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# The Use of Sonication Treatment to Completely Decellularize Blood Arteries: a Pilot Study

A. Azhim, K. Yamagami, K. Muramatsu, Y. Morimoto and M. Tanaka

***Abstract*—We have developed a novel sonication decellularization system to prepare completely decellularized bioscaffolds in a short treatment time. The aim of the study is to investigate the sonication decellularization efficiency and its relation with ultrasonic power output and dissolved oxygen (DO) concentration in different detergent solution. In the study, we used aorta samples to evaluate sonication decellularization efficiency, which assessed treatment duration, sonication power and SDS detergent with/without saline. The treated samples were evaluated histologically by Hematoxylin Eosin (HE) staining and scanning electron microscopic (SEM) photographs. The concentration of DO was monitored to identify the effect of sonication on cavitation-related DO concentration in the solution. From histological results, the sonication decellularization efficiency was better than the other preparation methods. Decellularization efficiency was tended to increase significantly when DO value decreasing after 6 hours of treatment. In conclusion, we conclude that sonication treatment can be used to prepare the complete decellularized scaffolds in short treatment time.**

## I. INTRODUCTION

Tissue engineering holds the potential for key technologies to the repair and reconstruction of atherosclerotic vascular, valvular heart, aneurysm and varices [1-3].

Decellularized tissue is promising as an ideal scaffold for cardiovascular tissue engineering. Decellularization techniques are classified by the chemicals used, such as acid or alkaline treatment, detergent treatment, or enzymatic digestion and the physical methods used, such as snap freezing and mechanical agitation [4-8]. Among these, detergent treatment is the most widely used. Decellularization of biological tissues by detergent treatment has the advantage of being easy to use, but its drawbacks include long treatment time, alteration of mechanical properties, and residual toxicity [9]. Researchers have developed specific treatment recipes to overcome each of these problems. Kishida et al. used high-hydrostatic pressure (HHP) method to produce a decellularized tissue that did not adopt any chemical agents [10, 11]. More recently, Karim et al. used a rotating perfusion bioreactor to decellularize and re-seed porcine heart valves [17], and Ott et al. decellularized whole rat hearts using a modified Langendorff apparatus [18].

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As another candidate for a new decellularization treatment, we have reported our study on the sonication decellularization method [12]. To maximize sonication decellularization efficiency, we hypothesize that the efficiency is depended on an independent factor of cavitation intensity. Even though cavitation phenomenon affected by several variables, in the study, we attempted to investigate its occurrence contributed by ultrasonic power, detergent with/without saline and concentration of DO.

The purpose of this study is to investigate the usefulness of sonication decellularization and determine its efficiency by related to ultrasonic power output and DO concentration in detergent solution with/without saline.

## II. METHODS

### A. Sonication Decellularization System

We have developed a decellularization system using sonication treatment as illustrated in Fig. 1. The decellularization system consisted of a commercially available ultrasonic horn (Sonifier 250A, Branson), a roller pump (RP-1000, Eyela), a constant temperature water bath (LTB-250, As-One), a temperature monitor (TR-71U, T&D), DO meter (DO-206, SAGA Electronic) and a custom-made reactor. Due to increase of solution temperature by sonication, we set a sensor beside the sample in the reactor to control temperature at constant. We performed the experiments at a constant temperature of 36±1°C [12]. The measurements of DO concentration in the solution were measured by DO meter.

We used porcine descending aorta in the preliminary study

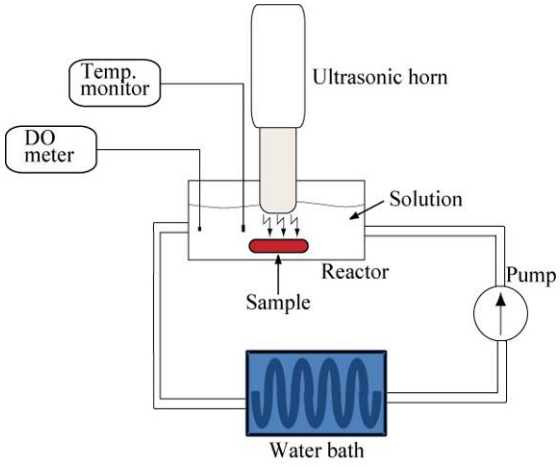


Fig. 1 Sonication decellularization system consisted of ultrasonic horn, pump, cooling water bath, reactor, temperature and dissolved oxygen (DO) sensors.

to investigate the sonication effects. The aorta was obtained from a local slaughterhouse (Tokyo Shibaura Organ Co. Ltd, Japan). The aorta samples were cut into approximately 15×15mm$^2$ and sonicated from the luminal side. We fixed the samples in the reactor at depth of 12 mm from the irradiated ultrasonic horn. We processed the samples at a constant circulating of detergent-based decellularization solution for 3~24 hours. We set ultrasonic power to 15 and 30 Watts of continuous oscillation. The frequency of ultrasound was 20 kHz. In the study, we used 2% SDS with/without 0.3% sodium chloride (NaCl) of solution (pH 5.6) to improve the efficiency of cell removal.

