Comparative Assessment of Various Concentration

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Comparative Assessment of Various Concentration and Exposure Time of Sodium Dodecyl Sulfate as Decellularization Agents for Small-Vessels Vascular Tissue Engineering

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Abstract

BACKGROUND: Finding the optimum vascular grafts (VG) to replace damaged blood arteries in cardiac surgery is still a work in progress. To be employed, a tissue-engineered VG (TEVG) must have the appropriate biological and mechanical qualities. Decellularized arteries may be a better TEVG than synthetic grafts because of their natural three-dimensional architecture.

AIM: The goal of this study was to compare different concentrations and times of sodium dodecyl sulfate (SDS) to decellularize tissue to find the best decellularized VG.

METHODS: In all decellularized scaffolds, which are 1% SDS-2 weeks group, hematoxylin and eosin and Masson's trichrome staining exhibited looser collagen networks and fewer nuclei.

RESULTS: The orientation of collagen fibers was identical to native vascular scaffolds. Collagen I deposition was seen in the immunohistochemistry assay. A tensile strength test revealed that the decellularized scaffold (0.5% SDS for 4 weeks and 0.5% SDS for 2 weeks) had exceeded the native arteries' maximal strength. In comparison to 1% SDS in 4 weeks treated groups, scanning electron microscopy following decellularization revealed no endothelial cells on the inner side of 1% SDS in 2 weeks group with minimum extracellular matrix damage. The endothelial cells remained marginally visible on the inner side of all 0.5% SDS treated groups. The 3-(4,5-dimethylthiazol-2yl)2,5-diphenyltetrazolium bromide test was used to determine the cytotoxicity of the decellularized scaffolds.

CONCLUSION: This study reveals that exposing a bovine mesenteric artery to 1% SDS for 2 weeks is an excellent procedure for extracting the most acellular VG, potentially serving as a biological scaffold for TEVGs.

Introduction

In the world, cardiovascular illnesses are the major cause of mortality and morbidity. One of the most common cardiovascular operations used to restore blood flow is coronary artery bypass grafting. From the previous study, more than 600.000 vascular grafts (VGs) are implanted to repair damaged blood arteries in cardiac patients [1]. In bypass surgery, autologous arteries such as the internal mammary artery and great saphenous vein, are the first choice for transplants. After 10–15 years, the internal mammary artery had a patency rate of about 90–95%, and the saphenous vein had a patency rate of about 50% [2]. However, some individuals are unable to get autologous grafts due to past diseases, surgery history, or insufficient sizes [3].

Meanwhile, synthetic grafts such as expanded polytetrafluoroethylene and Dacron grafts have shown some success in replacing larger coronary vessels (6–10 mm in diameter); however, thrombotic events have been linked to rapid led graft failure in coronary bypass surgery in vessels with diameters <6 mm. Tissue engineering (TE) is a new study topic, in which the manufacture of VGs using cutting-edge manufacturing processes has attracted the interest of a scientific society [4], [5]. The function of endothelial regeneration and the resemblance of the mechanical properties of tissue-engineered VG (TEVG) to natural blood arteries were the conditions for an optimal TEVG, whether large or tiny [6]. To be used in clinical settings, TEVG must have the right biological and mechanical qualities. To prevent inducing a persistent inflammatory response during the material degradation phase, the biological performance of TEVG should enable perfect integration of the graft in the human body. Furthermore, TEVG's mechanical qualities must be comparable to those of natural blood vessels, notably in terms of

deformability, compliance, and strength. The traditional method of vascular TE is gradually being replaced by an in situ approach, also called as guided endogenous regeneration [7]. Autologous vasculature substitutes are created by implanting cell-free scaffolds that are colonized and modified endogenously.

