# Characterization of a Decellularized Sheep Pulmonary Heart Valves and Analysis of their Capability as a Xenograft Initial Matrix Material in Heart Valve Tissue Engineering

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Posted Date: 22 May 2023

doi: 10.20944/preprints202305.1473.v1

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*Article*

# Characterization of a Decellularized Sheep Pulmonary Heart Valves and Analysis of Their Capability as a Xenograft Initial Matrix Material in Heart Valve Tissue Engineering

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**Abstract:** In order to overcome the disadvantages of existing treatments in heart valve tissue engineering, decellularization studies are carried out. The main purpose of decellularization is to eliminate the immunogenicity of biologically derived grafts and to obtain a scaffold that allows recellularization while preserving the natural tissue architecture. SD and SDS are detergent derivatives frequently used in decellularization studies. The aim of our study is to decellularize the pulmonary heart valves of young Merino sheep by using low-density SDS and SD detergents together, and then to perform their detailed characterization to determine whether they are suitable for clinical studies. Pulmonary heart valves of 4-6 month old sheep were decellularized in detergent solution for 24 hours. The amount of residual DNA was measured to determine the efficiency of decellularization. Then, the effect of decellularization on the ECM by histological staining was examined. In addition, the samples were visualized by SEM to determine the surface morphologies of the scaffolds. Uniaxial tensile test was performed to examine the effect of decellularization on biomechanical properties. The results showed DNA removal of 94% and 98% from the decellularized leaflet and artery portions after decellularization relative to the control group. No cell nuclei were found in histological staining and it was observed that the 3-layer leaflet structure was preserved. As a result of the tensile test, it was determined that there was no statistically significant difference between the control and decellularized groups in the UTS and elasticity modulus, and the biomechanical properties did not change. In conclusion, we suggest that the pulmonary valves of decellularized young Merino sheep can be used as a initial matrix in heart valve tissue engineering studies.

**Keywords:** decellularization; heart valve; tissue engineering; xenograft; biomaterial

## 1. Introduction

Valve replacement is performed when drugs are insufficient in the treatment of heart valve diseases, which are very common in cardiovascular problems and can cause death. Although these diseases are of genetic or environmental origin, modified valves; They are mechanical heart valves that do not have a growth feature and require lifelong anticoagulant medication, or short-lived bioprosthetic valves that can create an immune response in patients [1,2]. Heart valve tissue engineering is promising to overcome these disadvantages of current treatment methods [3].

In tissue engineering, it has been tried to produce tissue engineering heart valves using natural polymers such as collagen, hyaluronic acid, alginate or gelatin, and synthetic polymers such as polycaprolactone, polyglycolic acid, poly L-lactic acid, by printing methods such as 3D bioprinting, stereolithography, and electrospinning [4,5]. However, it has been reported that the artificial heart valves obtained due to the defects such as secondary structure change, great variability from batch to batch, and inadequate mechanical properties of the polymers used could not show sufficient efficiency in terms of both functional and bioresistance [6,7]. Therefore, decellularization studies of

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grafts of biological origin are carried out in order to obtain a scaffold that can be used as a starting matrix in the field of tissue engineering. In addition to having a natural ECM architecture, bioprosthetic valves are immunogenic, so cells are removed with decellularization applications and acellular, porous scaffold remains [8]. In heart valve tissue engineering, two different sources can be used: xenograft (graft obtained from different species) or allograft (graft obtained from the same species). The use of allografts as a source when designing human heart valves is limited to cadavers or organ donors. Therefore, xenografts without resource problems seem more attractive. However, the presence of xenoantigens found in the ECM after decellularization in xenografts and creating an immune response in humans may adversely affect graft success [9]. Therefore, the decellularization method, density and subsequent characterization processes are of vital importance.

Decellularization methods are divided into chemical, physical, biological (enzymatic) classes. Physical methods used in decellularization processes include mechanical forces, freezing and thawing, sonication, and radiation. Only cells close to the surface of the tissues can be effectively removed by mechanical forces. During freezing and thawing, intracellular ice crystals disrupt cell membranes, although chemical or enzymatic approaches must be used to remove intracellular materials [6,10]. Trypsin and endonuclease enzymes are generally used in enzymatic methods. While trypsin facilitates cell removal, it also affects the fibrous structural proteins of the valve ECM, causing damage to the histoarchitecture [11]. Chemical decellularization processes are mostly available in the literature, since physical and enzymatic approaches alone are not sufficiently effective in cell removal [12]. Chemical methods have been frequently studied to develop non-immunogenic and long-lasting grafts in heart valve tissue engineering [13,14]. Examples of chemical methods are acid/base solutions, hypotonic/hypertonic solutions, ionic/nonionic detergents. Especially ionic/nonionic detergents are mostly used in decellularization studies due to effective cell removal [11]. Triton X-100, sodium deoxycholate (SD), sodium dodecyl sulfate (SDS), and ethylenediaminetetraacetic acid (EDTA) are the most widely used detergents for decellularization. Except for low doses, SDS has been reported to cause degeneration of histoarchitecture and a cytotoxic environment for cells [15]. There are studies reporting that SD completely removes cells while preserving the histoarchitecture [16,17]. Triton X-100, which is one of the nonionic detergents, is a detergent that is frequently used in decellularization studies and is effective in cell extraction, DNA removal, and also in the protection of extracellular matrix components [18,19]. In our study, it was decided to use SD (0.8%), which did not damage the extracellular matrix (ECM) histoarchitecture as much as SDS and proved to be effective in cell removal, and SDS (0.2%), which was determined not to have low-intensity cytotoxic effects [20-22].

