In Vitro Recellularization of Aorta Scaffolds Prepared by Sonication Treatment

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Abstract— **Sonication treatment is used in the preparation of bioscaffolds that was able to support repopulation of vascular smooth muscle cells (VSMCs) upon cell-seeding. The aim of this study is to investigate the ability of sonicatedly decellularized tissue to repopulate VSMCs after 6 days of cell-seeding. In this study, sample of aorta tissues are decellularized by sonication treatment in 0.1% and 2% sodium dodecyl sulfate (SDS) detergent for 10 hours. It was followed by washing process with PBS solution for 5 days. Decellularized aorta tissues are then cell seeded with VSMCs by static seeding in 96-well plate containing Dulbecco's Modified Eagle Medium (DMEM) at 37°C. The infiltrations of VSMCs onto decellularized tissues are evaluated by comparison of Hematoxylin-Eosin (H-E) staining at 0 and 6 days of cell-seeding. The histological results of cell-seeding showed that VSMCs are able to infiltrate onto the decellularized tissues. From the results, sonicatedly decellularized tissue treated in 0.1% and 2% SDS, seeded with VSMCs showed infiltration depth of 0.43 mm and 0.35 mm, respectively. Hence, a sonicated decellularized tissue treated with 0.1% and 2% SDS was shown to support the repopulation of VSMCs.**

Keywords—decellularization, sonication, bioscaffold, aorta, cell-seeding

I. INTRODUCTION

Cell-seeding is one of the crucial steps for the development of tissue-engineered vascular grafts. Dissemination or lining of isolated cells within decellularized tissues are known as cell-seeding process [1]. Tissue-engineered grafts should be covered with specific population of autologous cells to reduce the possibility of graft thrombosis and intimal hyperplasia [2]. Lining of cells on the scaffolds lead to the production of cell's own matrix proteins that subsequently substitute the pre-seeded scaffolds [3]. These processes can be triggered through *in vitro* (tissue engineering) and extended to *in vivo* (guided tissue regeneration). There are several cell seeding techniques and cell sources that have been investigated for the preparation of tissue-engineered grafts. The most commonly used technique is static cell-seeding in which the concentrated cell suspension was pipetted into lumen or outside of the scaffolds. The seeded scaffold was then incubated for several hours or day to allow cellular attachment and migration [4]. Seeding of scaffolds using autologous vascular cells and stem cells was able to promote neotissues development and improve graft patency rates following *in vivo* implantation [5,6]. However, previous research showed that construction of thicker tissues has been impossible due to limited diffusion of nutrients and oxygen within decellularized tissues [7]. Moreover, the cells were only able to survive within an area of approximately 1-3 millimeters from the source of nutrients and oxygen. In our previous study, sonication treatment which applied the combination of ultrasonic and sodium dodecyl sulphate (SDS) has been reported to prepare complete decellularized tissue [8]. Hence, this study attempted to produce biological scaffolds from sonication treatment that can support the repopulation of vascular smooth muscle cells (VSMCs) by using modified static seeding method.

II. METHODS

A. Sonication Decellularization System

We have developed a decellularization system using sonication treatment as described previously [8, 9]. The decellularization protocol consists of two steps; 1) sonication treatment and 2) washing. The experiment was performed at a constant temperature of 36±1°C [10] with temperature sensor set beside the samples. Depending on the sonication power, the samples in the reactor was fixed at a constant depth of approximately 10 mm from sonicated ultrasonic transducers. The samples are set and controlled by actuators which move in XYZ axis contour.

Porcine descending aorta was obtained from a local slaughterhouse (Tokyo Shibaura Organ Co. Ltd, Japan). The aorta samples were cut into approximately 15x15 mm^2 and sonicated from the luminal side. The samples were processed at a constant circulating of detergent-based decellularization solution for 10 hours. The ultrasonic power was set to approximately 500 mV_{rms} of continuous oscillation. The frequency of ultrasound was 170 kHz. In the study, 0.1% (w/v) and 2% (w/v) SDS detergent were used.

B. Washing process

After the sonication treatment, samples were washed with Phosphate Buffer Saline (PBS) solution for 5 days. The PBS was replaced and the samples are stored at 4°C.