Despite recent advancements, the fundamental factors that must be solved in VTE technology are the same as those that must be overcome in other organ TE approaches, particularly in producing a good scaffold with body-compatible tissue into which to seed cells [4]. Decellularized arteries, as opposed to synthetic grafts, can be good VTE grafts because of their natural three-dimensional features that enable host cell migration and vascular remodeling [8]. In addition, decellularized arteries have favorable mechanical qualities that allow them to withstand the effects of artery pressure *in vivo*[7]. Commercially available decellularized xenogeneic grafts are currently available. However, they perform poorly due to alack of cellularity implantation. This can lead to increasing the risk of poor patency long-term, aneurysm formation, inflammation, and infection. Another downside of decellularized xenogeneic grafts is that they are more expensive than synthetic grafts. As a result, they have not been frequently employed in clinical trials [4], [5], [6]. As a result, several decellularized scaffolds generated from biological material such as bovine artery have been studied for use in small-diameter VTE continuously [1], [9]. Physical, chemical, and enzymatic procedures are commonly used in decellularization. Regrettably, no gold standard protocol exists for these treatments. sodium dodecyl sulfate (SDS) is a highly effective anionic detergent for decellularization that has been used to remove cells from a variety of tissues.

Furthermore, SDS-based methods are straightforward, requiring only a few readily available reagents, and are thus a practical starting point for decellularizing tissue [10]. As a result, SDS is more routinely utilized to decellularize a variety of tissue types [10], [11]. As a result, this study was planned to compare the time exposure of SDS to decellularized tissue and analyze the various SDS concentrations to find the best decellularized VG.

Materials and Methods

Artery preparation

A nearby slaughterhouse provided the bovine fresh mesenteric artery preparation from cows. The artery was dissected, and the surrounding tissues were removed, including adipose tissue and connective tissue. They were cleaned in 0.9% Natrium chloride saline and stored at −20°C in a sterile container with a streptomycin-penicillin solution. They were sliced into little (0.5 cm) and medium-sized pieces (5–6 cm).

Decellularization methods

The samples were divided into seven groups, with one control group and six experimental groups using (1) SDS 0.5% for 1 week, (2) SDS 0.5% for 2 weeks, (3) SDS 0.5% for 4 weeks, (4) SDS 1% for 1 week, (5) SDS 1% for 2 weeks, and (6) SDS 1% for 4 weeks. The artery was first washed with distilled water, then placed on an orbital shaker (TS-100, Stem Cells and Functional Tissue Laboratory Research at Dr. Soetomo Academic General Hospital Instrument Equipment Manufacturing, Surabaya Indonesia) for about 1 h (100 r/min), then incubated in a sterile container and soaked in distilled water for 8 h at room temperature with gentle agitation to demineralize the artery for group decellularization. They were treated with one of the six distinct decellularization techniques listed below after an additional 1 h of washing in distilled water, and the results were compared to the control group. The following phases in the decellularization technique were based on detergents. These arteries were immersed in either a 0.5 or a 1% SDS solution and continuously oscillated for each time in subgroups after an orbital shaker (1, 2, and 4 weeks). The decellularized arteries were then washed according to the technique to eliminate any remaining detergents. All of the procedures were carried out in a sterile, room-temperature environment. Finally, for immunohistochemistry (IHC) or standard hematoxylin and eosin (H and E) histology, all samples from each treatment group were preserved with 4% buffered formaldehyde or 10% buffered formaldehyde.

Histology examination

The formalin-fixed specimens were processed for imaging by dehydration. The process continues with embedding in paraffin sectioning into 5 m slices, and then staining with H and E. To assess the visibility of the scaffold's collagen, we utilize Masson's and Verhoeff-Van Gieson's trichrome staining. Under a light microscope, the stained tissue sections were evaluated using a digital image analysis system. The experienced histology physician from Dr. Soetomo Academic General Hospital examined the sections visually.

Scanning electron microscopy

Decellularized arteries were lyophilized for 48 h to remove all moisture before being sectioned with a scalpel, and scanning electron microscopy (SEM) stubs were mounted. Before imaging with an HITACHI FLEXSEM 100 scanning electron microscope, the samples were coated with 18nm osmium metal using

osmium plasma. Cross-sections of the sample interior and exterior were photographed to create representative photographs.