Pigs are generally used as donors in decellularization studies of heart valve tissue engineering. Among the reasons for this, it can be said that the biomechanical properties are good and the anatomy is similar to human valves [23]. However, considering the risk of porcine endogenous retrovirus (PERV) infection from porcine heart valves [24], the use of glutaraldehyde, which has a cytotoxic effect as a stabilizing agent [25], and the immunogenic effect in almost all studies according to clinical findings [26-28], a different source of xenografts should be found as an alternative to pigs. Decellularized sheep heart valves have been tested in in vivo implant models such as sheep, pig, rabbit [29–32]. It has been observed in animal trials that decellularized sheep heart valves do not induce an inflammatory response and recellularization occurs [33–35]. However, no clinical trial of decellularized sheep heart valves has been found. Virtually all characterizations of decellularized porcine heart valves have been performed, in vivo trials, clinical trials, and even commercial products have been created [26,36-38].

In the few studies in the literature on decellularized sheep heart valves; In vitro recellularization trials of sheep heart valves without detailed characterization after decellularization have been investigated for calcification tendencies and immunogenicity in various implant models [39–42]. Tudorache et al. (2013) decellularized sheep heart valves and then implanted them in sheep to investigate their function and morphological changes [39]. In this study, detergent-based method as well as cryopreservation procedures were applied for the decellularization process and characterizations were performed after transplantation into sheep to evaluate their efficacy. It was

 doi:10.20944/preprints202305.1473.v1



emphasized that the efficacy of the decellularized group after transplantation was good in the early term, but it was reported that cryopreserved samples caused calcification and immune response. However, the main purpose of heart valve tissue engineering is to design human-specific valves. The use of sheep as an implant model was originally made to determine allograft effectiveness. Therefore, detailed characterization procedures are required first for human implantation of decellularized sheep valves. Theodoridis et al. (2015) investigated the matrix-guided tissue regeneration potential of decellularized sheep heart valves in aged sheep as a transplantation model [40]. Since allografts were used in this study, detailed characterization processes for humans after decellularization were not performed. Converse et al. (2017) in a published study, after processing sheep aortic heart valve with detergent-based decellularization, they tried recellularization with human mesenchymal stem cells under bioreactor conditions without characterization processes and reported that they could not achieve full cellularity [42]. As can be seen, inadequate or no characterization of decellularized sheep heart valves may cause such problems. Therefore, detailed characterization tests are needed to evaluate sheep heart valves as a starting matrix in tissue engineering studies.

Our aim in this study is to characterize sheep heart valves, which are not yet ready for clinical trials, after decellularization with a detergent-based chemical method. Initially, pulmonary heart valves of 4-6 month old merino sheep were decellularized using ionic detergents such as SD and low concentration SDS. The efficiency of decellularization was determined by determination of residual DNA amount, histological staining, scanning electron microscopy (SEM) imaging, determination of swelling ratio and uniaxial tensile test. Thanks to our characterizations, it will be possible to determine the suitability of sheep pulmonary heart valves decellularized by detergent-based method as xenograft for clinical studies.

## 2. Experiments

### *2.1. Decellularization*

Hearts collected from a local slaughterhouse, approximately 20 minutes after the slaughter of 4–6 month old merino sheep, were brought to the laboratory in cold saline (0.9%). In sterile environment, pulmonary valves were isolated from hearts and cleared of external connective tissue and adipose tissue. It was then shaken in 1× PBS (Sigma, 524650) solution containing 0.8% SD (Sigma, 30970) and 0.2% SDS (Sigma, 71725) at 37 °C for 24 hours. They were then incubated in 1× PBS (Sigma, 524650) solution containing 0.8% SD (Sigma, 30970) and 0.2% SDS (Sigma, 71725) under shaking condition for 24 hours. They were washed 6 times for 12 hours each with PBS containing streptomycin and penicillin (100 IU/ml) (Gibco, 15140-122). Decellularized scaffolds were incubated with solutions of DNase I (200 µg/mL- Biomatik, A2442) and RNase A (50 µg/mL- Biomatik, A3806) prepared in 10 mM MgCl2 and 50 mM Trisma (pH: 7.5) buffer for 24 hours at 37 °C. After this time, it was washed with several rounds of ultrapure water. After washing, decontamination (with 70% ethanol) was applied for 5 hours. Decellularized scaffolds and non-decellularized native valves as a control were kept in PBS solution containing streptomycin and penicillin (100 IU/ml) at 4 °C until characterization was completed.