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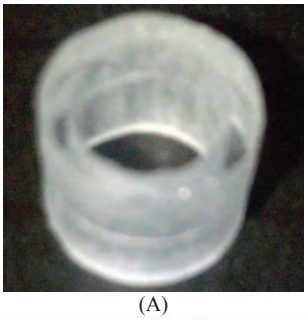
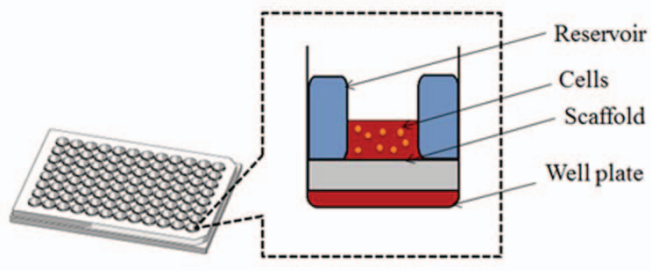


(A)



(B)

Fig. 1 Cell-seeding of VSMCs (A10 cell) onto decellularized aorta tissues. Self-designed O-ring shaped reservoirs (a), and illustration of A10 cell-seeding in 96-well plate for 6 days.

C. Vascular smooth muscle cells (VSMCs) seeding onto bioscaffolds

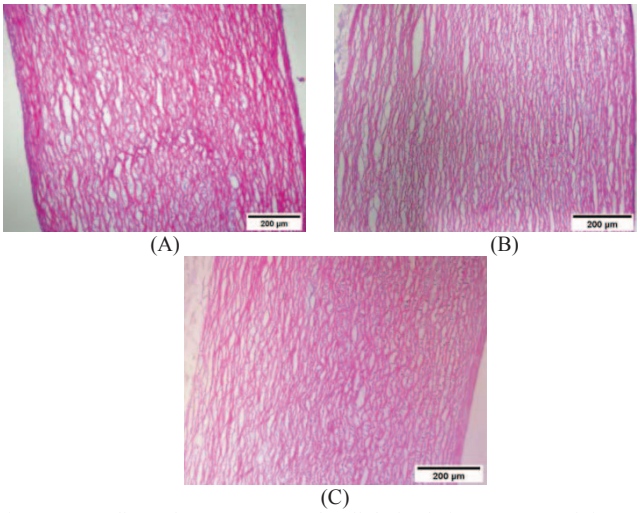
Self-designed O-ring shaped reservoirs were placed on top of decellularized aorta tissues in 96-well plate followed by seeding of VSMCs derived from A10 cell line as shown in Fig. 1. VSMCs derived from A10 cells (*ATCC,* Rockville, Maryland) were seeded at concentration of $1.5x10^4$ cell/cm^2/well. The scaffolds seeded with A10 cells were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 20% of fetal bovine serum (FBS), 150 units/ml of penicillin and 150 ug/ml of streptomycin. The scaffolds were then incubated at 37°C in 5% CO_2 and 95% air for 6 days with a change of culture medium at the first 24 hours and every 2 days thereafter.

D. Histological Analysis

Decellularized tissues were fixed, paraffin-embedded and sectioned following standard protocols of Hematoxylin-Eosin (H-E) staining [10]. The samples were cut into 8-µm sections, stained with Mayer's Hematoxylin-Eosin and photographed on a microscopic CCD camera (Olympus DP-71). The VSMCs infiltration was evaluated by comparison of H-E staining at 0 and 6 days of cell-seeding. Image J (National Institute of Health, USA) was used to measure the infiltration depth of VSMCs onto decellularized tissues. The results were expressed as mean ± standard deviation (SD).

E. Scanning electron microscope (SEM) analysis

Samples were fixed with 1% glutaraldehyde for 1 hour at room temperature. The tissues were then dehydrated with a series of ethanol solutions of increasing concentrations,



(A) (B)

(C)

Figure 2 Seeding of VSMCs onto decellularized tissue. H-E staining of sonicatedly decellularized tissue on 0 day (A), 3 days (B), and 6 days (C) after cell-seeding.

starting from 50%, 60%, 70%, 80%, 90% and 100% for 20 minutes each. The tissue was 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. The samples were then immersed in 100% TBA and carefully dissected into regions of interest, keeping one dimension of the tissue at ~3 mm and then froze for 30 min at 4°C. Later, the samples were freeze-dried for approximately 3 hours with a freeze-drying device (JFD-310, JEOL) and visualized with a scanning electron microscope (JSM-5310LVB, JEOL).