Cell viability study on scaffolds

Gamma rays of 25 kGy were used to sterilize all of the decellularized scaffolds. The decellularized vascular scaffold was placed in each well of a 24-well cell culture plate, and the MG63 cell line (Pasteur Institute) was cultivated on each scaffold for 1, 3, and 5 days at a concentration of 2×10^3 cells per scaffold. By observing the color change from yellow-colored tetrazolium salt 3-(4,5-dimethylthiazol-2yl)2,5-diphenyltetrazolium bromide (MTT) to purple-colored formazan, the MTT assay was used to measure cell metabolic activity. The extract was prepared after a 24–26 h incubation period at 37°C in a 1 mL culture medium containing serum protein. The extract solution was diluted to 12.5%, 25%, and 50% in the same culture medium. A subconfluent monolayer of L929 cells was incubated at 37°C for 24 h with an equivalent amount (100 mL) of HGPR05 extract, a negative control (high-density polyethylene), a positive control (dilute phenol), and a cell. The grown cells were incubated for 4 h at 37°C in a humidified, 5% CO_2 atmosphere after being treated with 50 mL of MTT. Excess MTT was removed by aspiration, and 100 mL isopropanol was added to dissolve the formazan crystals. The cytotoxicity tests were done three times. The color exchange was determined using a spectrophotometer to measure absorbance at 570 nm.

Biomechanical test: Tensile strength test

To recover the grant texture, samples with a length of 5–6 cm were preincubated in 0.9% NaCl for 15 min. The tests were carried out on an autograph universal testing machine with a 10N load cell and a crosshead speed of 1 mm/min. The clamps were separated by 10 mm. The distance between the valley and the mountain was 12 mm. For each scaffold, the measurements were taken at least four times. When a sample broke in the middle, the measurement was still valid. It was determined what the maximum strength was. The thickness of the specimen varies depending on the scaffold type. A computerized caliper was used to measure the thickness with an accuracy of 0.1 mm.

Statistical analysis

The data were analyzed using SPSS v25 (SPSS, Chicago, IL, USA). In the statistical analysis, one-way ANOVA and the *post hoc* Tukey test were performed. To express quantitative data, the mean value standard error of the mean was utilized. A statistically significant $p < 0.05$ was also considered.

Results

Overall appearances of the scaffolds

Images of decellularized vascular scaffolds are shown in Figure 1. The decellularized scaffold was chosen at random from the experimental groups, with the negative control on the left (Figure 2). Each vessel's breadth, thickness, and length (n = 6) were also measured. The decellularized and control groups, on the other hand, showed no significant differences in breadth, thickness, or length (Table 1).

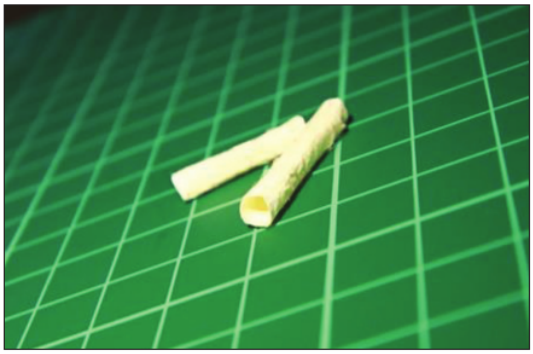

Figure 1: A gross view of decellularized bovine mesenteric artery

Histological analysis of the decellularized scaffolds

The cross-sections of the scaffolds in each group were evaluated using H&E staining (Figure 2a-g) and Masson's trichrome staining (Figure 2h-n). H and E examination of native arteries revealed dense cells embedded in the arterial wall (Figure 2a). The native artery exhibited fewer nuclei and looser collagen networks than the decellularized arteries. According to Masson's trichrome staining, the native arteries were largely made up of thick collagen fibers (blue) and smooth muscle cells (red). Decellularization indicated that the pores in each of the experimental scaffolds were large and generated light Masson's Trichrome staining (Figure 2h-n), suggesting considerable elastin loss. The blue line in Masson's trichrome staining represents the collagen in the ECM. The regular orientation of collagen fibers in the 1% SDS 2 weeks-treated group was identical to that of the intact vasculature. The