### *2.2. Residual DNA Measurement*

Manual DNA isolation was performed to determine the amount of residual DNA after decellularization. Sections of 1 cm × 1 cm were taken from the arterial wall and leaflet parts of the control group and decellularized scaffolds under sterile conditions. The samples were kept in a solution containing 10 mM Trisma, 50 mM KCl, 1.5 mM $MgCl_2$, 0.5% Tween 20 and 20 mg/mL Proteinase K (NZYTech, MB01902) at 55 °C for 48 hours. Then, the solution was centrifuged at 3000 g at +4 °C for 15 minutes and the supernatant was collected. Phenol: chloroform: isoamyl alcohol (25:24:1 by volume, respectively) was added on the supernatant and centrifuged at +4 °C at 3000 g speed for 15 minutes. 200 µl of 3 M sodium acetate (pH: 5.5) and 500 µl of 95% ethanol were added to the collected supernatant and incubated at −80 °C for 20 minutes. Afterwards, it was incubated at 37 °C for 30 minutes and centrifuged at 10000 rpm at 25 °C for 10 minutes and the supernatant was

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discarded. After adding 100 µl of distilled water on the remaining pellet and dissolving the DNA, the amount of double-stranded DNA (260/280 nm) was measured with nanodrop (SHIMADZU, BioSpec-nano) and compared with the non-decellularized control group. Data were found in ng/µl and DNA removal was calculated as %.

### *2.3. Histological Characterization*

Histological staining was performed to observe the effects of decellularization on the pulmonary artery wall and leaflet portions and to confirm acellularity. As a preliminary preparation, decellularized leaflet and artery samples and control group samples were fixed with 10% neutral buffered formaldehyde solution. Then, dehydration was achieved by passing through increasing ethanol series (60-70-80-90-96-100). After the samples were embedded in paraffin, they were cut into 5 µm sections with a microtome (Leica, RM2245). It was then treated with xylene after incubation at 55 °C to remove paraffin from the samples. After the deparaffinization process, rehydration was achieved by passing the samples through decreasing series of ethanol. Subsequently, the samples were stained with H&E (Vector, H3502) and Movat pentachrome (Abcam, ab245884). H&E stains the cytoplasm of cells and ECM proteins pink, while the nucleus stains blue-violet. Movat pentachrome also stains collagen yellow, elastin black, glycosaminoglycans blue, and nuclei black. Following the staining process of the preparations, they were observed with a light microscope (Olympus, BX53) and their photographs were recorded.

### *2.4. SEM Imaging*

Scanning electron microscopy (SEM) (Zeiss EVO, LS10) was used to observe the surface morphology of the pulmonary leaflet and artery wall samples of the control and decellularized groups. The samples were fixed for at least 24 hours with a solution containing 2.5% by volume glutaraldehyde prepared in PBS. Then, the scaffolds were washed with ultrapure water and passed through increasing series of ethanol (60-70-80-90-96-100%) for 5 minutes each and left to dry at room temperature. The dried scaffolds were coated with gold (80%) - palladium (20%) by the spray coater for 90 seconds. Then, imaging was started with SEM at 15.00 kV 1000× magnification.

### *2.5. Tensile Test*

Uniaxial tensile test was performed to determine the effect of decellularization on sheep heart valve biomechanical properties. After the arterial wall parts of the control group and decellularized scaffolds were moistened with PBS, they were cut into 1–2 cm strips and placed in a general tensile testing device (Instron). It was stressed at a rate of 5 mm/min with an initial load of 0.1 MPa at room temperature [43]. The initial thickness, length and width of the samples were also noted, and the final tensile strength (UTS), modulus of elasticity (Young's modulus) values were recorded with the data obtained after the test.

### *2.6. Swelling Ratio*

Pieces of 1 cm × 1 cm were cut from the arterial wall and leaflet parts of the control group and decellularized samples and their initial dry weights were weighed. The cut samples were dipped into tubes containing PBS. Samples kept in the tube at 37 °C for 4 hours were collected and excess PBS was carefully wiped away. The final weights were then weighed and the % swelling ratio was calculated according to the formula [43];

$$\%\ swelling\ ratio = \left(\frac{Wet\ weight - Dry\ weight}{Dry\ weight}\right) \times 100 \quad (1)$$

### *2.7. Statistical Analysis*

The analysis was performed in 3 replicates for each group (n = 3). Data are given as mean ± standard deviation. IBM SPSS Statistics (Version 23) software was used for statistical analysis.

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Student's t test was used to compare the mean of two groups, and one-way ANOVA test was used to compare the means of groups with more than two. A value of $p < 0.05$ was considered statistically significant.

### 3. Results and Discussion

When the pulmonary heart valves of Merino sheep were decellularized, macroscopically examined, it was observed that especially the transparent leaflet parts turned dull color, and the muscle-related region in the pulmonary root part turned from red to brown, as in Figure 1.

