Decellularization of Sheep Heart Tissue: A Biocompatible Platform for Tissue Regeneration

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Abstract

Tissue engineering (TE) is an evolving discipline aimed at the repair, replacement, or regeneration of injured tissues and organs through the integration of biomaterials, living cells, and bioactive substances. Among the various strategies, the use of acellular scaffolds derived from extracellular matrix (ECM) has shown great promise. These scaffolds, which are devoid of cellular components, retain the structural and biochemical cues necessary for supporting cell adhesion, proliferation, and differentiation. Their inherent biocompatibility and bioactivity make them highly suitable for tissue regeneration applications. In the this study, we aimed to develop and evaluate a biocompatible scaffold from sheep heart tissue using a chemical decellularization process. The efficiency of decellularization and preservation of the ECM were assessed through histological staining, DNA quantification, biochemical assays, and scanning electron microscopy (SEM). Results confirmed the effective removal of cellular material while maintaining the ECM architecture. To investigate the scaffold's biocompatibility, adipose-derived mesenchymal stem cells (Ad-MSCs) were seeded onto the decellularized matrix. Cell viability and adhesion were evaluated using MTT assays and SEM imaging. The scaffold supported a high rate of cell viability (88%) and demonstrated favorable cell attachment. These findings highlight the potential of decellularized sheep heart tissue as a reliable, bioactive, and structurally supportive platform for stem cell integration and tissue regeneration. Given its mechanical strength, preserved ECM composition, and excellent

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cytocompatibility, this scaffold may serve as a promising candidate for applications in regenerative medicine and TE, particularly in cardiac and soft tissue repair.

Keywords: Tissue Engineering, Adipose-Derived Stem Cells (ADSCs), Heart Tissue, Decellularization, Cardiovascular Regeneration

1. Introduction

Cardiovascular diseases remain a leading health concern, significantly impacting both life expectancy and quality of life despite advances in medical treatments and surgical interventions. Recognized as an emerging epidemic since the mid-1990s, heart failure rates have escalated due to an aging and growing global population (1,2). Current therapeutic approaches include organ transplantation, surgical reconstruction, mechanical devices, and metabolic product injections. However, these methods face challenges, such as immune rejection, the necessity for lifelong immunosuppressive therapy, and complications arising from device malfunctions or infections (3,4).

In recent times, tissue engineering has gained attention as a potential solution for treating cardiovascular diseases. Decellularized extracellular matrix (d-ECM) is central to regenerative medicine and cardiac TE, to repair, replace, and regenerate damaged or defective heart tissue. Developing a three-dimensional scaffold with properties that can modulate cellular activity and behavior for effective regeneration requires carefully balancing three primary components: cells, scaffolds, and biosignals (5–7). The extracellular matrix (ECM), produced by cells, forms a complex three-dimensional network of macromolecules, including structural proteins like collagen, polysaccharides, enzymes, and growth factors. This network not only defines the tissue’s mechanical, chemical, and physical properties but also influences cellular function (8–11).

The cardiac extracellular matrix (ECM) consists of polysaccharides and glycosylated proteins, including fibrous components such as laminin, fibronectin, and tenascin, each fulfilling a distinct function. This natural ECM, produced by the heart's cells, facilitates communication between cells and their surroundings, influencing vital processes such as cell movement, development, and death. However, replicating this complex ECM in a laboratory setting is challenging. To address this, researchers use tissue decellularization, a technique that removes cells but preserves the ECM's structure, reducing the risk of immune rejection (4,6,12).

Decellularization protocols often use a enzymatic, chemical, and physical techniques. Standard physical methods include freeze-thaw cycles and mechanical forces to break down cell membranes; however, these

can damage the ECM due to ice crystal formation or mechanical stress. Enzymatic methods, which use nucleases and proteases to degrade cellular components such as RNA, DNA, and proteins, may also disrupt the ECM. Chemical methods widely involve the use of non-ionic or ionic detergents for cell removal. For example, Triton X-100 (non-ionic) preserves protein-protein interactions, while SDS (ionic) effectively solubilizes cell membranes, though prolonged exposure can alter ECM structure. Tailoring decellularization protocols to each tissue type is essential to maintain ECM integrity across different organs (13–15).

In this study, we evaluated chemical methods for preparing decellularized sheep heart scaffolds, focusing on the importance of optimized washing steps. By comparing the molecular and cellular properties of the prepared scaffolds to untreated controls, we aimed to verify the effectiveness of the decellularization process in preserving the extracellular matrix (ECM) structure while ensuring complete cell removal. We conducted detailed analyses on the samples to confirm ECM integrity and the absence of cellular remnants. Additionally, we performed MTT assays to assess the in vitro biocompatibility of the decellularized scaffolds, examining cell viability and survival when cultured on the ECM. This comprehensive approach ensures that the decellularized ECM provides a safe and supportive environment for future cell seeding and tissue engineering applications.

