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Engineered Meniscus Scaffolds using Sonication Decellularization Treatment System

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Abstract: Meniscus located in the weight-bearing area responsible for the movement and functions of the knee. However, the frequent injuries within the avascular region of meniscus have lack of healing capability. Thus, the emerging decellularized scaffolds serve as one the interventions for the regeneration of new tissues to treat early degenerative joint disease. The aim of this study is to investigate the effectiveness of sonication treatment system in decellularization of meniscus tissues. The decellularization process was conducted in 40 kHz frequency with 0.1% SDS solution for 10 hours and proceeds with five days washing process. The decellularization efficiency was evaluated through histology, gel electrophoresis and biochemical assays to observe the cellular components removal and preservation of extracellular matrix (ECM). Compared to the control group, the histological evaluation of sonication decellularized scaffolds based on staining van Gieson showed complete removal of cellular components. Picrosirius red and Safranin O/fast green staining revealed the well preservation of the distribution of collagen and glycosaminoglycan networks (GAGs) in sonication decellularized scaffolds and no visible of DNA bands in the electrophoresis of agarose gel. Biochemical assessment for DNA quantification illustrated a significant decrement of DNA residues and GAGs for sonication decellularized scaffolds while maintained in collagen content. Based on the results, it can be deduced that sonication decellularization treatment system successfully prepared scaffolds with low cellular contents and maintained extracellular matrix components. Therefore, sonication decellularization treatment system can serve as one of the potential physical decellularization method in tissue engineering and regenerative medicine fields.

Keywords: meniscus; sonication; decellularization; extracellular matrix; scaffolds

1. Introduction

The meniscus tissues in the knee include medial and lateral meniscus has been constantly subjected to extreme forces between femur and tibia [1]. In maintaining normal knee function, meniscus performs essential roles. In particular, meniscus helps to lubricate joint, absorb shocks and even distribute compressive forces during movement [2]. Based on statistics recorded, there are 66 per 100 000 of people in general population suffering from meniscus injury annually due to the intense forces that are constantly applied to the meniscus especially in physically active people.

Biochemical components in complex meniscus structures consisting primarily of collagen interlacing networks interposed with cells and other extracellular matrix [4-5]. Collagen Type I predominates in collagen networks in meniscus tissues. However, the region within the tissue structure varies based on the different percentage of extracellular matrix content and cellular phenotype presence. Meniscus has three unique regions are identified as

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external (red-red), central (red-white) and internal (white-white) areas that make meniscus known as a non-homogenous tissue. It is highly vascularized for the outer region and therefore has a high regenerative capacity that differs from the inner region, which has no accessibility to vascularization [6]. For inner regions, it has limited self-repair capability due to the absence of blood vessels [6-7].

In this present era with the evolution of technology, the treatment approach for meniscus repair had progressively developed from total and partial meniscectomy to meniscus allograft transplantation. It is due to the partial meniscectomy does not consistently generate the desired affirmative outcomes after the treatment that can lead to permanent degenerative changes [8]. In Europe and United States, there is currently a clinical product for meniscus implant had been approved as a replacement for the severely injured meniscus that known as Menaflex^{TM} collagen [9]. However, it is not extensively characterized and the biomechanical benefits of the implant are still unknown. Thus, numerous impressive progresses on scaffolds fabrication research for meniscus replacement have been discovered until today.

The tissue engineering and regenerative medicine field emerges as a gold standard platform for the production of tissue substitutes as a great alternative for implants to replace the intolerably injured meniscus due to disease, trauma or aging [10].Tissue engineering has evolved from the development of biomaterials involving the production of functional tissue through the combination of three components, namely scaffolds, cells and biological signaling molecules referred to as tissue engineering triad [11]. A scaffold is known as three-dimensional porous solid biomaterials and plays a unique role as a template for the migration and adhesion of cells for the tissue formation. For the scaffolds, there are biological and synthetic scaffolds. As for the biological scaffold, it is obtained primarily from the tissues of humans and animals that are decellularized [9].