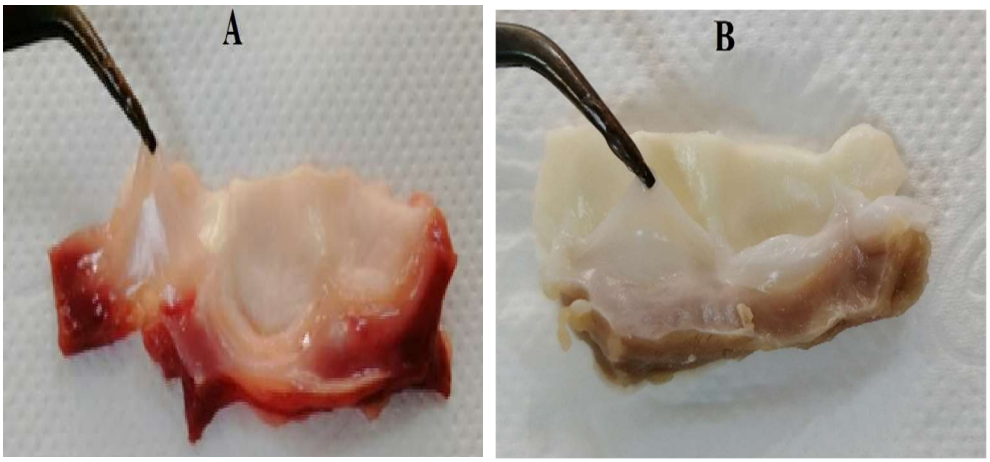


**Figure 1.** Native (**A**) and decellularized (**B**) sheep pulmonary heart valve.

The swelling rate of the scaffolds was analyzed to determine the effectiveness of decellularization. With decellularization applications, the cells separated from the ECM are replaced by spaces and the porosity increases. Therefore, the swelling rate of the scaffolds increases. As a result of our measurements, the % swelling rate of leaflet samples in the control group was 288.4 ± 85.6, while this rate increased to 638.5 ± 60 after decellularization. The swelling rate of the arterial wall specimens of the control group was 81.9 ± 16.3 and increased to 124.3 ± 9.3 after decellularization (Figure 2). Compared to the control group, the % swelling rates of the decellularized leaflet and arterial wall samples were increased by 121% and 52%, respectively.

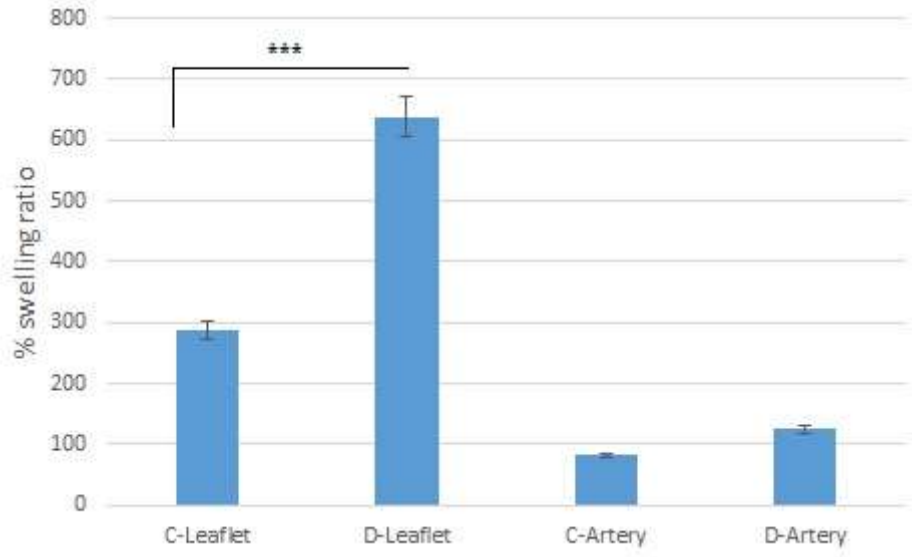


**Figure 2.** % swelling ratios of control and decellularized artery wall and leaflet samples. ***p <0.001 statistically significant (C: control, D: decellularized).

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In order to determine the residual DNA amount, DNA isolation was performed and the measurement results in ng/µl were obtained with nanodrop. According to the nanodrop measurement results, the DNA amount of the leaflet samples of the control group was 982 ± 131.3 ng/µl, and 1783.3 ± 110.4 ng/µl in the arterial wall samples. After decellularization, the amount of double-stranded DNA in leaflet samples decreased to 54.22 ± 9 ng/µl and the amount of DNA in artery samples decreased to 26.04 ± 13 ng/µl (Figure 3). DNA removal in decellularized samples compared to control groups was 94% and 98% in leaflet and artery parts, respectively. In most of the studies in the literature, approximately 95% of DNA is cleared by decellularization [35,44]. In our study, DNA removal was similar to the literature data and detergent-based decellularization process with at least 94% DNA removal provided effective decellularization in sheep heart valves. In order to say that the decellularization process is effective, it has been reported that in addition to the high level of DNA removal, no cell nuclei and stained cells should be detected in histological stainings [45].