2. Methods and material

2.1. Ethics approval

All experimental procedures were performed in accordance with the European Union Council Directive of November 24, 1986, and were approved by the Ethics Committee of the University of Mohaghegh Ardabili (IR.UMA.REC.1400.084).

2.2. Scaffold Preparation

For this study, sheep heart tissue (n = 3) was harvested immediately after slaughter from a local slaughterhouse, following ethical protocols. The heart tissue, rich in vascular structures, was selected for its suitability for decellularization. A chemical decellularization approach was utilized, involving 0.5% sodium deoxycholate (Sigma-Aldrich), 0.5% sodium dodecyl sulfate (SDS, Sigma-Aldrich), and phosphate-buffered saline (PBS, Gibco). The heart tissue was sectioned into 3 mm thick slices, measuring approximately 2x2 cm. These sections were washed with distilled water, followed by three 10-minute washes in PBS to remove residual blood and debris. The tissue samples were then immersed in a solution containing 0.5% SDS and 0.5% sodium deoxycholate for 24 hours to facilitate cellular removal. Following decellularization, the tissues underwent six successive washes with PBS, spaced 12 hours

apart, to eliminate any remaining detergents and cellular debris. After decellularization, the samples and control tissues were stored in PBS and examined morphologically to assess ECM integrity and confirm effective cell removal.

2.3. The Histological Analysis

To assess the histological characteristics of the decellularized scaffolds, including cell removal and extracellular matrix preservation, both the control (n = 5) and scaffold (n = 5) samples were fixed in 10% formalin (Sigma-Aldrich). Each sample was sectioned into 5-micrometer slices and embedded in paraffin. Hematoxylin and eosin staining (H&E staining kit, Sigma-Aldrich) was performed on the sections to evaluate the efficiency of cell elimination and the structural integrity of the ECM following decellularization. The effectiveness of DNA removal was assessed using 4',6-diamidino-2-phenylindole staining (DAPI staining kit, Sigma-Aldrich). DAPI binds to adenine-thymine (A-T) rich regions of double-stranded DNA, allowing for clear visualization of any remaining nuclei in the decellularized samples. To verify the preservation of collagen filaments, the most abundant protein filaments in the cardiac ECM, Masson's trichrome staining (Masson's trichrome staining kit, Sigma-Aldrich) was employed. This staining method highlights the collagen structures within the scaffold, providing a detailed assessment of ECM integrity post-decellularization.

2.4. Ultrastructural Evaluation

The ultrastructural preservation of the decellularized scaffolds was evaluated by SEM (SEM, SU4800, Japan). Samples from both the control group (n = 4) and the decellularized scaffolds (n = 4) were first fixed in 2.5% glutaraldehyde (Sigma-Aldrich) for 24 hours. Following fixation, the samples were dehydrated through graded ethanol (Sigma-Aldrich) series (30%, 50%, 70%), with a final immersion in 100% ethanol. Once fully dehydrated, the samples were air-dried to ensure complete moisture removal. The dried samples were coated with a thin layer of gold. The ultrastructural features of the scaffolds were then captured using SEM, allowing for a detailed evaluation of the scaffold's surface morphology and structural preservation following the decellularization process.

2.5. DNA Quantification

To quantify the DNA content, 20 mg of both control (n = 3) and decellularized (n = 3) tissue samples were precisely weighed. The tissue was then digested by adding 5 μL of proteinase K (Qiagen) per milliliter of digestion buffer. The scaffold and control samples were homogenized and incubated in a bain-marie at 55°C overnight.

After incubation, the supernatant was carefully transferred to fresh tubes. Sodium acetate (Merck) was added to each sample, thoroughly mixed by inversion, and incubated at −20°C for 15 min to facilitate protein and cell debris precipitation. The samples were centrifuged at maximum speed for 2 minutes using an Eppendorf centrifuge, after which the supernatant was carefully collected into fresh tubes.

Two volumes of 98% ethanol were added to the supernatant to precipitate nucleic acids, followed by gentle inversion to mix. The samples were incubated at −20°C for 15 min, then centrifuged at 6000 rpm for 20 minutes at 4°C. Then were washed with 98% ethanol and twice with 70% ethanol for air-dry.

The dried pellets were resuspended in injection-grade water, and the DNA concentration in the decellularized and control samples was measured using a Nanodrop spectrophotometer (Termo scientific USA-Termo One C) at a wavelength of 260 nm, with results expressed in ng/mg (16).

2.6. Glycosaminoglycan (GAG) Quantification

A Glycosaminoglycan Quantification Kit (Kazist, Iran) was used to evaluate the preservation of glycosaminoglycans (GAGs) in the decellularized scaffold samples. Scaffold samples (n = 3) and control samples (n = 3), each weighing 15 mg, were prepared and placed in 1.5 mL microtubes. Following the kit's instructions, 400 µL of enzyme solution was added to each microtube, and the samples were incubated at 65 °C in a bain-marie for 16 hours.

