Decellularized Bovine Meniscus in Morphological Assessment Prior to Bioscaffold Preparation

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***Abstract*— Decellularization is a process of tissue treatment targeting cell removal. Sonication system was developed in order to decellularize meniscus tissues. The samples were sonicated in 0.1% sodium dodecyl sulphate (SDS) for 10 hours and at 40 kHz ultrasound frequency. All the samples were structurally examined using van Gieson, Picrosirius red, Safranin-O/Fast green staining, and scanning electron microscopic (SEM) observation. Histological analysis of sonication treated-samples by van Gieson staining demonstrated complete nuclei removal compared to the control samples. The Picrosirius red and Safranin-O/Fast green staining indicate the preservation of collagen and glycosaminoglycans (GAGs) structure, respectively. In addition, the morphological observation by SEM shows the availability of micropores on the surface of decellularized sample. Consequently, the sonication decellularization treatment did not affect extracellular matrix (ECM) properties, while forming micropores on the surface of meniscus tissues. This made it possible to proceed in other fulfillment of bioscaffold preparation.**

***Keywords*— Decellularization; sonication; meniscus tissues; bioscaffold material**

I. INTRODUCTION

Meniscus tissues comprised of medial and lateral components that are important in load bearing and distribution, shock absorption, joint lubrication and stabilization of the knee joint [1]. However, the meniscus tissue function can be deteriorated by sports-related injuries in young people or by long-term degeneration in elderly people [2]. Meniscus tear injury is normally located in the inner avascular part of meniscus tissue with tenuous blood supply, making it impossible to undergo normal healing process. The most common available clinical treatment for meniscus tear is partial meniscectomy. However, this treatment only resolves the short-term clinical problem and in the longer-term, it will still lead to cartilage degradation, increase in pain as well as the loss of joint function [3].

Recently, tissue engineering approaches has been proposed in order to develop a replacement meniscus using natural scaffold which also known as bioscaffold materials [4]. The sources of samples for bioscaffold materials can be isolated from human or animal, depending on the purpose of study [5]. A process called "decellularization" is required in the step toward bioscaffold preparation, followed by pre- and post- decellularization assessment. Typically, the decellularization treatments can be categorized into physical, chemical, and enzymatic methods [6]. The efficiency of decellularization is dependent on the specific methods and origin of targeted tissues. Each type of treatment's methods may affect the biochemical composition, tissue structure and mechanical behavior of the ECM scaffold [7]. Nowadays, physical methods such as sonication, or freezing and thawing can be used to facilitate the decellularization by chemical methods using SDS solution [7]. In our previous study, the decellularization using sonication at 20 kHz frequency with an aid of 2% SDS concentration had resulted in the highest removal of cells [8]. However, the presence of cells in meniscus could still be observed after the treatment. An attempt to increase cell removal efficiency with the preservation of ECM composition could be studied by increasing the sonication frequency, while reducing the SDS concentration.

The aim of the present study is to investigate the effects of 40 kHz sonication and 0.1% SDS onto the morphological properties of meniscus tissues. In particular, the histological and SEM evaluation were done in order to observe the cell removal efficiency while maintaining the ECM composition, prior to bioscaffold material preparation.

II. METHOD

A. Meniscus Tissues Preparation

The bovine meniscus tissues were obtained from a local slaughterhouse (Tokyo Shibaura Organ Co. Ltd, Japan), placed into sterile and chilled phosphate buffer saline (PBS), and

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transported to the laboratory. The specimens were then stored temporarily at -20°C until further usage. Prior to decellularization, the meniscus tissues were thawed and cut into approximately; 10mm width and 3 mm thick to prepare 10mm x 10mm x 3mm sample.

B. Sonication Decellularization System

A decellularization system was developed using sonication method. The decellularization system consists of a commercially available ultrasonic device (Fx-500-35/72/110/170K, Flexonic), a roller pump (RP-1000, Eyela), a constant temperature water bath (LTB-250, As-One), a temperature monitor (TR-71U, T&D), and a custom made reactor. In a reactor, the samples were fixed at 12 mm depth from the irradiated transducer and the ultrasonic power supplied was 15 Watts/cm^2. The decellularization process was carried out for 10 hours, where an ultrasound frequency at 40 kHz was applied in a constant circulation of 0.1% SDS solution. This experiment was performed at a constant temperature of 35±1°C. The usage of SDS solution aids in cells removal process. Additionally, a control experiment was performed in an immersion decellularization condition, and without the sonication exposure.