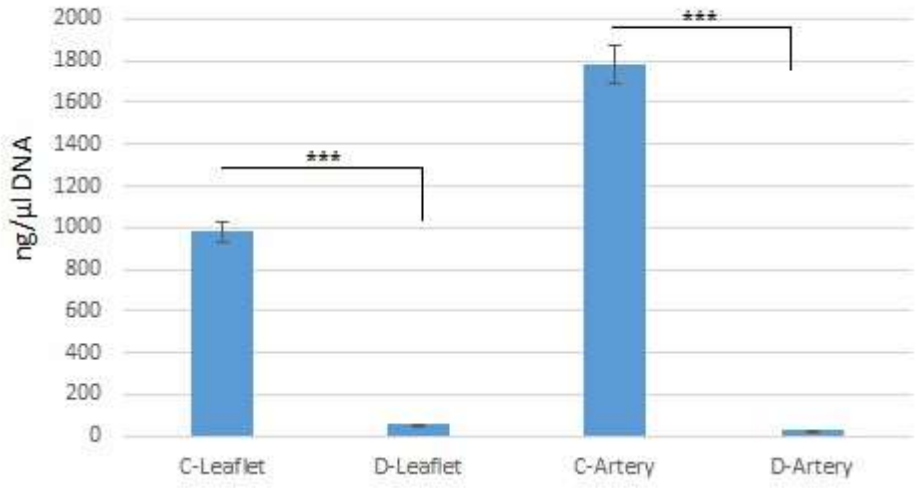


**Figure 3.** Nanodrop measurement results of DNA obtained from control group and decellularized leaflet/arterial wall samples (ng/µl). ***p <0.001 statistically significant.

SEM images showed that the endothelial layer on the surfaces of the decellularized samples was destroyed and collagen fibrils were exposed when compared to the control group (Figure 4). It also revealed that the subendothelial structure was preserved after decellularization.

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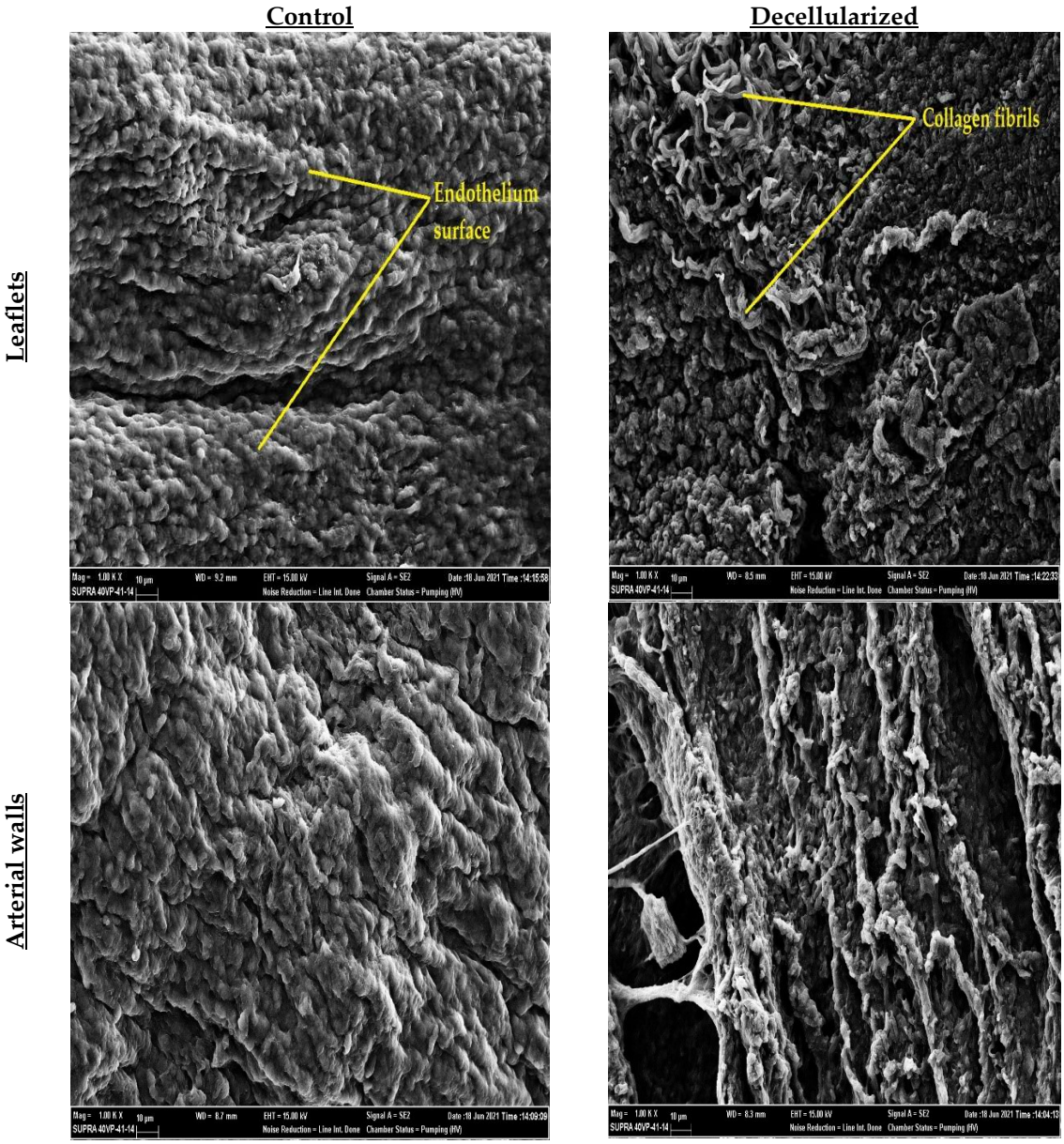


**Figure 4.** SEM image (1000X) of control and decellularized leaflet/arterial wall samples. An almost smooth endothelial surface was observed in the control groups, whereas decellularized samples showed a porous surface where the endothelium was removed and fibrils were exposed.