After incubation, the microtubes were individually weighed for alignment in the centrifuge and subsequently centrifuged at 6000 rpm for 15 minutes at 4°C. The supernatant was then carefully transferred to new microtubes, and 50 µL of a protein precipitant was added, followed by an additional centrifugation step under the same conditions. After centrifugation, 30 µL of each sample was pipetted into the wells of a 96-well plate, followed by the addition of 200 µL of GAG reagent to each well. Optical absorbance was measured at 560 nm using an ELISA reader (BioTek EIA reader), according to the manufacturer's protocol. A standard curve was created using Excel software, and the GAG concentration in the samples was calculated in mg/µg.

2.7. Total protein quantification

For total protein quantification, Coomassie Brilliant Blue reagent (Sigma-Aldrich) was prepared by dissolving 10 mg of the dye in 5 mL of 95% ethanol, followed by the addition of 10 mL of 85% orthophosphoric acid (Sigma-Aldrich). The mixture was then diluted to a final volume of 100 mL with distilled water and stored refrigerated. A bovine serum albumin (BSA) standard solution (Sigma-Aldrich) was prepared by dissolving 1 mg of BSA in distilled water to a total volume of 10 mL, yielding a concentration of 10 mg/mL. For experimental analysis, 0.2 g of decellularized scaffold (n = 6) and control

(n = 6) tissue were weighed and washed with PBS. The samples were centrifuged at 4000 rpm, and the buffer was replaced after each spin. In the next step, 150 µL of PBS containing 0.1 M DTT (Sigma-Aldrich) was added to each sample, and they were subjected to three freeze-thaw cycles using liquid nitrogen to disrupt the tissue. After each thaw, samples were vortexed to ensure complete tissue homogenization. The supernatant was collected for protein analysis following centrifugation at 1200 rpm and 4°C.

Protein quantification was performed by mixing 20 µL of each supernatant with 180 µL of Bradford reagent in a 96-well plate. Absorbance was measured at 595 nm using an ELISA reader, and protein concentrations were determined by comparing the readings to a BSA standard curve prepared in Excel.

2.8. Cell culture

Adipose-derived mesenchymal stem cells (Ad-MSCs), obtained from the Iranian Biological Resource Center, were incubated (Memmert IPP750plus) at 37 °C with 95% humidity and 5% CO2. After 24 hours, cells were passaged using 0.25% Trypsin-EDTA (Gibco) and divided into three flasks. The flasks were monitored daily, with the medium replaced every 48 hours to maintain optimal conditions and remove dead cells or debris. The cells were grown in a low-glucose DMEM medium (Gibco), supplemented with 20% FBS (Gibco) and 1% penicillin-streptomycin solution (Sigma) to ensure optimal proliferation and sterility. Adipose-derived mesenchymal stem cells (Ad-MSCs) at the second passage were utilized for biocompatibility testing. Cell viability was assessed via the MTT assay, and cell adhesion was examined using scanning electron microscopy (SEM), providing valuable information on scaffold compatibility (17).

2.9. Assessment of Cell Viability

The cell viability assay is based on reducing MTT tetrazolium salt (Sigma, MO, US) to purple formazan crystals by the oxidoreductase enzyme present in the mitochondria of viable cells. This color change indicates cellular viability and the functional presence of the oxidoreductase enzyme.

For the cell viability assay, circular segments of the decellularized heart scaffolds, each measuring 0.5 cm in diameter, were prepared. These scaffolds were sterilized using a 0.5% penicillin-streptomycin solution in phosphate-buffered saline (PBS), followed by ultraviolet (UV) irradiation for 20 minutes. To verify sterility, scaffolds were incubated in DMEM medium enriched with 10% FBS for 24 hours at 37°C , 5% CO_2. After confirming sterility, the scaffolds were transferred into a 96-well culture plate and seeded with Ad-MSCs at a density of 10^4 cells per scaffold. For the control group, an equal number of Ad-MSCs were cultured directly in wells without scaffolds. The plates were then incubated at 37°C, 5% CO_2 for 24 hours.