I. RESULTS

Upon 6 days of cell-seeding, the seeded decellularized tissues were evaluated by H-E staining to observe infiltration of VSMCs. Fig. 2B show the H-E stained samples for sonicatedly decellularized aorta tissues after 3 days of cell-seeding. The VSMCs were observed to infiltrate the decellularized tissue and repopulated the niche of native tissue. Following the 6 days of cell-seeding, high density of VSMCs were present on the samples and indicating that seeded cells were distributed evenly throughout decellularized tissue as shown in Fig. 2C.

The infiltration depth of VSMCs onto decellularized tissues were analysed and compared between each samples as shown in Fig. 3. The decellularized aorta tissues treated with sonication treatment in 0.1% and 2% SDS seeded with VSMCs show infiltration depth of 0.43 mm and 0.35 mm, respectively.

SEM was performed to observe the adherence of VSMCs on decellularized tissues for 0, 2, 3 and 6 days of cell-seeding as shown in Fig. 4. The VSMCs were observed to adhere tightly on the adventitial (Fig. 4C) and endothelial surface (Fig. 4D) of decellularized tissues after two days of cell-seeding. As cell seeding reached day 3, the SEM show round VSMCs on the adventitial and endothelial surface of decellularized tissues as shown in Fig. 4E and Fig. 4F,

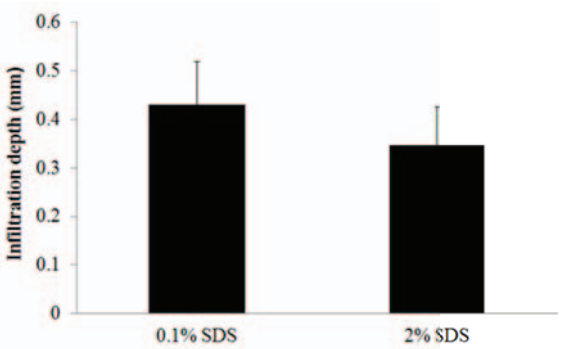


Figure 3 Infiltration depths of VSMCs onto decellularized tissues after 6 days of cell-seeding. Sonication treatment was performed in two SDS concentration, 0.1% SDS and 2% SDS.

respectively. The density of seeded cells adhered to decellularized tissue show increase in cell along with the seeding incubation time. The SEM of decellularized tissue show high density of VSMCs on adventitial (Fig. 4G) and endothelial (Fig. 4H) surface after 6 days.

II. DISCUSSION

Seeding cells into decellularized matrix is one of the fundamental techniques of tissue engineering. Various methods have been proposed for seeding cells into decellularized tissue, including static seeding. Static seeding is the most commonly used method to seed a scaffold due to its simplicity, minimal technical requirements, feasible in laboratory application and minimal hydrodynamic damage to the cells [11]. However, this method resulted in limited cells infiltration, inhomogeneous distribution and low seeding efficiency [12].

Previous study has demonstrated pure static seeding on the surface of dermis as the reference method [13]. These pure static seeding methods showed no cellular infiltration upon 12 days of cell-seeding. This is further confirmed with the immunofluorescence image of seeded scaffolds as a thin cell monolayer was observed to deposit on the surface of human acellular dermis. Prior to limitation of static cell-seeding, previous literature has demonstrated a modification of static seeding with the use of silicon ringlets [14]. The use of silicon ringlets during seeding of decellularized ovine carotid arteries have improved the exchange of nutrients and oxygen while preventing the collapsing and narrowing of scaffolds after absorption of culture medium. On other hand, semi-static seeding has been introduced to improve the uniformity of cell-distribution for seeding of chondrocytes into polyglycolic acid scaffolds [15]. These new method was developed involving periodic manual inversion of scaffolds in well plates followed by restricted agitation in conventional T-flasks. This simple and easy-to-perform modification of standard static method has resulted to substantial improvements in seeding efficiency, homogenous cell distribution and greater uniformity of ECM deposition in cultured constructs [15].