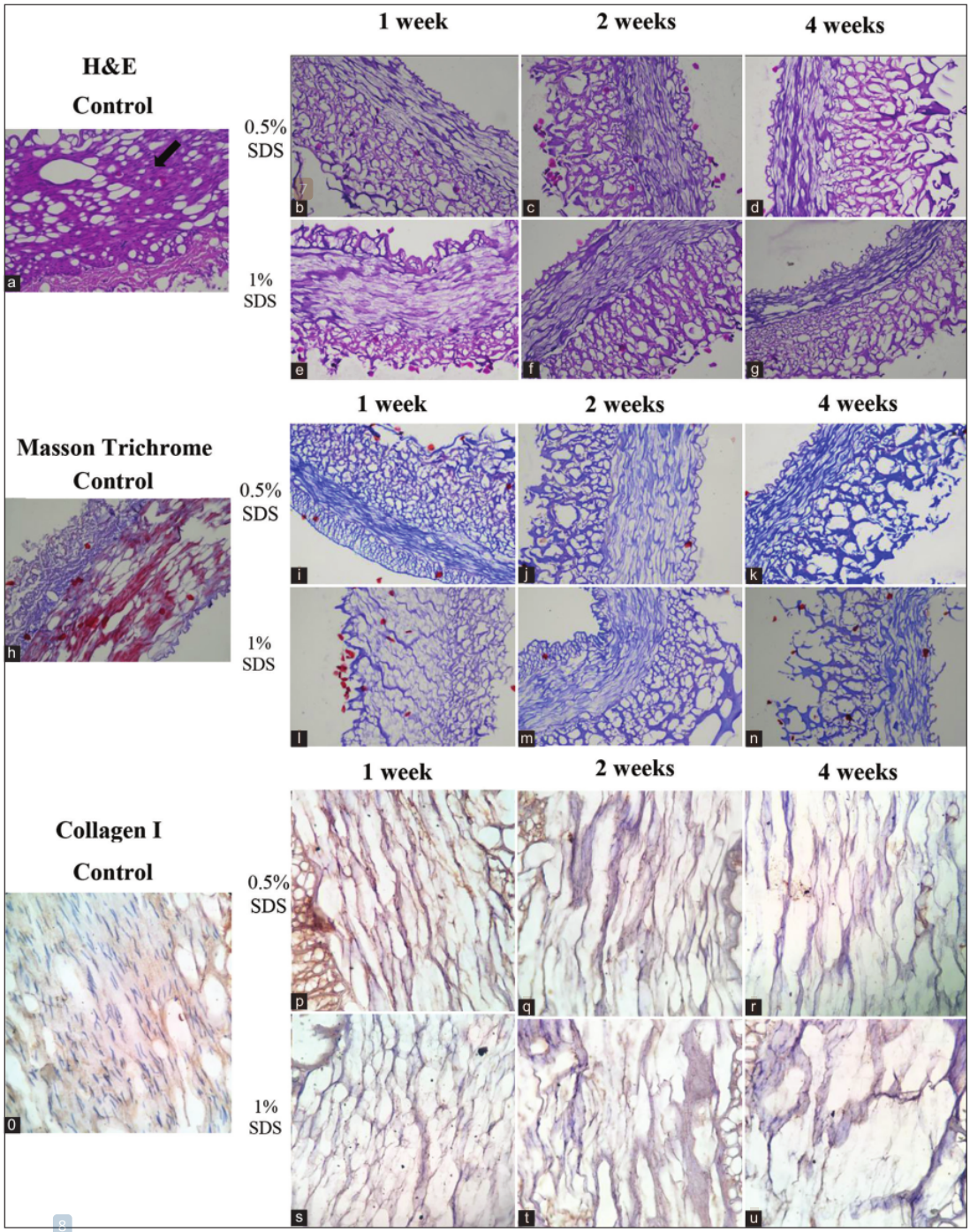

Figure 2: Visual characterization of decellularized blood vessel scaffolds. Various microscopic and histological staining techniques were employed to compare the following characteristics between six experimental decellularized scaffolds and fresh vascular controls. (b-g) Hematoxylin and eosin staining (×400) compared to control (a) and (i-n) Masson's trichrome staining results compared to control (h) (×400). And to evaluate the collagen thickness with immunohistochemical study with collagen I antibody (p-u)

