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Technical papers

Structural Integrity of Aortic Scaffolds Decellularized by Sonication Decellularization System

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***Abstract*—Sonication decellularization technique has shown effectiveness to remove all the cellular components by the disruption of the cell membranes and removal of the cell debris to prepare the bioscaffolds. However, it is important to confirm whether this technique does not have a detrimental effect on elastin and collagen in bioscaffolds. The objectives of this study are to evaluate the structural integrity of bioscaffolds using histological staining and quantitatively collagen and elastin measurement. Aortic tissues were sonicated in 0.1% SDS for 10 hours at the frequency of 170 kHz with the power output of 15W and washed in Phosphate Buffer Solution (PBS) for 5 days. Then the sonicated aortic tissues were evaluated by Hematoxylin & Eosin (H&E) staining for cell removal analysis, Verhoeff-van Gieson (VVG) staining for visualizing elastin and Picrosirius Red (PSR) staining for visualizing collagen. The collagen and elastic fibres were semi-quantified by ImageJ software. The results showed that sonication decellularization system can remove all the cellular components while maintaining the structural integrity of elastin and collagen on bioscaffolds. This study indicates that sonication decellularization system could remove all cellular components and maintain the structure of the extracellular matrix.**

Keywords—sonication, decellularization, bioscaffolds, aorta, elastin, collagen.

I. Introduction

Numerous ECM based bioscaffolds have been constructed through a varied approach to mimic entirely the native tissues. The important of bioscaffolds characteristic is not only free from cells but also should have similar to the mechanical properties of the native tissues. The mechanical properties of tissues are determined by the tissue's components such as elastin, collagen and smooth muscle cells composition, spatial organization and interaction [1][2].

Elastin and collagen are the main component of the extracellular matrix in a blood vessel. In the aorta, the elastin is more abundant than collagen so that it is most compliant vessel in the vascular system [2]. Collagen found in aorta are type I and III that account about 80-90% of the total collagen. Elastin provides elasticity in the aorta to resume their shape after stretching or contraction, while collagen provides the tensile strength and stiffness [3][4].

ECM based scaffolds have an advantage as mechanical properties are the closest to native tissues since it is biologically derived materials. The challenge to develop the ECM based scaffolds lies on decellularization process that required completely cellular components and preservation of mechanical properties. Sodium Dodecyl Sulphate (SDS) is the most commonly used for decellularization process as their effectiveness to remove the cells. Nevertheless, the use of SDS alone could disrupt the integrity structure of ECM as the need to lengthen the exposure of SDS to the tissues. To shorten the treatment time of SDS, sonication is incorporated to facilitate the SDS penetration.

Previous work has shown the effectiveness of sonication decellularization system to remove completely cellular components [5][6]. However, preservation of the elastin and collagen integrity as the main component structure in ECM is critical to the mechanical properties of the aorta as it modulates the pressure and flows in the entire cardiovascular system. Hence, the objective of this study is to investigate the effect of sonication decellularization system on the integrity of elastin and collagen of bioscaffolds.

II. Methodology

A. Tissue Preparation

The porcine aorta was obtained from a local slaughterhouse in Gambang, Kuantan. Aorta tissues were cleaned by removing blood and adherent fats. Aortic tissues were cut into 15x15mm^2 and stored in -20^{o}C until use.

B. Decellularization Treatment

1) Sonication Decellularization

Sonication decellularization system consists of an ultrasonic generator (Fx-500, Flexonic), roller pump (RP-1000, Eyela), water bath (LTB-250, As-One), temperature monitor (TR-71U, T&D), hydrophone (TC4013, Eastek), multiparameter meter (HI 9828, Hanna instruments) and reactor. Aorta tissues were placed 10mm from the ultrasonic transducer and sonicated in 0.1% Sodium Dodecyl Sulphate (SDS) at constant temperature 36± for 10 hours. The ultrasound frequency was set at 170 kHz with the power output of 15W. The tissue was washed in PBS for 5 days after decellularization [7]–[9].

2) Immersion Decellularization

Aorta tissues were immersed in 0.1% SDS for 10 hours at ±36^{o}C under mechanical shaking. The tissue was washed in PBS for 5 days after decellularization treatment.