For a control, a typical decellularization: immersion and shaking decellularization was performed using that similar detergent solution.

### *B. Histological Analysis*

Histological analysis was performed to evaluate tissue integrity and cell removal. We fixed, paraffin-embedded and sectioned decellularized samples following standard protocols. We cut samples into 8-um sections, stained them with Mayer's Hematoxylin-Eosin (HE) and photographed the sections on a microscopic CCD camera (Olympus DP-71) as shown in Fig. 2. In sonication decellularization, there was clearly a complete decellularization at irradiated specific region in the extra-cellular matrix (ECM). Therefore, we evaluated the region as decellularization depths which were measured from luminal side (see Fig. 2).

We fixed samples sections with 4% paraformaldehyde (PFA) for 24 hours at 4°C before staining. We dehydrated the samples with sequential alcohol gradient (50, 60, 70, 80, 90 and 100% for 1 hour each gradient) and xylene (3×1 hours), embedded in paraffin for 3×1 hours at 60°C, and sliced by microtome.

### *C. Scanning Electron Microscopy*

We fixed the treated tissue with 1% glutaraldehyde for a hour at room temperature. We dehydrated the samples with a series of ethanol solutions of increasing concentration, beginning with 50% and progressing through 60%, 70%, 80%, 90% and 100% for 20 minutes each. We transferred from 100% ethanol to 50% (v/v) tert-butyl alcohol (TBA) or 2-Methyl-2-propanol in 100% absolute ethanol for 20 min at 37°C and transfer to 2×100% absolute TBA every 20 min at 37°C. We immersed the sample in 100% TBA, carefully dissected it into pertinent regions of interest from the anterior apex, keeping one dimension of the tissue at ~3 mm and then froze it for 30 min at 4°C. We freeze-dried the sample for approximately 3 hours with a freeze drying device (JFD-310, JEOL). We sputter coated sample with gold (Au) for 60s using a Fine Coater (JFC-1200, JEOL). We then visualized samples with a scanning electron microscope (JSM-5310LVB, JEOL) as shown in Fig. 6.

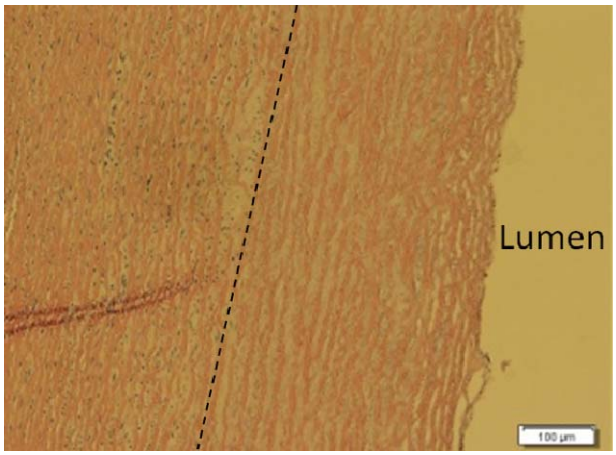


Fig. 2 Representative of HE staining photomicrographs by sonication treatment (30W output) in 2% SDS, 0.3%NaCl for 15 hours. Dash lines indicate the complete decellularization boundary in the extracellular matrix.

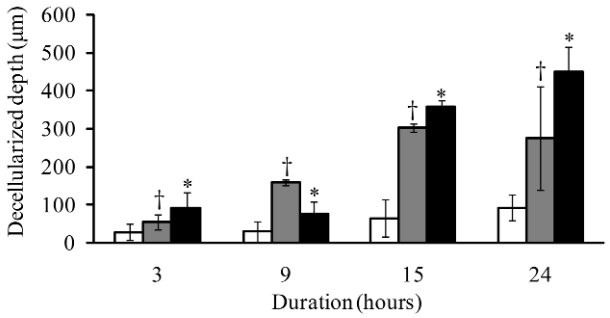


Fig. 3 Decellularization depth after performing for 3, 6, 15 and 24 hours using immersion (white bar) and sonication (15W (gray bar) and 30W (black bar) power output) treatments, respectively in 2%SDS, 0.3%NaCl detergent solution. *P<0.05 significances compared with the power output of 15W, †P<0.05 significances compared with immersion ones.

## III. RESULTS

Fig. 3 presents the complete decellularization depth in immersion and sonication for 3-24 hours-treatments. The decellularized depth increased significantly in sonication treatments after 9 hours ($P< 0.05$).