Table 1: Result of width, thickness, length and tensile strength of decellularized artery with control

Variables (n = 6)	Mean width (mm ± SD)	Mean thickness (mm ± SD)	Mean length (mm ± SD)	Mean tensile strength (Mpa ± SD)
Control	5.50	2.44	52.05	4.35
SDS 0.5% 1 week	5.67	2.50	51.21	3.24
SDS 0.5% 2 weeks	5.55	2.51	50.28	3.20
SDS 0.5% 4 weeks	5.43	2.56	52.01	3.29
SDS 1% 1 week	5.52	2.27	52.00	3.23
SDS 1% 2 weeks	5.54	2.67	51.04	3.25
SDS 1% 4 weeks	5.51	2.43	52.06	3.12

SD: Standard deviation, SDS: Sodium dodecyl sulfate.

irregularity in collagen fibers orientation was higher in the 1% SDS 4 weeks-treated group than in the 1% SDS 2 weeks-treated group. Collagen irregularity is wavy and densely distributed in collagen fibers.

Other histological investigations indicated that the inner surface of the original arteries was made up of a smooth internal elastic lamina known as the basement membrane, which is necessary for endothelial cell adherence during the stem cell seeding phase. This structure is well retained in the original arteries and all decellularized groups (Figure 2).

Immunohistochemical study: Type 1 collagen antibody

Anti-collagen I IHC was done in formalin-fixed and paraffin-embedded scaffold sections according to the manufacturer's instructions. To show the collagen I deposition and expression within the cytoplasm of the cell, light microscopic analysis of anti-collagen I IHC stained decellularized artery scaffold section (Figure 2o-u) was performed. Statistical evaluations of decellularized scaffolds groups show a substantial loss of collagen control after decellularization ($p = 0.000$), although the 0.5% SDS 1 week-treated group was found to be the most group that maintained collagen content after the decellularization protocols (17.8 ± 2.31) (Table 2). 0.5% SDS and 1 SDS 2 weeks-treated groups, on the other hand, show a comparable result (13.8 vs. 13.8, Table 2). 0.5% SDS and 1% SDS 4 weeks, on the other hand, had the lowest level of type 1 collagen.

Table 2: Result of type 1 collagen in immunohistochemical staining of decellularized artery with control

Variables	n	Type 1 collagen		p
		$\bar{X}$ ± SD	Minimum–maximum	
Control	6	18.5 ± 1.04^{d}	17–20	0.000*
SDS 0.5% 1 week	6	$17.8 \pm 2.31^{c,d}$	15–21	
SDS 0.5% 2 weeks	6	13.8 ± 2.63^{b}	10–17	
SDS 0.5% 4 weeks	6	5.5 ± 1.04^{a}	4–7	
SDS 1% 1 week	6	$15.0 \pm 1.41^{b,c}$	13–17	
SDS 1% 2 weeks	6	13.5 ± 2.07^{b}	11–17	
SDS 1% 4 weeks	6	6.3 ± 2.06^{a}	4–9	

^{a,b,c,d}Different superscripts indicate groups with significantly different mean. SD: Standard deviation, SDS: Sodium dodecyl sulfate.

Scanning electron microscopy

SEM revealed the ultrastructure of an intact endothelial cell layer on the lumen of healthy arteries (Figure 3a). While no endothelial cells were visible on the inner side of 1% SDS in 2 weeks-treated groups (Figure 3j) with minimum extracellular matrix degradation, no endothelial cells were evident on the inner side of 1% SDS in 4 weeks-treated groups (Figure 3n and o). The leftover endothelium cells remained faintly visible on the inner side in all 0.5% SDS treatment groups (Figure 3i-k). The matrices in the six decellularized groups, on the other hand, exhibited a similar appearance: the exterior microporous structures were sufficiently open for seeded cell migration and ingrowth, and all of the decellularized groups were more porous than the control vessels (Figure 3). All cellular components were eliminated from the arterial wall of decellularized arteries, and the porous marrow was exclusively made up of preserved-well collagen and elastin fiber (Figure 3a).