Following incubation, 20 µL of MTT reagent was added to each well, and the plates were shielded with aluminum foil and incubated for another 4 hours. After this, the supernatant containing the MTT reagent was removed, and 200 µL of dimethyl sulfoxide (DMSO; Merck) was introduced to dissolve the resulting formazan crystals. The plates were then left for 10 minutes before absorbance was measured at 630 nm using a microplate reader to assess cell viability. The percentage of viable cells was calculated relative to the control (monolayer culture) using the formula:

Cell viability (%) = (Mean OD of sample / Mean OD of control) × 100

2.10. Assessment of Cell Adhesion

After 24 hours of stem cell culture on the scaffolds, tissue fixation was initiated using a 2.5% glutaraldehyde solution in a saline buffer adjusted to a pH of 7.4. The culture medium was carefully removed, and the glutaraldehyde solution was applied to the tissue samples, which were then incubated for 48 hours to ensure effective preservation. To achieve thorough fixation of cellular and scaffold structures, this step was repeated, followed by an additional 24-hour immersion in the fixative.

Subsequent to the fixation process, the glutaraldehyde solution was drained, and the samples were rinsed three times with the same solution to remove excess fixative. The tissues then underwent a graded ethanol dehydration sequence, with sequential immersions in 30%, 50%, and 70% ethanol for 15 minutes each. The complete dehydration was achieved through a final immersion in 100% ethanol. The samples were then allowed to air-dry completely.

Once dried, a thin layer of gold was applied to the samples to enhance surface conductivity, a necessary step for imaging. The prepared samples were analyzed using scanning electron microscopy (SEM) at an accelerating voltage of 15 kV and a magnification of 500x to examine detailed surface morphology and the interactions between the scaffolds and the seeded cells.

2.11. Data Analysis

Results are expressed as mean ± SEM. Statistical differences among multiple groups were assessed using one-way analysis of variance (ANOVA), while comparisons between two groups were made using the t-test. A p-value below 0.05 ($p < 0.05$) was considered indicative of statistical significance. All statistical tests and graphical representations were conducted using GraphPad Prism version 8.2.1.

3. Results

3.1. Scaffold Characterization

DAPI (4′,6-diamidino-2-phenylindole) staining was used to detect residual nuclear material. DAPI, a fluorescent dye that binds specifically to adenine-thymine-rich regions of DNA, is often used in fluorescence microscopy. Control samples exhibited bright blue fluorescence, indicating nuclear presence, whereas the absence of fluorescence in treated samples confirmed the effective elimination of nuclear material during decellularization (Fig. 1 A, B).

To further examine the extracellular matrix (ECM), Masson's trichrome staining was performed to highlight collagen fibers, a primary structural element of the ECM. Results showed that the collagen network remained intact, suggesting that the decellularization process preserved ECM architecture without significant disruption (Fig. 1 C, D).

Additionally, Hematoxylin and eosin (H&E) staining confirmed the absence of cellular material within the scaffolds. H&E is a widely used histological technique, where eosin, an acidic dye, stains the cytoplasm and other acidophilic components pink, and hematoxylin, a basic dye, stains basophilic structures such as DNA and RNA blue or purple. In the analyzed samples, cytoplasm appeared pink and nuclei purple, confirming successful cell removal from the scaffolds (Fig. 1 E, F).