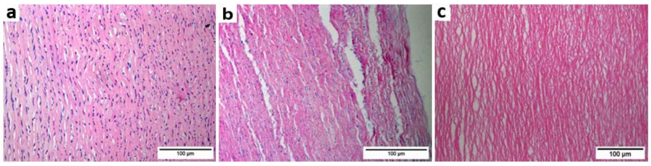

Fig. 1. H&E staining of (a) native aortic tissue, (b) immersed aortic tissue and (c) sonicated aortic tissue.

C. Histological Analysis

Decellularized tissues were fixed in 4% paraformaldehyde for 24 h [10]. Tissues were dehydrated with increased alcohol concentration of 70%, 80%, 90%, 100% for 1 h each, followed by washing with three times of Xylene and Xylene/paraffin (1:1) overnight. Then, tissues were embedded in paraffin and sectioned 8-µm thick for Hematoxylin & Eosin (H&E), Picrosirius Red (PSR) and Verhoeff-Van Gieson (VVG) staining.

D. Semi-Quantitative of Collagen and Elastic Fibres

The percentage area of collagen and elastic fibres were quantified using ImageJ software (National Institute of Health, USA) version 1.51j8. All images were randomly selected with an objective lens magnification at x20 and the image pixel was measured using the measure tool. First, the background subtract was performed to remove any noise in the images. Then, the colour deconvolution was performed to separate the RGB channel. The blue channel was adjusted with the threshold of 0-250.

E. Statistical Analysis

Values are shown as means ±SD. Significance differences between the aorta tissues were analyzed by Student's t-Test using the SPSS for Windows version 10.0 (SPSS GmbH Software, München, Germany). P values less than 0.05 were considered significant.

III. Results

A. Cell Removal Analysis

The effectiveness of sonication treatment in preparing bioscaffolds was investigated based on the cell removal efficiency and collagen and elastin preservation as the main component of the extracellular matrix.

Cell removal analysis was studied through the Hematoxylin & Eosin (H&E) staining as shown in Figure 1. The staining demonstrated the even distribution of nuclei cells (blue colour) in native tissues. Complete removal of nuclei was obtained after sonication treatment with no nuclei present throughout the samples. Meanwhile, the nuclei cells still detected in aortic tissues decellularized by immersion treatment.

B. Structural Integrity Analysis

The collagen and elastin preservation in aortic scaffolds were determined by Picrosirius Red (PSR) and Verhoeff-Van Gieson (VVG) staining respectively. The collagen fibres integrity was studied through the PSR staining as shown in Figure 2.

The staining with the bright field images showed the collagen fibres as a red colour for type I and pink for type III.

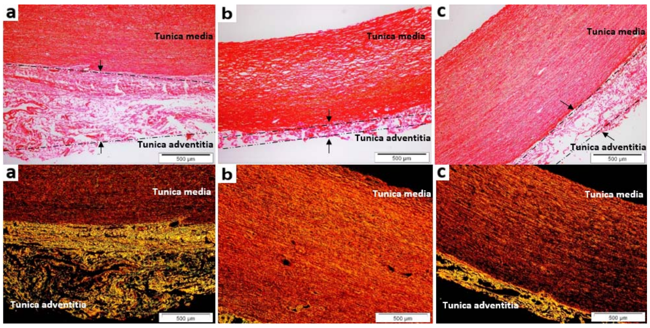

Fig. 2. Picrosirius Red staining with bright field (top panel) and polarized (bottom panel) images of (a) native aortic tissue, (b) immersed aortic tissue and (c) sonicated aortic tissue. Arrow indicates the thickness of tunica adventitia in aortic scaffolds.

Meanwhile, the staining with a polarized light image showed the collagen fibres as yellow to red colour for type I and green for type III. For bright field image, in native aortic tissues, the collagen fibres were arranged densely in media and adventitial layer. The preservation of collagen structure was obtained in sonicated aortic tissue with the dense arrangement of collagen fibres. Meanwhile, the loose arrangement with increasing the interfibrillar space of collagen was obtained in aortic tissue decellularized by immersion treatment. For a polarized light image, two distinct layers could be observed from the polarized images. The inner layer which is the tunica intima and tunica media demonstrated the coarse bright red or yellow with thin green fibres. The peripheral layer which is the tunica adventitia contained the bright yellow. Besides the arrangement of collagen fibres, the thickness of the adventitial layers was different. The highest thickness is in native aortic tissues and was decreased with the decellularized treatment. The thinnest layer was demonstrated in aortic scaffolds decellularized by immersion treatment.