Tensile strength test

The tensile strength test revealed that the decellularized scaffold 1% SDS 1 weeks-treated group had the highest maximum strength among the other decellularized groups, as indicated in Table 1. After that, a group of SDS 0.5% treated for 4 weeks with 3.29 MPa was added. The maximal strength of 0.5% SDS in a 1-week-treated group and 1% SDS in a 2-week-treated group was found to be identical (3.24 MPa vs. 3.25 MPa). Meanwhile, the maximum strength of the tensile strength test was lowest in the SDS 0.5% in 4 weeks treated group (3.12 MPa).

Cytotoxicity of the decellularized scaffolds in vitro

The MTT bromide assay was used to assess the cytotoxicity of the decellularized scaffolds, as previously described. In addition, the RGRs of HUVECs grown in the presence of leach liquid from the scaffolds at varied concentrations were examined after 1, 3, and 5 days of culture. SDS 1% 2 weeks had tended to be the most viable decellularized scaffold among the other decellularized scaffolds, according to the results of the cytotoxicity MTT assay. Table 3 reflected this. On the cytotoxicity MTT assay, the SDS 1% in 2 weeks treated group had 121.09% viable cells compared to the control group.

Discussion

Decellularized VGs can serve as a suitable TEVG, potentially addressing the issue of graft scarcity in bypass surgery. The decellularized vascular scaffold benefited from the structure and mechanical performance while avoiding allergic reactions