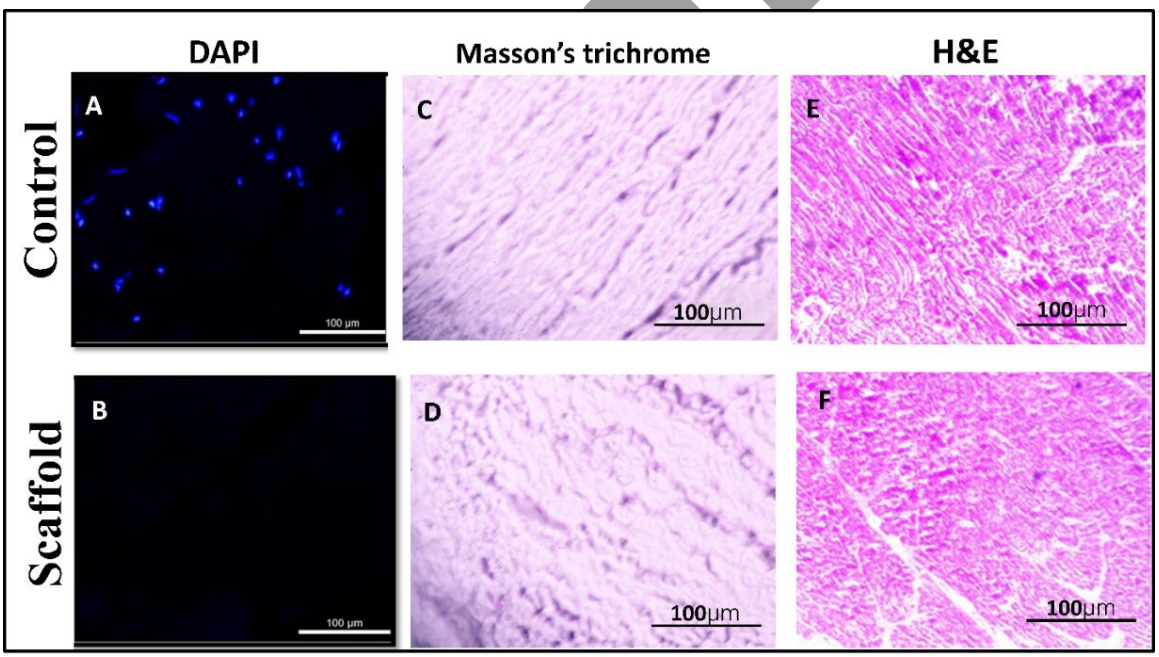

Fig. 1. Evaluation of cellular content removal and extracellular matrix preservation in decellularized sheep-heart scaffolds. (A, B) DAPI staining of nuclei, where A shows the control sample with visible nuclear staining (blue fluorescence) and B shows the scaffold after decellularization, indicating the absence of nuclear material. **(C, D)** Masson's trichrome staining, highlighting collagen fibers. C depicts the control sample, while D shows the

scaffold, confirming the retention of collagen structures with no observable cellular content. **(E, F)** Hematoxylin and eosin (H&E) staining, demonstrating the general tissue morphology. E illustrates the presence of cells in the control sample, while F shows the scaffold with the removal of cells, yet preservation of the extracellular matrix. Scale bars: 100 μm.

3.2. DNA Quantification

The DNA quantification data from the control tissue sample indicated a DNA content of 3.33 ± 0.58 ng/μl, reflecting the presence of substantial cellular and nucleic acid material. In contrast, the scaffold tissue sample demonstrated a significant reduction in DNA content, measuring only 0.56 ± 0.58 ng/μl ($P = 0.0089$). This substantial decrease highlights the efficacy of the decellularization process, which successfully removed nearly all cellular components and nucleic acid residues (Fig. 2 A).

3.3. GAG and Total Protein Quantification

Quantifying glycosaminoglycans (GAGs) and total protein content is crucial for scaffold characterization, given their roles in cytokine production, activation of signaling molecules, providing extracellular matrix elasticity and strength, and facilitating nutrient and metabolite diffusion. The control tissue samples, contained 3.01 ± 0.38 μg/mg of GAGs, reflecting the intact extracellular matrix structure. The decellularized scaffold sample exhibited a slightly lower GAG content of 2.28 ± 0.38 μg/mg, with no significant reduction observed. This minor decrease indicates substantial GAG retention following the decellularization process (Fig. 2B).

Total protein quantification of the control tissue revealed a protein content of 173.1 ± 1.696 μg/mg. In comparison, the scaffold sample indicated a comparable protein content of 178.2 ± 1.696 μg/mg, indicating no significant difference between the two. These results suggest that the decellularization method preserved a considerable amount of protein content within the scaffold tissue (Fig. 2C).