Fig. 4 shows HE staining photomicrographs of native aortic (A) and those of treatments using 2% SDS with/without 0.3%NaCl. Due to sonication at specific region on ECM, cells were completely removed, but did not in immersion treatment. Therefore, effects on decellularization

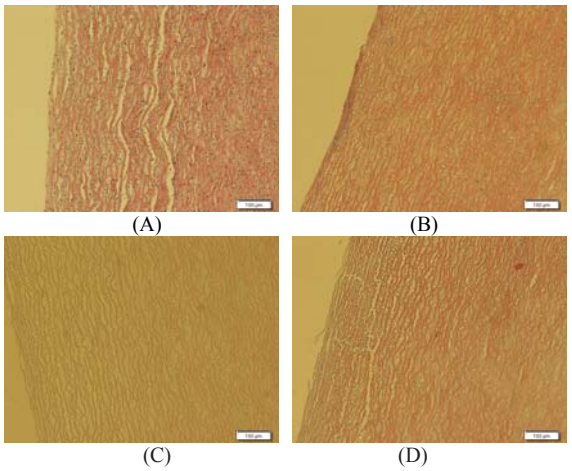


Fig.4 Photomicrographs of HE staining of native aortic (A), immersion treatment in 2%SDS solution (B), ultrasonic treatment in that buffer for output of 15W (C) and 30W (D). All treatments were performed for 24 hrs.

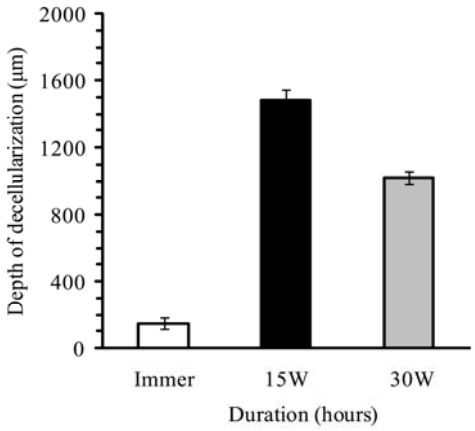


Fig.5 Depth of complete decellularization by immersion (white bar) and sonication treatments (black and gray bars for power output of 15 W and 30 W, respectively) with 2 % SDS solution for 24 hours

were assessed by measurement of complete decellularized depth from luminal to dashed line boundary in the ECM (see Fig. 2).

Fig. 5 represents complete decellularization depth which sonicated for 24 hours in 2%SDS solution with different power output of 15W and 30W. The depth was measured from their HE staining photographs as shown in Fig.4C and 4D. There are differences in 15W (black bar) and 30W (gray bar) because of the different thickness of the used samples.

Fig.6 shows the SEM photographs observed from intimal surface view. There were no significant changes on basement membrane surface between native (A1 and A2) and immersion treated samples (B1, B2, C1 and C2). However, using sonication treatments, ECM fibers were found barely formed.

Fig. 7 presents DO concentration in the 2% SDS with/without 0.3%NaCl when sonication decellularization treatment for 24 hours. After 6 hours of treatment, both DO values decreased and constantly changed at 11.67± 0.79 and 11.98± 0.50, respectively.

## IV. Discussion

A novel application of ultrasonic technology affords the possibility to efficiently decellularize aorta tissues for bio-scaffold preparation as discussed in the paper.

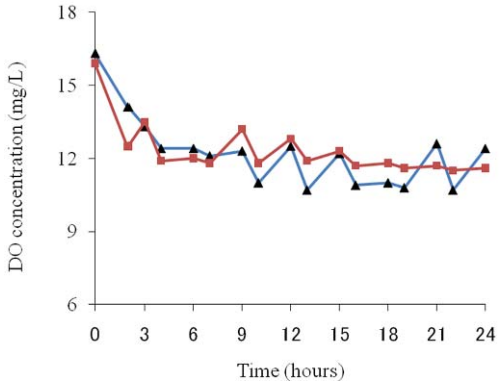


Fig. 7 Concentration of DO in sonication treatments for 2%SDS, 0.3%NaCl (▲) and 2%SDS (■).