For Verhoeff-Van Gieson staining as shown in Figure 3, elastic fibres were stained as dark blue while collagen fibres were stained as pink. The elastic fibres in aorta were arranged in wavy whip-like appearance and have a branching. In native aortic tissues, elastic fibres were stained densely and evenly distribution throughout the section with intact collagen. The densely and evenly distributed of elastic fibres was preserved in aortic scaffolds decellularized by sonication treatment. Meanwhile, the elastic fibres were not evenly distributed in the aortic scaffolds decellularized by immersion treatment. The elastic fibres in centre of media layer were loosely arranged while in the peripheral of media layer were compacted with the loss of collagen fibres.

C. Semi Quantification of Collagen and Elastic Fibres

The semi-quantification of collagen and elastic fibres in samples were quantified by ImageJ in Figure 4. The percentage area of collagen fibres in native aortic tissues was 60%. The number of collagen fibres in sonicated and immersed aortic tissues was significantly increased than

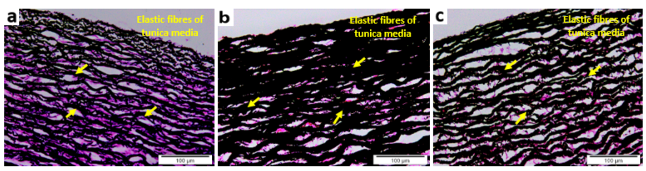

Fig. 3. Verhoeff-Van Gieson staining of (a) native aortic tissue, (b) immersed aortic tissue and (c) sonicated aortic tissue. Arrow indicates the elastic fibres in tunica media of aortic scaffolds.

native aortic tissues which account about 79% and 90% respectively. The percentage area of elastic fibres in aortic tissues was 66% before decellularization and 74% and 72% after decellularization by immersion and sonication treatment respectively. This represented a 10% of increase elastic fibres in decellularized aortic scaffolds.

IV. DISCUSSION

Several decellularization methods were developed with the aim to remove the cells while maintaining the three-dimensional structure of ECM. However, all the methods are recognized to lead some disruption to ECM structure. The proprietary methods used in this study incorporated the ultra-sonication and SDS which have shown the effectiveness to remove cells completely [11]–[13]. The structural integrity of decellularized aortic tissues was examined to assess the effect of sonication on collagen and elastin as the main component of the extracellular matrix (ECM). The preservation of structural configuration and components of ECM based scaffolds is highly desirable to provide biological activity as well as mechanical strength and elasticity as closest to native tissues.

The structural integrity of the aortic scaffolds is important in the evaluation of decellularized samples as it affects the normal function and stability of the vessels. Both collagen and elastin are crucial for the determination of the tensile strength, stiffness, and elasticity of the aorta. Elastic fibres in the aorta have an important role to provide elastic distention with minimal energy lost during diastole and allow intrinsic recoiling to their original position during systole. While collagen limits the distention of the aorta to prevent the damage. Both elastin and collagen determine the passive mechanical behavior of the aorta that subsequently determines the load on the heart that in turn determines the cardiac function [14]. Therefore, both elastin and collagen structure are critical to the mechanical behavior and function of the aorta in the cardiac cycle.

Histological analysis confirmed that cells were completely removed from the aortic bioscaffolds. In addition, the SEM (unpublished) analysis confirm the structure of collagen and elastin in aortic bioscaffolds was well preserved. Naturally, we expect the percentage area of collagen and elastic fibres in native aortic tissue to be greater than in decellularized tissues. Since the collagen and elastic fibres content will not be increased by decellularization.