Therefore, in the present study, a modified static seeding method was performed to recellularize aorta scaffold. During

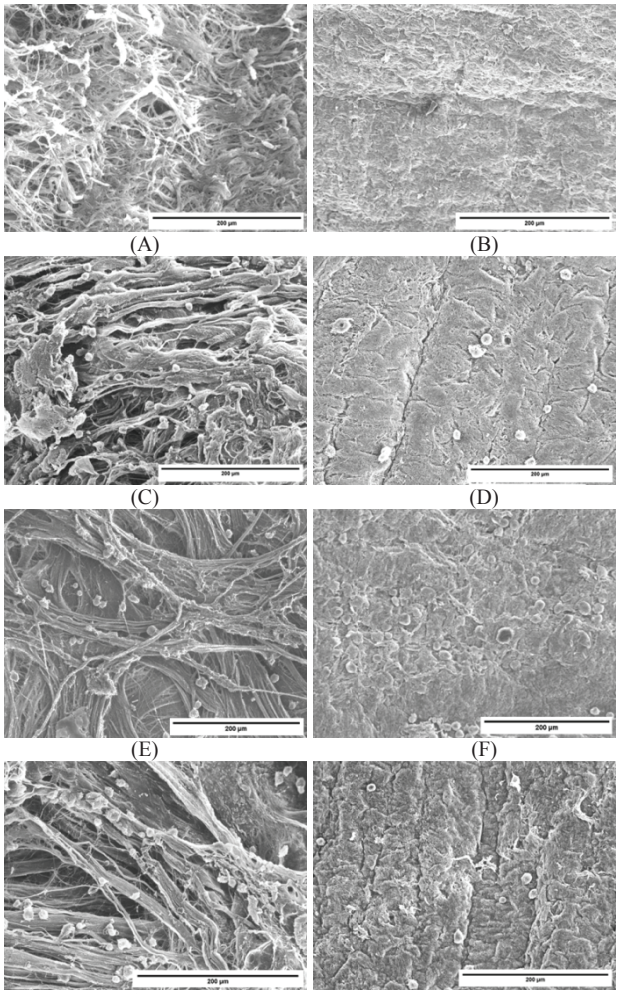


Figure 4 Scanning electron micrographs of VSMCs seeded on adventitial surface for 0-day (A), 2-day (C), 3-day (E), 6-day (G), and endothelial surface for 0-day (B), 2-day (D), 3-day (F), 6-day (H).

cell seeding, self-designed O-ring shaped reservoir was fixed on the scaffold to improve the seeding efficiency of static seeding method. It is followed by seeding of VSMCs on decellularized tissues within the reservoir. The use of self-designed O-ring shaped reservoir has increased the efficiency of cell infiltration onto decellularized tissue. This is because the cell suspension was maintained on the scaffolds which are within the area of O-ring shaped reservoir. These reservoirs prevent the cell suspension from draining outside the region of cell-seeding. In addition, the use of reservoir enables exchange of nutrients and oxygen freely by passive absorption as there was a very small distance between culture medium and seeded scaffolds [14]. Hence, it is shown that with the addition of self-designed O-ring shaped reservoir to static cell-seeding, the efficiency of cell infiltration and adhesion is improved.

Compared to other seeding methods involving pure static seeding, cell suspension injection, incision and low-pressure seeding method from previously published literature [13], our proposed self-designed O-ring shaped reservoir showed higher infiltration depth. Our study reported penetration depth of 350 to 430 μm using self-designed O-ring shaped reservoir upon 6

days of cell-seeding. The infiltration depth of our study was observed to be higher than the previously mentioned pure static seeding which showed penetration depth of 0 μm, followed by cell suspension incision and low-pressure seeding method [13]. This comparison has proved the potential of self-designed O-ring shaped reservoir in the repopulation and infiltration of cells into the scaffolds.

Furthermore, the result of cell infiltration has successfully eliminated the hypothesis reported by previous studies which showed that construction of decellularized tissues are impossible due to limited diffusion of nutrients and oxygen within scaffolds [7]. The use of self-designed O-ring shaped reservoir has overcome the hurdle of improving static seeding efficiency in these studies.

The use of ultrasonication has been reported to aid in the recellularization of scaffolds. Previous study of ultrasonication with intensity of 360 W for a total of 1 min has demonstrated attachment of cells on the surface of scaffolds forming monolayer culture after 24 hours of cell-seeding [16]. However, a fine balance between cells removal and sonication intensity are required to preserve the architecture of extracellular matrix (ECM). Hence, this study was performed in such a way that sonication intensity used to decellularize aorta tissue was able to prepare adequate open porous matrix to support repopulation of VSMCs.

III. CONCLUSION

Sonication treatment holds the potential of preparing complete decellularized tissues that was able to support the infiltration and adherence of VSMCs. The findings show that bioscaffolds engineered from sonication treatment have great potential to be used in the future biomedical implant.

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