The leaflets are composed of three layers: fibrosa, spongiosa, and ventricularis, respectively. Collagens in the fibrosa layer, glycosaminoglycans in the spongiosa layer and elastins in the ventricularis part are predominantly located. Depending on the method used in the decellularization process, the basic ECM components in this 3-layer structure are slightly affected. To determine this damage, H&E and Movat pentachrome staining were performed. According to the H&E staining images, it was shown that there was no difference in the amount and orientation of collagen in the fibrosa layer of the leaflets (Figure 5).

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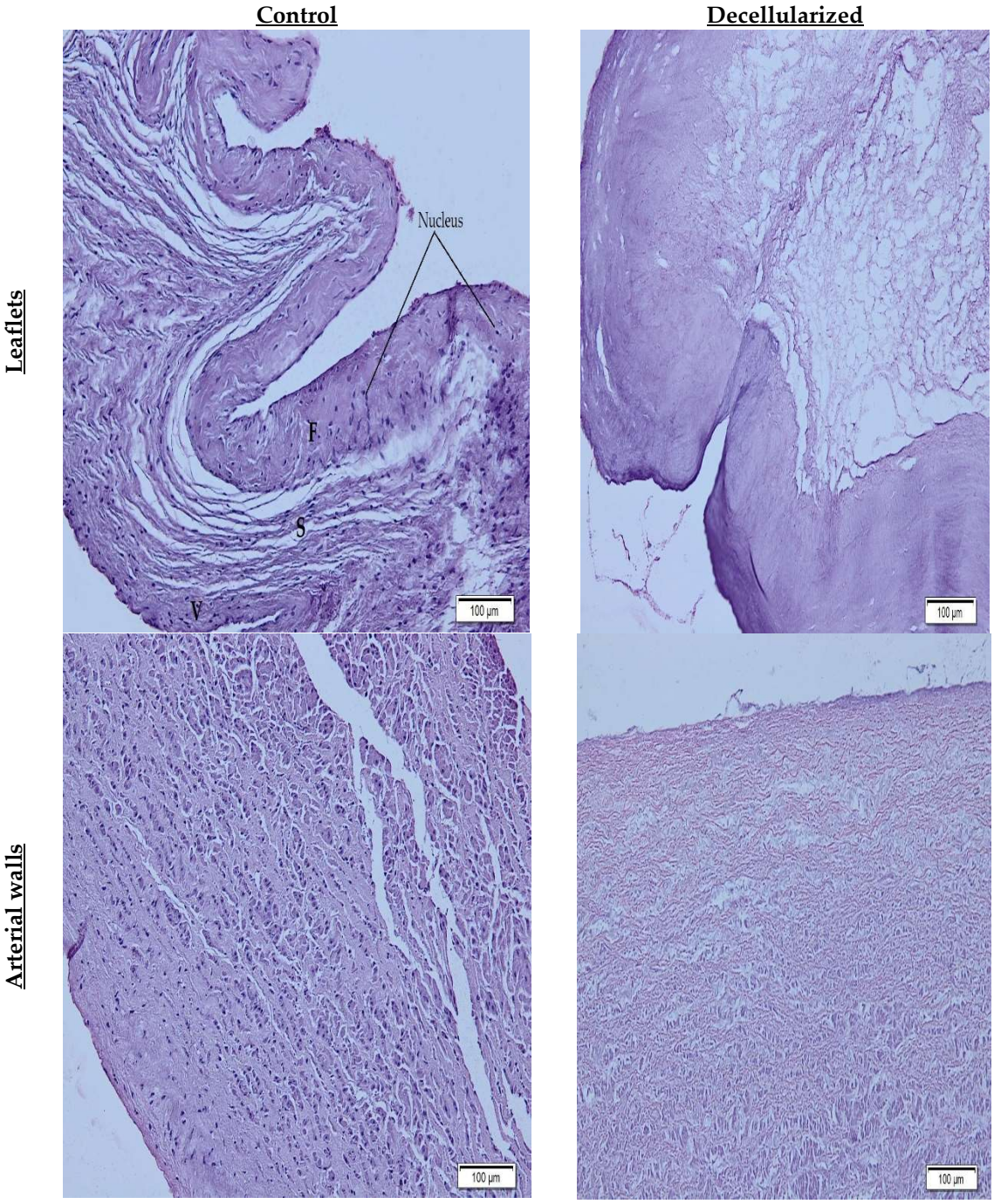


**Figure 5.** H&E stained control and decellularized leaflet/arterial wall images, F: fibrosa, S: spongiosa, V: ventricularis layers. While black-stained nuclei were observed in the control groups, no nuclei were found in the samples after decellularization.