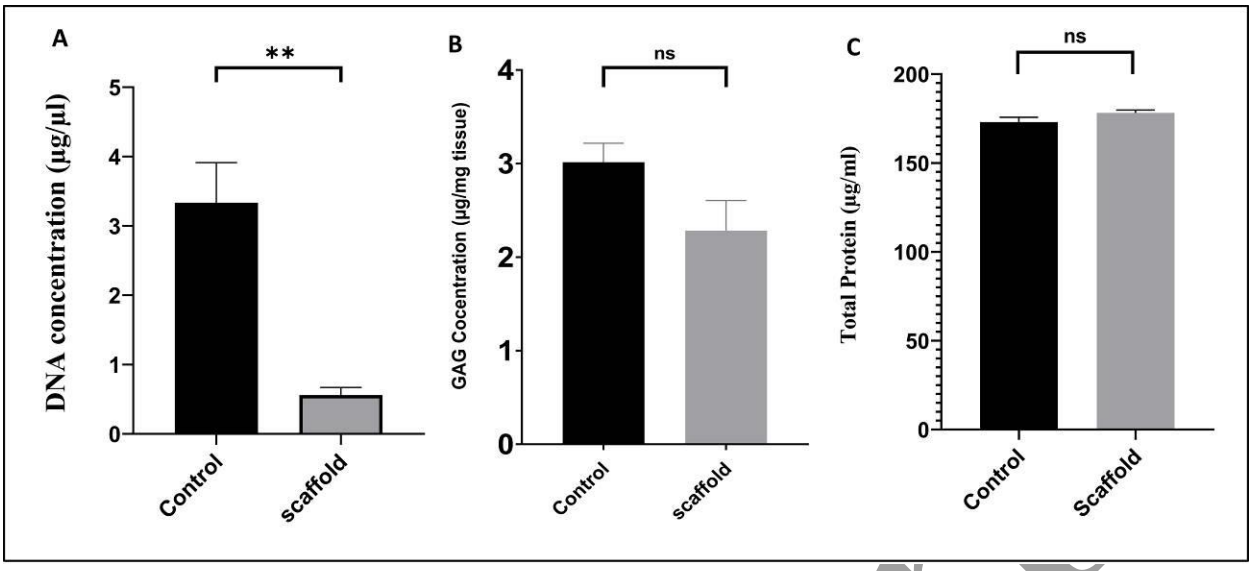

Fig. 2. Quantitative analysis of DNA, glycosaminoglycan (GAG), and total protein content in control and decellularized sheep-heart scaffolds. (A) DNA concentration was measured in three independent experiments (n=3). The results hows a significant reduction in the scaffold group compared to the control, indicating effective removal of cellular DNA during the decellularization process (** $P < 0.01$). **(B)** GAG concentration reveals no significant difference (ns) between the control and scaffold groups, suggesting preservation of glycosaminoglycans. **(C)** Total protein content analysis indicates no significant difference (ns) between control and scaffold groups, confirming the retention of key protein components. Data are presented as mean ± SEM.

3.4. Biocompatibility Evaluation

Biocompatibility of the decellularized sheep-heart scaffolds was evaluated by performing an MTT assay to measure the viability of AD-MSCs seeded onto the scaffolds at 24, 48, and 72 hours after seeding. The results demonstrated an increase in cell viability over time, with 83% viability at 24 hours, 85% at 48 hours, and 88% at 72 hours. These outcomes indicated that the scaffolds provided effective support for cell attachment, proliferation, and growth. Moreover, no significant differences in cell viability were observed when compared to the control group (monolayer cell culture in media), underscoring the high biocompatibility of the decellularized scaffolds. These findings demonstrated the potential of these scaffolds as a supportive and conducive matrix for stem cell activity, which is critical for applications in tissue engineering (Fig. 3).

Further evidence from scanning electron microscopy (SEM) imaging demonstrated strong adhesion of adipose-derived mesenchymal stem cells (AD-MSCs) to the surfaces of the decellularized scaffolds (Fig.

4). This observation underscores the scaffold's capacity to support effective cell attachment, highlighting its potential as a highly biocompatible environment conducive to stem cell development, proliferation, and sustained viability.

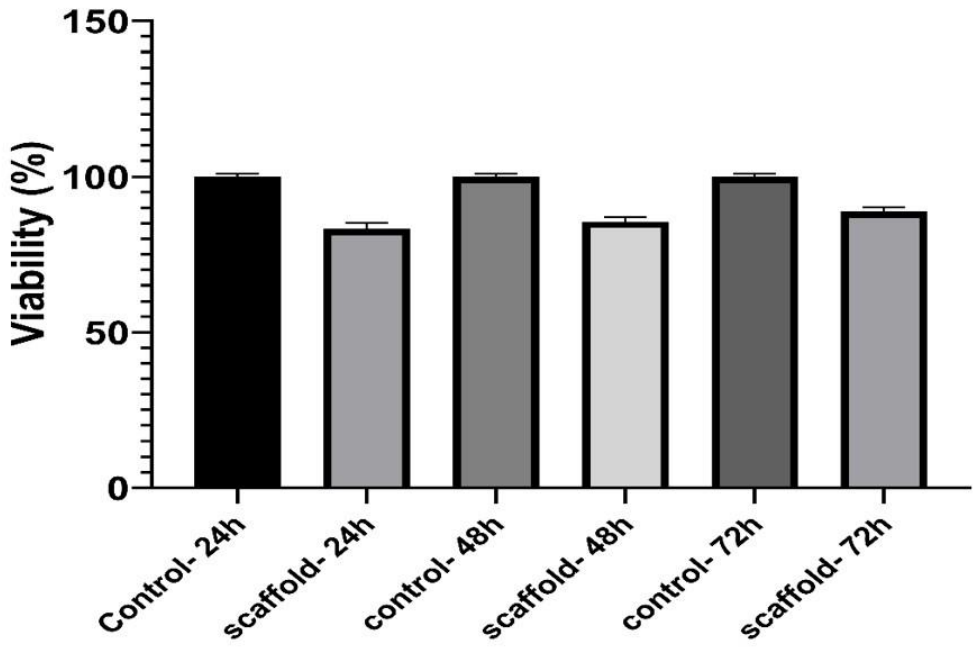

Fig. 3. Biocompatibility assessment of decellularized sheep-heart scaffolds using the MTT assay. The viability of adipose-derived mesenchymal stem cells (AD-MSCs) seeded on the decellularized heart scaffolds was measured at 24, 48, and 72 hours post-seeding. The results showed an increase in cell viability over time, with 83% at 24 hours, 85% at 48 hours, and 88% at 72 hours. No significant differences in cell viability were observed when compared to the control group (monolayer cell culture in media).