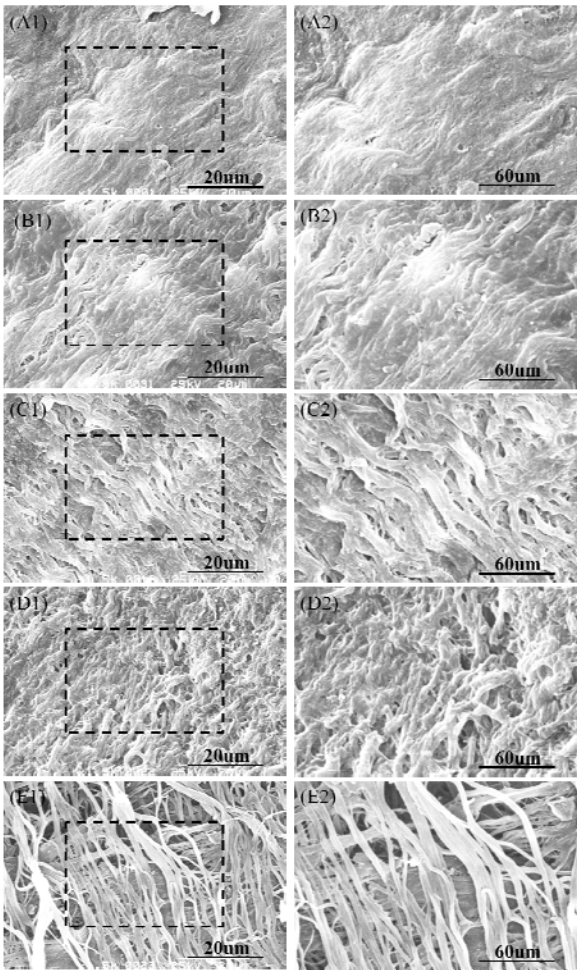


Fig.6 Representative SEM images from the intimal surface view for native tissue (A1), 24 hours immersion treatments in 2%SDS, 0.3%NaCl (B1) and in 2%SDS (C1), 24 hours sonication treatments in 2%SDS, 0.3%NaCl (D1) and 2%SDS (E1). Original magnifications indicate ×1,500. Dashed line boxes are the enlarge views (×5,000) as shown in A2, B2, C2, D2 and E2.

Ultrasound has been used for a variety of purposes that includes areas as diverse as the detection of flaws in concrete buildings, the synthesis of fine chemicals and the diagnosis of disease. By the 1960s the industrial uses of ultrasound power were accepted and being used in cleaning and plastic welding which continue to be major applications. Nowadays there are research groups and industries with expertise in a much wider range of activities that involve the uses of ultrasound in electrochemistry, food technology, chemical synthesis, materials extraction, nanotechnology, phase separation, surface cleaning, therapy and water and sewage treatment [15].

Ultrasound has been divided broadly into those who use it as a measurement device with no effect on the medium (high frequency low power ultrasound e.g., in non-destructive testing) and those who use it to produce physical or chemical effects in a medium (higher power low frequency ultrasound e.g. in sonochemistry). In the study, we demonstrated that sonication using low frequency ultrasound is capable to completely decellularize aorta. However, we must take sonication power into account as the related destruction on its

ECM (see Fig. 6). ECM had no significant function consequence when scaffolds are lyophilized. The optimization of decellularized preparation without effect on collagen matrix should be more evaluated by circular dichroism spectroscopy in further study.

We have studied the feature of ultrasonic pressure field as presented previously [12]. According to an understanding of the ultrasonic pressure fields, samples were fit at an optimal distance of irradiation. As ultrasonic beam has higher directivity, spatial decellularization was depended on diameter of horn. In the study, spatial homogeneity of our treatment was limited to approximately $10cm^2$ of decellularization, corresponding to its diameter of horn.

It is known that the micelle size is various, depending on detergent species, nature of added salt, and temperature, and different models have been proposed for surfactant micelles [13-14]. The concept of micelle formation is relevant to solubilization and reconstitution studies of membrane proteins since it appears that there is some correlation between the ability to form micelles and the concentration of detergent required for solubilization. The optimal solubilization of the membrane protien can be achieved with pre-micellar concentration of surfactant under high salt concentration [13]. However, adding salt in the SDS detergent solution found that did not contribute to the increase of sonication decellularization efficiency in this study. The relations between micellar concentration and DO concentration have to be investigated in the further study.

According to the knowledge of ultrasonic cleaning process, cavitation is one of important factors must be to consider to maximize the effective of the processes. The cavitation intensity are affected by temperature, viscosity, the solubility of gas in the liquid, the diffusion rate of DO in the liquid and vapor pressure. For most effective cavitation, the used liquid must contain as little DO as possible [15]. Regarding to Fig.3 and Fig. 7, we could explain that decreases of DO concentration in the detergent were contributed to the significant increases of decellularization efficiency.

There are some limitations. The obtained aorta samples were size was not stable, therefore, there are approximately 1.0-1.5 mm thickness of samples (Fig. 5). Sonication treatment can be used to assist decellularization however, it must be noted that increased sonication power would be disrupting cell membranes and rinsing the cellular material away. It must be taken into account both decellularization and disruption of cell membrane influences to prepare bio-scaffolds for tissue engineering applications.

In conclusion, we conclude that sonication treatment can be used to prepare the complete decellularized scaffolds in short treatment time.

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