknowledged. The comparative analysis was limited to a specific set of decellularization methods and focused primarily on cardiac tissues, which may not fully represent other tissue types with different structural and compositional characteristics. Additionally, while the study provides significant findings on the trade-offs between cellular removal efficiency and ECM preservation, the long-term functional outcomes of these decellularized tissues *in vivo* were not assessed. Future research should expand the scope of decellularization methods and tissue types, and evaluate the long-term biological and functional performance of the decellularized scaffolds in relevant animal models. This will help to optimize decellularization protocols further and ensure the development of tissue scaffolds that are both structurally sound and functionally viable for clinical applications.

4. Conclusions

This study compared chemical and physical decellularization methods for cardiac tissues, revealing that while chemical methods like SDS are highly effective in removing cellular components, they significantly compromise the ECM, crucial for tissue functionality. Conversely, physical methods, particularly freeze-thawing, better preserve ECM integrity but are less efficient in cellular removal. The choice of decellularization method should therefore be based on the specific application needs, with chemical methods suited for scenarios requiring complete decellularization and physical methods favored when ECM preservation is essential. Future research should focus on optimizing these methods to balance cellular removal with ECM preservation, thereby enhancing the development of functional tissue scaffolds for regenerative medicine.

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