No significant difference was observed in terms of collagen arrangement and amount in the 3 layers of leaflets stained with Movat pentachrome after decellularization compared to the control group (Figure 6). It is known that the decellularization process leads to collagen reduction in these layers [46]. However, when compared with the control group, both leaflets and arteries appear to be similar in terms of collagen organization and amount [47]. In addition, the amount of elastin was slightly affected by the decellularization process in the arterial sections, but no critical change occurred. In a study investigating the change of glycosaminoglycans (GAG), which is located in the spongiosa layer of heart valves obtained from humans and sheep, which is effective in the viscoelastic behavior of tissues after decellularization, a report was presented that showed greater loss of GAG in sheep leaflets, and this was due to the greater initial cellularity in sheep leaflets [48]. In the same

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study, according to the quantitative measurement results of sulfated GAGs, a decrease of approximately 89.6% was observed in sheep heart valves after decellularization, while this rate was 57.4% in human valve leaflets. In addition, no significant change was observed in the blue-stained GAGs after decellularization in our study.

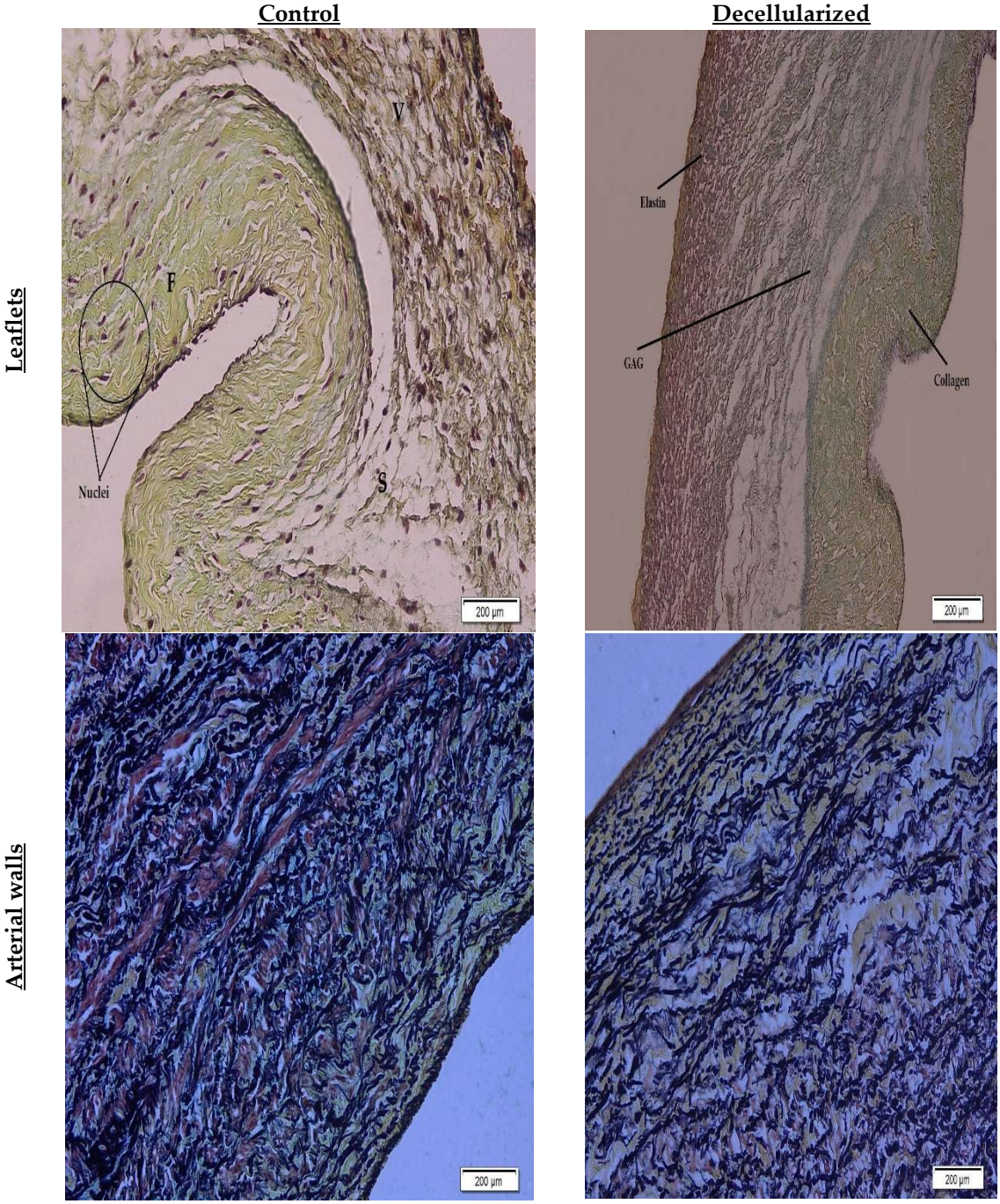


**Figure 6.** Movat's pentachrome stained control and decellularized leaflet/arterial wall images. While black stained elastins and nuclei, yellow stained collagens and blue stained GAGs were visible in the control groups, no nuclei remained after decellularization and other ECM components were preserved.