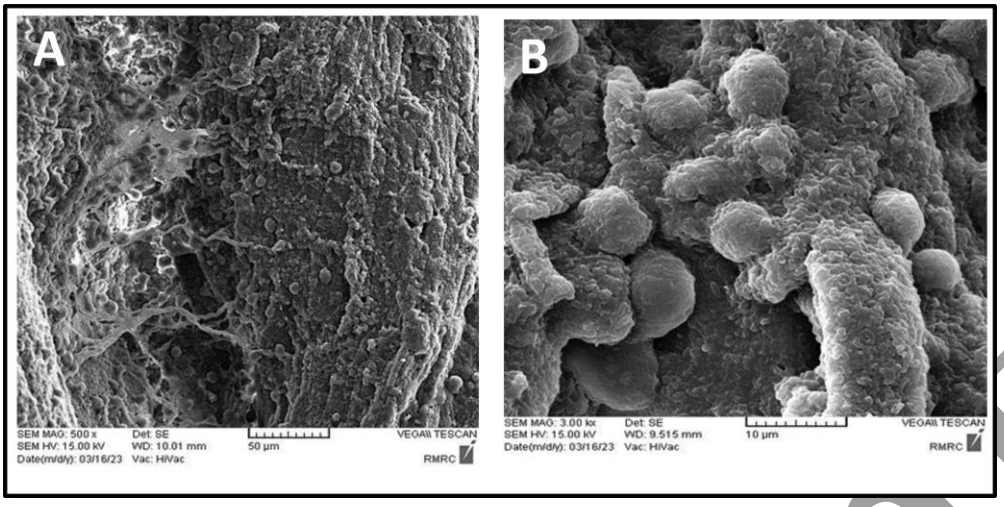

Fig. 4. Scanning electron microscopy (SEM) images showing the adhesion of adipose-derived mesenchymal stem cells (AD-MSCs) on the surface of decellularized sheep-heart scaffolds. (A) SEM image taken 24 hours after cell seeding at 500x magnification, with a scale bar of 50 µm, demonstrating initial cell attachment on the scaffold surface. (B) SEM image taken 48 hours after cell seeding at 500x magnification, with a scale bar of 10 µm, showing enhanced cell adhesion and spreading. Both images were captured at a voltage of 15 kV.

4. Discussion

In cardiac tissue engineering and regenerative medicine, the utilization of decellularized extracellular matrix (d-ECM) has garnered significant attention as a viable strategy for the repair, replacement, and regeneration of damaged or dysfunctional cardiac tissue. Central to this approach is the fabrication of a three-dimensional scaffold designed to modulate cellular behavior and promote functional tissue restoration. The success of this process hinges upon the synergistic integration of three fundamental components: the scaffold framework, appropriate cellular populations, and bioactive signaling factors (18,19). The ECM is a sophisticated and dynamic three-dimensional scaffold, primarily made up of structural proteins like collagen, alongside various enzymes, signaling molecules, and polysaccharides. This matrix is synthesized and released by local cells, and it creates a unique microenvironment that facilitates cell attachment, growth, movement, and specialization. Furthermore, the ECM contributes to the tissue's unique physical, chemical, and biomechanical features that are crucial for its proper function (20,21). The cardiac ECM predominantly consists of glycosylated proteins and polysaccharides, encompassing fibrous proteins such as laminin, tenascin, and fibronectin, each of which plays distinct and critical roles in maintaining the structural and functional integrity of the tissue microenvironment (22).

The native ECM, synthesized by resident cardiac cells, provides a biologically active and naturally functionalized scaffold that facilitates critical cell–cell and cell–matrix interactions. These interactions are essential for regulating cellular processes such as migration, differentiation, and apoptosis, ultimately influencing tissue homeostasis and repair (23). Due to the intricate biochemical and structural composition of natural ECM, replicating its full complexity through synthetic means remains a significant challenge. Consequently, a growing body of research has turned toward the use of decellularized tissues to generate biologically derived ECM scaffolds. These scaffolds aim to preserve the native three-dimensional architecture and biochemical cues of the ECM while effectively removing immunogenic cellular components to minimize the risk of immune rejection in regenerative applications (24).

However, several challenges remain in optimizing the decellularization and recellularization processes. Complete removal of cellular antigens is necessary to minimize immune responses, while maintaining ECM integrity is crucial for mechanical stability and biological functionality. Additionally, the repopulation of decellularized matrices with appropriate cell types, including endothelial cells, cardiomyocytes, and supporting stromal cells, remains a complex and tightly regulated process. Advancements in stem cell technologies, bioreactor systems, and gene editing tools may help overcome these limitations and enhance the functionality of bioengineered cardiac tissues (25).