C. Cells removal evaluation in decellularized meniscus tissues

The decellularized samples were processed according to standard protocols. The samples were fixed with 4% paraformaldehyde (PFA) for 24 hours at 4°C. Then the fixed samples were mounted on Surgipath FSC22 (Leica, USA), and froze in dry ice before freeze dried. The samples were cut using a cryostat CM1510S (Leica, USA) into 8 µm sections and were stained by van Gieson. The samples were dehydrated with sequential ethyl alcohol gradient (50, 60, 70, 80, 90 and 100%×2 for 5 minutes each gradient) and xylene (2×5 minutes). Samples were observed from the cross-section. The cells removal was statistically analyzed using Student t-test. The data were analyzed using SPSS software, with data comparison at n=3. Results were considered significant at $p<0.05$. The results were represented as mean ± standard error (SE).

D. Collagen and Glycosaminoglycans (GAGs) evaluation in decellularized meniscus tissues

The decellularized samples were processed according to the previous method, (referred: part C). The collagen and GAGs structures morphology were determined by Picrosirius red staining and Safranin-O/ fast green staining, respectively.

E. Scanning Electron Microscopy (SEM) observation

The ultrastructure of the decellularized meniscus tissues was observed using the SEM (JSM-5310LVB, JEOL). Prior to observation, the decellularized samples were fixed with 4% paraformaldehyde (PFA) for 24 hours at room temperature. The fixed samples were then dehydrated with increasing concentrations of ethanol solution; 50%, 60%, 70%, 80%, 90% and 100% for 20 minutes respectively. The samples were transferred from 100% ethanol to 50% (v/v) tert-butyl alcohol (TBA) or 2-methyl-2-propanol in 100% absolute ethanol for 20 minutes at 37°C and transfer to 2×100% absolute TBA every 20 minutes at 37°C. The samples were immersed 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 were frozen for 30 minutes at 4°C. The samples were freeze dried for approximately 3 hours with a freeze dryer (JFD-310, JEOL). Lastly, the samples were subjected to gold (Au) sputter coating for 60 seconds using a Fine Coater (JFC-1200, JEOL).

III. RESULTS

A. Cells Removal in Meniscus Tissues

The decellularized meniscus tissues were analyzed using van Gieson staining in order to assess the presence of nuclei. Fig. 1(G) shows that the decellularization of meniscus tissue was successfully achieved as there is no nucleus detected within the sonication treated-tissue. The effective removal of cells by sonication method was further justified through a comparison with van Gieson staining of native tissue and immersion treated tissue. Fig. 1B, C, E and F shows the presence of nuclei at central and surface part of the native tissues and the immersion-treated tissues. The details of nuclei numbers are shown in Fig. 2. The central and surface parts of native tissues (Fig. 1B and C) were comprised of approximately 284.09 ± 49.21 nucleus/mm^2 and 1207.12± 353.10 nucleus/mm^2, respectively. Furthermore, the number of nuclei within the central and surface part of immersion-treated tissues (Fig. 1E and F) was approximately 170.45± 56.82 nucleus/mm^2 and 257.18 ± 99.37 nucleus/mm^2,

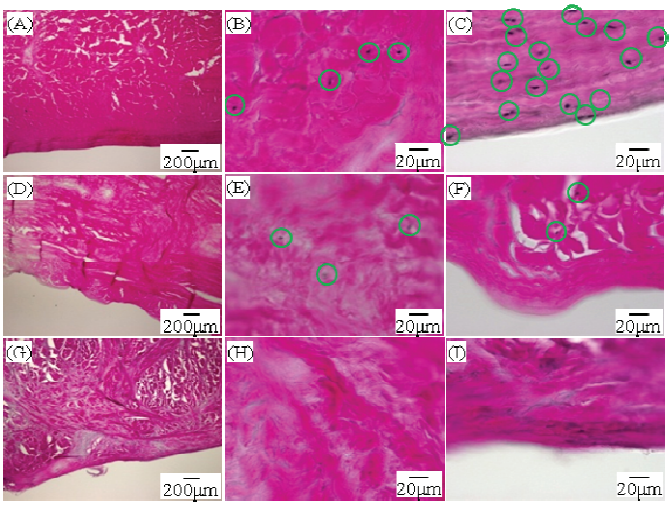

Fig. 1. Photographs of van Gieson staining for native tissues (A-C), immersion-treated tissues (D-F), sonication-treated tissues (G-I)

respectively. These values were lower than the aforementioned number of nuclei in native tissues, thus indicating an incomplete decelullarization process had occurred. Besides, it was shown that the number of nuclei were higher at the surface part of meniscus tissues and vice versa (Fig. 2).