No stained nuclei or cellular residues were found in the images obtained as a result of H&E and Movat pentachrome staining. Although the 3-layer leaflet structure was preserved, there was no significant change in basic ECM components such as collagen, elastin and GAG (Figures 5 and 6). Cell extraction was successfully performed in the pulmonary leaflet and arterial portions

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decellularized with SD and low-intensity SDS, as SEM images also confirmed the absence of endothelium on the surface.

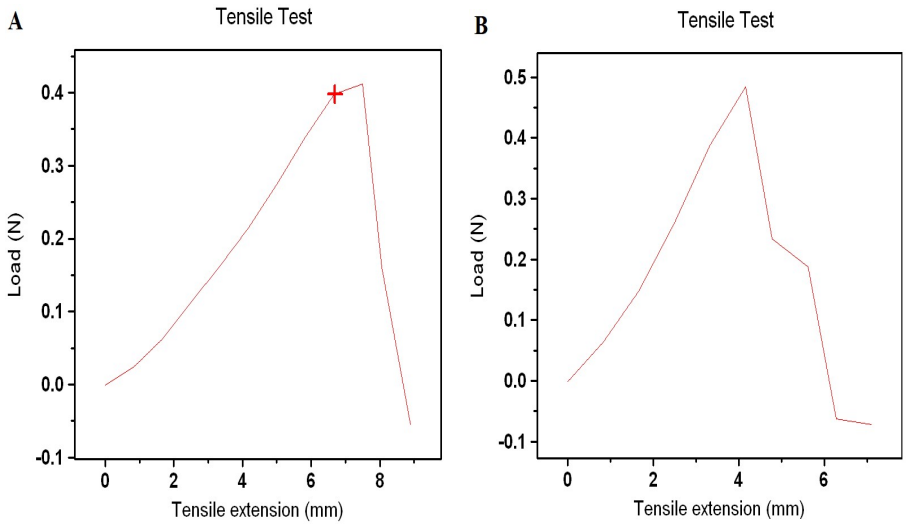


**Figure 7.** Tensile test plots of the control (**A**) and decellularized (**B**) arterial wall groups.

To examine the effect of decellularization processes on the biomechanical properties of tissues, the ultimate tensile strength (UTS) and modulus of elasticity (Young's modulus) of arterial sections taken in the circumferential direction were analyzed in a conventional tensile testing device. The initially measured width of the samples was 8 mm, the thickness was 2 mm, and the length was recorded as 18 mm. Based on the data obtained, it was observed that there was no statistically significant change in the UTS and modulus of elasticity values (Table 1). It has been reported that after detergent-based decellularization, there is no change in stretching properties, especially UTS, in sheep heart valves compared to the control group [49]. As observed in histological staining, minimal changes in collagen, elastin and GAG components and preservation of the 3D architecture ensured the preservation of mechanical properties. Similar findings were obtained in porcine heart valves, whose biomechanical properties are known to be preserved after decellularization, and no significant differences in tensile properties were observed when compared with control groups [50].

**Table 1.** Tensile test results of control group and decellularized artery samples. Since $p>0.05$, there is no statistically significant difference between groups.

| | UTS (MPa) | Modulus of Elasticity (MPa) |
|---|---|---|
| **Control** | 0,033 ± 0,02 | 0,0037 ± 0,002 |
| **Decellularized** | 0,047 ± 0,015 | 0,011 ± 0,003 |

## 4. Conclusions

In this study, sheep pulmonary heart valve was decellularized using SD and low-density SDS to obtain a starting matrix suitable for applications in heart valve tissue engineering. The scaffolds obtained were subjected to various characterizations in order to determine the efficiency of this process following decellularization and to determine the changes occurring in the samples. Histological images, % swelling ratio, which indirectly indicate porosity, and residual DNA analysis results showed us that we obtained a completely acellular scaffold. In addition, SEM and histological images showed that the natural architecture and 3-layer leaflet structure were significantly preserved. In the tensile test for biomechanical characterization, it was observed that there was no significant change after decellularization and the detergent-based decellularization method provided efficient cell removal. These results indicate that the decellularized merino sheep pulmonary heart valve has a porous structure suitable for recellularization. In our future studies, we plan to recellularize the

decellularized sheep heart valves with human cells in vitro and investigate their regeneration potential in this scaffold. As a result, this study has finally proven that decellularized sheep heart valves are a promising candidate in heart valve tissue engineering as a candidate for xenograft, and baseline values that can be considered as reference values as a starting matrix have been revealed.

**Institutional Review Board Statement:** Not applicable.

**Informed Consent Statement:** Not applicable.

**Data Availability Statement:** The data presented in this study are available within the article.

**Acknowledgments:** This study was supported by Yıldız Technical University Scientific Research Projects with the project number FCD-2021-4687.

**Conflicts of Interest:** The authors declare no conflict of interest.

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