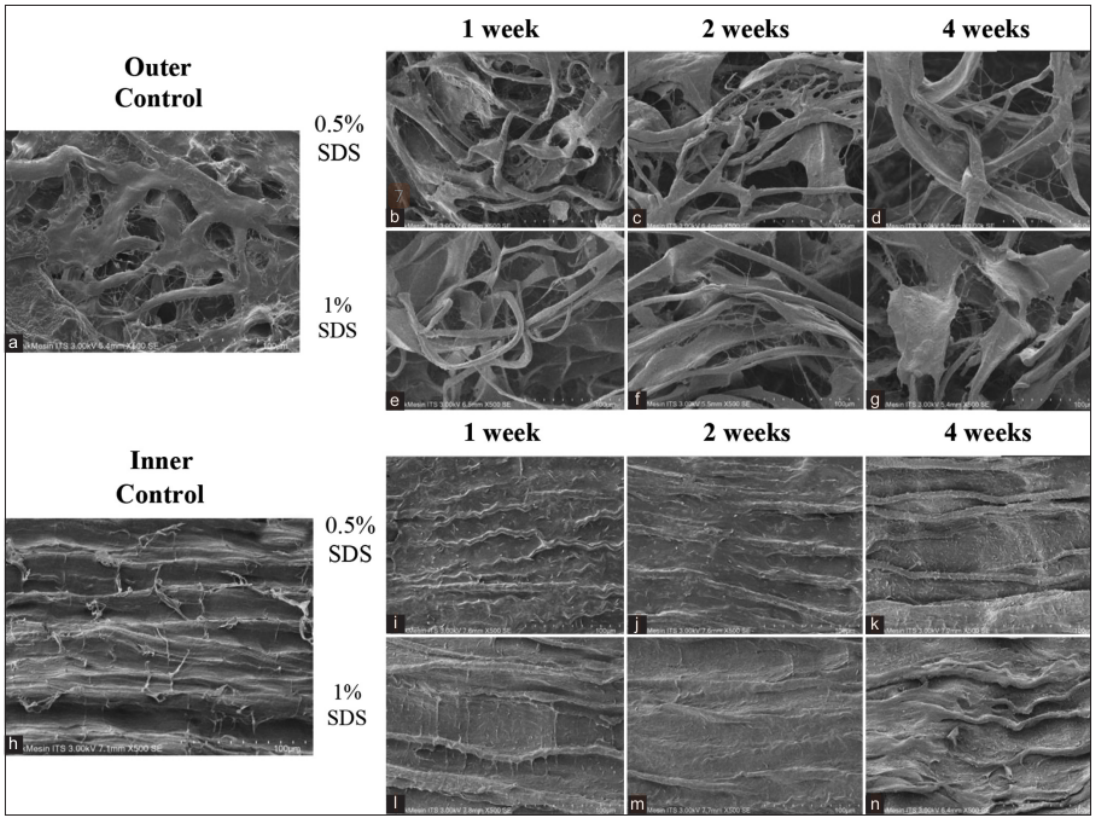

Figure 3: Visual characterization of decellularized blood vessel scaffolds. Scanning electron micrographs showing the outer (b-g) (×500) surfaces in six experimental groups compared to the control (a). The inner surfaces appearances showed (i-n) in the experimental group compared to the control (h) (×500)

associated with their development [11]. This research is the first step toward TE a small-diameter bypass graft. Our ultimate goal, like with previous engineering paradigms, is to create an acellular conduit scaffold that can be seeded with autologous vascular cells before implantation. The tissue source for such a product in this study was bovine mesenteric arteries.

Table 3: Cytotoxicity 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay of decellularized artery with control

Variables	Percentage of viability cells	Mean of percentage of viability cells
Control	101.81	100
	98.19	
SDS 0.5% 1 week	104.47	116.46
	128.46	
SDS 0.5% 2 weeks	110.87	111.43
	111.98	
SDS 0.5% 4 weeks	108.8	111.48
	114.16	
SDS 1% 1 week	114.13	112.99
	111.85	
SDS 1% 2 weeks	120.67	121.09
	121.51	
SDS 1% 4 weeks	107.75	108.31
	108.87	

SDS: Sodium dodecyl sulfate.

As indicated in a previous study, the artery was discovered to have a difference in the mechanism of intimal thickening, resulting in VSMC proliferation being less than the vein. As a result, there is less atherosclerosis than in the veins [1]. Morrison *et al*. in Brazil discovered that the bovine mesenteric artery was less likely to develop problems such as thrombosis and neointimal hyperplasia [12]. In the creation of a vascular transplant, the artery of the mesenteric bovine is preferable to the vein in terms of graft patency [13], [14]. To build the most efficient acellular conduit scaffold, we compared different doses and duration exposures of SDS on the bovine mesenteric artery.

SDS is one of the most often used decellularization solutions because it is so successful at separating nuclear and cytoplasmic waste. SDS dissolves both the outer and nuclear membranes, denatures proteins, and changes the normal structure of the matrix. Due to its advantages, mechanical decellularization, such as SDS, was preferred in TE techniques. The vascular component was reduced to a minimum after decellularization, and the donor (bovine) cell was greatly reduced. SDS's procedure effectively reduced wall thickness following decellularization, similar to the prior work [8]. Decellularized arteries' cell signaling components also help with cell adhesion, migration, proliferation, and differentiation [15]. Decellularization ensures that no cellular components

are left behind, preventing immunological reactions while yet preserving biochemical and mechanical capabilities. Free-thawing, solvents, osmotic gradients, physical methods, detergents, chelating agents, enzymes, and combinations are all used to decellularize cells. Decellularization can be treated with detergents, which has a number of benefits, including being superficial, causing minimal damage, and allowing for simple control of experimental conditions [8], [13], [14].

This experimental investigation used SDS to prepare decellularized vascular scaffolds. Each group's decellularization resulted in a significant reduction in the loss of cells from the artery wall. The absence of visible cells in all groups of decellularized arteries in Figure 1 was confirmed by histological inspection using H and E staining. This indicated that our decellularization protocols had successfully removed cells. Figure 2 shows an SEM analysis. The big pores inside the decellularized groups were visible, indicating that the minimal decellularization criteria had been met.