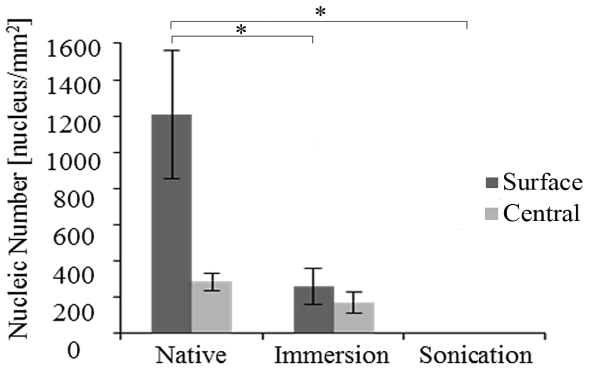

Fig.2. Nucleic number of before and after decellularization (native meniscus, immersion-treated, sonication-treated meniscus), (*$P < 0.05$).

B. Collagen morphology after decellularization

Picrosirius red staining is shown in Fig. 3 and reveals a highly dense nature of collagen network in the native tissue (Fig. 3A). The yellow arrow in Fig. 3(A) shows a bundle of collagen. The immersion-treated tissue reveals a much more porous architecture (Fig. 3D) compared to the sonication-treated tissue. Furthermore, the overall collagen network in sonication-treated tissues appeared fully intact, resembling the native tissues where the collagen Type I (yellow stained) and Type III (green stained) were preserved.

C. Glycosaminoglycans morphology after decellularization

The structure of meniscus tissues was further studied through safranin-O/fast green staining. This study revealed that there is a relatively homogeneous distribution of glycosaminoglycans (GAGs) throughout the tissue. Representative images of safranin O/fast green staining are shown in Fig. 4. Interestingly, the GAGs content (red color) in sonication-treated sample at central part of meniscus was still present. This shows the capability of sonication treatment to decellularize the meniscus tissues without affecting its structural properties.

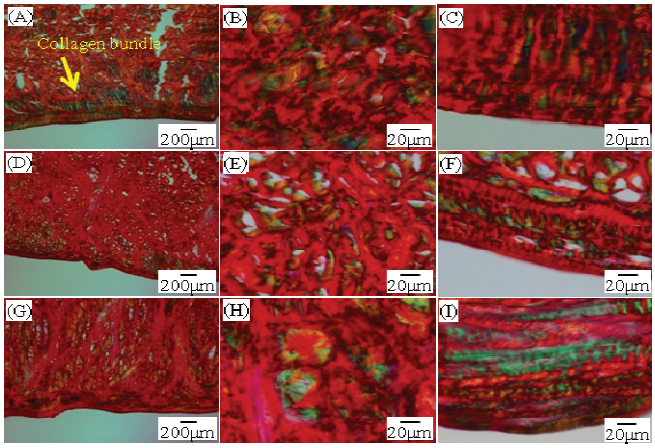

Fig. 3. Photograph of picrosirius red staining of native tissues (A-C), immersion-treated tissues (D-F), sonication-treated tissues (G-I)

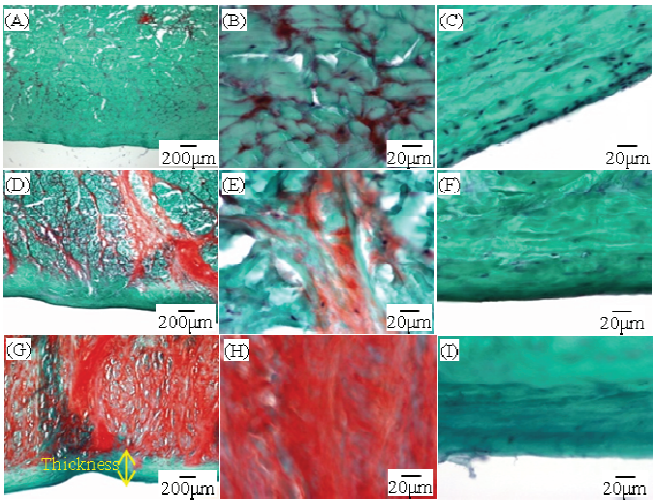

Fig. 4. Photograph of safranin-O/fast green staining of native tissues (A-C), immersion-treated tissues (D-F), sonication-treated tissues (G-I).