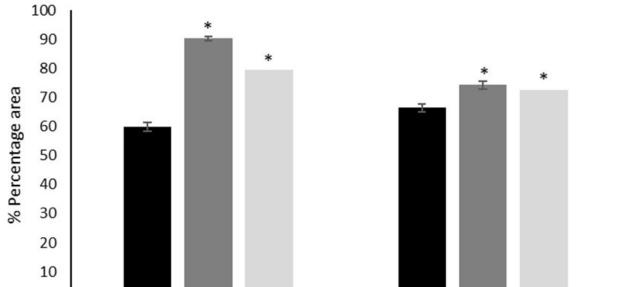

Fig. 4. Percentage of collagen and elastic fibres in (A) native aortic tissue, (B) immersed aortic tissue and (C) sonicated aortic tissue.

However, a significant increase of collagen and elastic fibres were observed in aortic bioscaffolds compared to native aortic tissues. The loosely packed of decellularized aortic tissues with absent of cellular components allow of the dye to stain easily. These results are supported by the previous study that describes the collagen detection with Sirius red which found that the blocking of amino group reduced the uptake of Sirius red by the collagen [15].

The disruption of the spatial organization of collagen and elastin strongly affects the cellular response and mechanical properties of the aorta in response to pulsatile blood flow from the heart [16][17]. Cellular process in tissues is activated by the interaction with the cell binding sites such as integrin, discoidin, GPVI, and mannose which regulate the cellular activities including adhesion, proliferation, and migration. These cell binding site could not bind to the denatured of the ECM structure [18]. The previous study by Grazer *et al.* have investigated the effect of structure alteration after decellularization of ECM based scaffolds limit the cell repopulation [19]. The low repopulation of the cells might due to the loss of cell binding site in consequence of the ECM structure alteration. However, the previous study by Caralt *et al.* demonstrated that cells were repopulated on the decellularized rat kidney despite the loss of collagen [20]. Similarly, Syazwani *et al.* demonstrated the successful repopulation of VSMCs on slightly altered the decellularized porcine meniscus structure [21]. Recently, Zhou *et al.* successfully recellularized hepatocytes and endothelial progenitor cells (EPCs) in the rat liver with the slight change of architecture and composition of extracellular matrix [22]. Collectively, these results suggest the slight change of ECM structure and integrity do not necessarily hinder cell repopulation.

Several studies have previously found the disruption of ECM structure caused by SDS. According to Courtman *et al.*, after the development of pericardial acellular matrix by using different detergent demonstrated that the SDS could alter the internal charged state of the biomaterials structure. These charged increases after SDS bind effectively to the protein that can lead both swellings due to increasing water content and loss of thermal stability and finally, disrupts the hydrogen bonding of the collagen triple helical domain. The hydrophobicity of elastin is expected to reduce, opening the molecular structure that in turn, increasing the affinity of elastases [23]. Poornejad *et al.* reported the 1% SDS cause caused significant damage to GAGs, growth factors, and collagen fibers in the ECM of the porcine renal tissue scaffold [24]. Similar to study a by Woods and co-workers, which reported the disruption of collagen in porcine anterior cruciate ligament and altered its tensile-stiffness ligaments after decellularization with 1% SDS [25]. He *et al.* suggested that SDS exposure to decellularized should be optimized in terms of concentration and duration to improve the preservation of both structural and functional components of the whole organ bioscaffolds [26]. Taking into this consideration, we sonicated the aorta tissues with a lower concentration (0.1%) of SDS. Based on our results, decellularization using sonication decellularization system is effective to remove all cellular components and preserve the major structure of elastin and collagen of ECM. The sonication decellularization system enhances the penetration of SDS into deep tissue location with shorter time of SDS exposure to the tissues which might mitigate the SDS impact on the collagen and elastin structure on ECM. The higher concentration and prolonged exposure time of SDS to tissues may compromise the ECM integrity.

There is some limitation of this study. First, the thickness of aorta samples was not stable which affects the sonication power to disrupt the cell membranes and wash the cellular materials away. The approximately of the sample thickness was 1.0-1.5mm. Second, the biochemical and biomechanical properties of the bioscaffolds were not analyzed and conducted as functional tested to affirm the study. The slight change of the ECM structure might not affect the functionality of the bioscaffolds.

V. CONCLUSION

In conclusion, the sonication decellularization system is effective to remove all the cells while preserving the structural integrity of collagen and elastin on decellularized aortic tissues in a short treatment time. Therefore, this sonication decellularization system can be applied to obtain well-preserved bioscaffolds for the biomedical implant as promising decellularization protocol.

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