Histology using Masson's and Verhoeff-van Gieson's trichromes (Figure 1) also shows that arteries subjected to the proposed treatments of decellularization can maintain their native structure without losing significant elastic fiber and total collagen, implying that the ECM structure is not severely damaged. The most scaffold that may show preserve the structure of ECM was found in SDS 1% 2 weeks. Similar to the previous study, SDS 1% group was shown to be the most successful in removing cellular material, which was expected given that ionic detergents are more effective in removing nuclear remains than non-ionic Triton X-100. In comparison to the SDS 1% group, the structure of the ECM was lost the most after 4 weeks. We discovered that as the concentration of SDS increases, the thickness of the ECM structure decreases, resulting in increased artery stiffness. The lack of smooth muscle cell layers causes the stiffness to grow. All of the groups saw a decrease in vascular wall thickness. In addition, histological photos show that the inner and outer elastic laminae, which are known to be important structures for endothelial cell attachment and thrombus resistance, have been preserved [16].

Small pores within the scaffolds may inhibit cellular penetration. Smooth muscle cells must colonize the inner part of the matrix and cause ECM scaffold remodeling in functioning TEVGs; hence, the ECM scaffold pore size is crucial [17]. The porous structure of the scaffolds is due to the extra space provided by removing cell layers, and the formation of large pores is most likely caused by the decomposition of small collagen fibers, which is unlikely to affect the scaffolds' mechanical properties [17], [18]. To determine the size of the scaffolds, we used SEM visualization. Large pores can be seen inside the ECM structure (Figure 2). It does, however, necessitate striking a compromise between obtaining appropriate porosity while preserving the scaffold's microstructure and mechanical qualities.

The cytotoxicity test is widely accepted as a reliable means of determining the safety of biomaterials [19]. The cytotoxicity test was performed on human mesenchymal stem cells using the extremely sensitive and repeatable MTT assay. A biomaterial with an RGR score of Grade 0–1 can be classified non-cytotoxic according to the cytotoxicity criterion. In the PU VG cytotoxicity test, the RGR value was above 80%, indicating that the electrospun PU synthetic blood vessel is biosafe. The cytotoxicity MTT assay was used to determine the scaffold's vitality after the cells were implanted. After decellularization, all decellularized scaffolds were shown to be viable for cell implantation [20], [21]. The extracellular matrix of the three-dimensional decellularized vascular scaffolds is composed of primary collagen, elastin, and glycosaminoglycan (GAG), as previously indicated [7]. Collagen and elastin fibers might give tensile strength to tolerate pulsatile blood flow *in vivo* [16], [20]. Decellularization, on the other hand, may cause some metabolic and mechanical harm [22], [23]. When compared to native arteries, the mechanical properties of decellularized arteries were considerably inferior in terms of the maximum tensile strength (Table 1). The loss of GAGs following decellularization resulted in a drop in maximum tensile strength, which was consistent with a reduction in the viscoelasticity of decellularized arteries [11], [23]. However, among the other concentrations, SDS 1% 2 weeks demonstrated the best mean tensile strength, despite the fact that a recent study found that higher SDS concentrations impaired ECM composition, lowering mechanical strength.

Collagen and elastin, two key ECM components, are critical for the mechanical integrity of arteries. The ECM structure must be preserved in order for the vascular transplant to function properly [24], [25], [26]. As a result, we tested type 1 collagen in this work to see if it might reduce collagen components in the decellularized scaffold (Table 2). In all groups of decellularized arteries, there is a decrease. It has to do with the considerable loss of collagen that occurs as a result of the decellularization process.

There were a few flaws in this research. Due to the COVID-19 pandemic's restrictions, the quantification of DNA examination was halted in this experimental study. Animal experiments involving VG scaffold implantation are required to better evaluate the scaffolds' efficiency. To attain the greatest result of the tiny diameter of TEVGs, we propose to perform decellularized arteries with a combination of stem cell seeding to the graft in a future study.

Conclusion

The bovine mesenteric artery was shown to yield the highest acellular VG while also maintaining the ECM structure when treated with 1% SDS for 2 weeks. A biological framework for TEVGs made from decellularized bovine mesenteric arteries was shown to have a well-preserved structure and adequate biocompatibility.

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Ethical approval

This study was authorized by the Dr. Soetomo Academic General Hospital's health research ethics committee based on the regulations of the US Department of Health and Human Services (HHS) (0157/LOE/301.4.2/X/2020).

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