D. Scanning Electron Microscopy-Surface morphology

The SEM images of meniscus tissues are shown in Fig. 5. From this morphological observation, the samples of native tissues were filled with cells (asperities) (Fig. 5C). The sonication-treated samples showed a dense extracellular matrix (ECM) permeated by micropores. The numbers of micropores (green circle in Fig. 5G) were approximately 26±8 circumscribed channel/μm^2 and the pores areas (red arrow in Fig. 5I) were approximately 0.50±0.32μm^2. There were also cavities in immersion-treated samples (Fig. 5F).

IV. Discussion

Meniscus tissues are mainly composed of fibrocartilage and collagen with a tightly packed ECM [9], therefore it is much more difficult to decellularize, and the usage of chemical reagent alone is insufficient. A novel decellularization method using an ultrasonic energy could enhance SDS reaction in decellularization process of meniscus tissue. Thus, sonication contributes to cell membranes disruption and cell content release, while SDS solution aids in removing cell residues from tissues [10,6]. The efficiency of decellularization through sonication technique is hypothesized to be dependent on cavitation intensity. Acoustic cavitation is said to be the primary mechanism for tissue erosion, which might be correlated to the decellularization of meniscus tissues [11]. Based on the van

Gieson staining, decellularization by SDS immersion treatment shows that small number of nuclei still remained in the scaffold, thus treatment in SDS alone is insufficient for a complete decellularization of meniscus tissue. In spite of its weakness, treatment using sonication in the presence of SDS solution should increase the decellularization efficiency. An attempt to increase the sonication frequency to 40 kHz, while reducing the

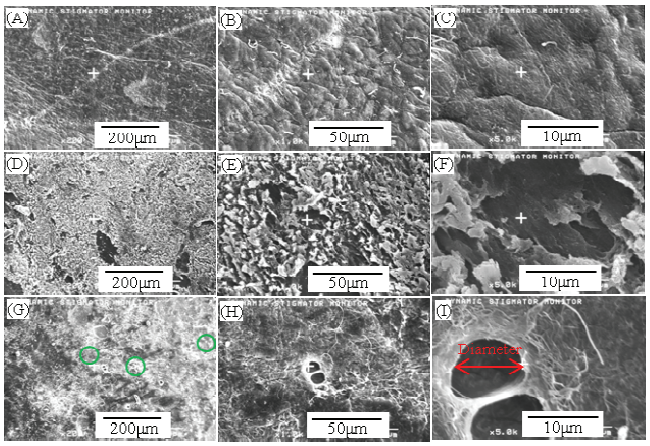

Fig. 5. Representative SEM images from native tissues (A-C), immersion-treated tissues (D-F), sonication-treated tissues (G-I).

SDS concentration to 0.1 % had successfully improved the cell removal efficiency. The period of decellularization used had also been increased to 10 hours, since the period of treatment in previous study is insufficient to completely decellularize meniscus tissue [8]. The improvement in decellularization outcome had proved that the aforementioned technique can be efficiently applied onto bovine meniscus tissues. However, decellularization efficiency of meniscus tissue should be further evaluated in term of their collagen and GAGs preservation.

Picrosirius red staining of sonication-treated tissues showed the preservation of collagen structure (Type I and III), and this could be useful for cells growth or recellularization process. Therefore, sonication-treated samples have the potential of bioabsorbable scaffold. One of the biochemical contents of meniscus tissue is GAGs [2]. The GAGs play an important role in maintaining the optimal visco-elastic behavior, compressive stiffness, tissue hydration due to high water content (~78%) and further facilitating in frictionless movement of the menisci over articular surfaces of the tibia and femur [12, 13]. In this study, the GAGs structure of sonication-treated tissues remained and thus, maintains the ability of meniscus to preserve water. From SEM analysis results, there was no asperity observed on the surface of sonication-treated tissues. In summary, sonication-treated tissues have better structural organization compared to immersion-treated tissues. Besides, SEM of the sonication-treated samples showed presence of micropores, which play an important role for future recellularization study [12].

V. Conclusion

In this study, bovine meniscus samples were completely decellularized using the combination of sonication and SDS treatment without negatively affecting the ECM properties. Therefore, these cell-free constructs could serve as an excellent bioscaffold material.

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