Effective decellularization protocols commonly integrate enzymatic, chemical, and physical methods to ensure thorough cellular removal while minimizing damage to the extracellular matrix (ECM). Physical techniques—such as freeze-thaw cycles and mechanical agitation—are typically employed as supportive measures to facilitate cell lysis but can compromise ECM integrity due to ice crystal formation and mechanical stress (26). Enzymatic approaches, utilizing nucleases and proteases, are efficient at degrading nucleic acids and cellular proteins; however, if not precisely controlled, they may inadvertently disrupt ECM components (27). Chemical methods remain the most widely applied, particularly those employing detergents such as Triton X-100 and SDS, due to their effectiveness in solubilizing cell membranes. Nevertheless, extended exposure to these detergents—especially SDS—can result in protein denaturation and ECM degradation . To preserve the biochemical and mechanical properties of the matrix, it is therefore advisable to limit the duration of detergent exposure (28).

In this study, we adopted a chemical decellularization strategy using sheep heart tissue, leveraging the anatomical and physiological similarities between sheep and humans. Compared to smaller animal models, sheep hearts offer a more clinically relevant platform for translational research, and their comparable size facilitates the development of scalable tissue engineering protocols (29,30). Moreover, we propose an optimized decellularization protocol for cardiac tissue based on a synergistic combination of Triton X-100 and SDS, applied over a shortened incubation period to minimize ECM disruption.

The efficacy of the decellularization protocol was validated through both histological and biochemical assessments. DAPI staining confirmed the complete absence of nuclear material, while hematoxylin and eosin (H&E) staining demonstrated effective removal of cellular components with preservation of the extracellular matrix (ECM) architecture. Masson's trichrome staining further revealed intact collagen fibers, underscoring the structural integrity of the decellularized scaffold. Biochemical analyses corroborated the histological findings. Quantification of glycosaminoglycans (GAGs) showed a slight, non-significant reduction in content, indicating that essential biomechanical characteristics were largely preserved. Total protein analysis similarly revealed no significant differences between native and decellularized tissues, highlighting the protocol's ability to maintain key structural proteins critical for supporting recellularization and tissue regeneration. These findings are consistent with previous studies demonstrating the potential of decellularized scaffolds to support recellularization and functional tissue regeneration. For instance, in the study by Lu et al. (2013), the decellularized heart matrix provided a biologically relevant environment that facilitated the engraftment, differentiation, and organization of human cardiovascular progenitor cells, ultimately promoting the formation of functional cardiac tissue (31).

Biocompatibility is crucial for scaffold evaluation, and our results showed that the decellularized sheep-heart scaffolds provided a conducive environment for cell viability and growth. The MTT assay revealed no significant difference in stem cells viability between the scaffold and control samples. Furthermore, SEM imaging confirmed successful cell attachment, reinforcing the scaffold’s potential for supporting cell adhesion, proliferation, and differentiation. These findings indicate that our decellularized scaffolds are well-suited for use in cardiac tissue engineering. This results are consistent with previous studies. Ott et al. (2008) were pioneers in demonstrating the functional recellularization of decellularized cardiac scaffolds, achieving pump-like activity with reseeded murine heart tissues (32). Weymann and colleagues expanded this work by developing decellularized porcine heart scaffolds, preserving crucial ECM features and achieving partial recellularization. Although challenges such as incomplete endothelialization remain, advancements continue to refine decellularization and recellularization strategies (33).

Nevertheless, certain limitations must be acknowledged. While detergents like SDS are highly effective at removing cellular content, they can disrupt the ECM ultrastructure if not carefully controlled. Optimizing detergent concentration and exposure time is crucial to minimizing this damage. research should explore alternative decellularization techniques. Additionally, we did not perform mechanical testing in this study, but we recommend that future research include such assessments. In conclusion, our study demonstrates that decellularized sheep-heart scaffolds retain essential ECM components, exhibit high biocompatibility, and provide a robust platform for cardiac tissue engineering. Continued research into scaffold

optimization and effective recellularization strategies will be crucial for advancing these constructs toward organ repair and regeneration, bringing them closer to clinical application.

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Authors' contributions

A. Ab collaborated on the conception, design, and planning of the study. A. Ab, A. As conducted the literature review, while Z. Ch , M. J, S. Z, reviewed the selected studies, assessed their quality, and gathered the required data. S. Z, A. Ab, A. As, M. J, Z. Ch, H A.G performed the statistical analyses and drafted the initial manuscript. All authors contributed to the interpretation of the results, provided critical revisions, and approved the final version of the manuscript for publication.

Conflict of Interest

The authors declare no conflict of interest in this study

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This research received no specific grant from any funding agency in the public, commercial, or not-for-profit sectors.

Data availability

All data generated or analyzed during this study are included in this article. Further enquiries can be directed to the corresponding author.

Ethics

All experimental procedures were performed in accordance with the European Union Council Directive of November 24, 1986, and were approved by the Ethics Committee of the University of Mohaghegh Ardabili (IR.UMA.REC.1400.084).

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