Decellularization is a process that removes the cellular antigens and nuclear content within the xenografts tissues and at the same time preserved the extracellular matrix compositions and biomechanical strength for preparation of bioscaffold. Decellularization strategies are classified into chemical substances (detergents, acids, alkaline), biological (trypsin, ribonuclease, protease enzyme) and physical techniques (perfusion, high hydrostatic pressure, freeze-thaw). This kind of prepared bioscaffolds would have low immunogenicity and hence might cause very low inflammatory response once implanted into the recipients [12]. According to Yu et al., an ideal prepared bioscaffolds through decellularization should have no residual cellular components, preserved extracellular matrix, low immunogenicity and maintain biomechanical strength [13]. The preservation of extracellular matrix is vital for cells to adhere, proliferate and differentiate during recellularization process for regeneration of new tissues [13].

The purpose of this research is to characterize the scaffolds prepared using the sonication treatment system for 10 hours. In order to analyze the removal of cellular components and the preservation of extracellular matrix compositions in the tissue, the success of meniscus decellularization process was determined through histological, biochemical and gel electrophoresis assessments.

2. Methodology

2.1 Meniscus tissues procurement

All fresh meniscus tissues derived from bovine was purchased from the commercialized sources. The unwanted surrounding tissues were removed using sharp blade. Tissue specimens were washed using 1x chilled Phosphate Buffer Saline (PBS) and stored in -20℃ for future usage.

2.2 Decellularization process

For decellularization process, the sonication decellularization treatment system was utilized to compare with immersion treatment as the control in this research. This system was operated as a closed system which was improved from the previous study that includes the 40 kHz frequency with 0.1% SDS aqueous solution [**14-18**]. First, the meniscus tissues were placed on the sample holder which was constantly fixed 12 mm from the transducer. The level of dissolved oxygen, pH, conductivity and temperature were timely monitored. The temperature of the water bath was maintained to 36±1°C. As a control, all parameters for immersion treatments were set correspondingly to the sonication decellularization treatment system with the exception of frequency. To ensure a constant SDS solution circulation throughout the process, there was 70 rpm included in the treatment.

2.3 Washing Process

The decellularized scaffolds were continuously immersed in 1X Phosphate Buffer Saline (PBS) for 5 days with 70 rpm agitation for the washing process. There was a need to change the buffer solution every 24 hours.

2.4 Microscopic evaluations

Microscopic evaluations were carried out by histological analysis of native tissues and decellularized. In 4% paraformaldehyde, the native tissues and decellularized samples (n= 3) were fixed at 4°C for 24 hours. The scaffolds were embedded in paraffin wax and then froze on cold plate for one hour (Leica EG1150 C). The chilled paraffin-embedded tissues were sectioned into 8µm thin ribbon using manual microtome (Leica, USA), placed on microscope slides and let dried for overnight. The slides were deparaffinized, rehydrated with gradient alcohol concentration and stained using van Gieson to evaluate the removal of cells, Picrosirius red staining to observe the distribution of collagen networks and Safranin O/Fast Green to evaluate the distribution of GAG networks. Samples of stained tissue were dehydrated with a graded gradient of alcohol ranging from 50% to 100% (5 minutes per gradient), followed by xylene (2 x 5 minutes).

2.5 Genomic DNA Quantification and Fragments detection

The tissue samples were sliced into small pieces and freeze-dried for a day. The tissues were weighed in a 1.5ml centrifuge tube, incubated in 20 mg/ml Proteinase K with 200 µl of lysis buffer. For DNA extraction, the Bioneer AccuPrep Genomic DNA Extraction Kit (Korea) was utilized by following the standard manual instructions. This Accuprep kit included the usage of glass fibers that is fixed in a binding column that particularly allows the binding of DNA in the presence of a chaotropic salt. The unwanted proteins and other contaminants were eliminated throughout the process during short washing and spin steps. Lastly, the collected DNA on the glass fibers was diluted with 200µl of Elution buffer. The DNA extracted were quantified using Nanodrop spectrophotometer (Thermo Scientific) at 260 nm and 280 nm optical density. The gel electrophoresis was conducted to observe the DNA fragments of the purified DNA. The ethidium bromide dye was incorporated in the prepared 1% gel as the fluorescence tag during the process. The 6X loading dye was mixed with purified DNA and loaded into the wells together with DNA ladders. The gel electrophoresis was completed for 45 minutes at 90V and the fragments obtained were viewed under Gel Imager.

2.6 Collagen and GAGs quantification

The collagen and glycosaminoglycans content within the tissues were quantified using the assay kit from Biocolor (UK). For collagen content, the lyophilized samples were digested with cold acid-pepsin for two days and proceeds with the standard extraction instructions from Collagen Assay Kit manual (Biocolor Ltd., UK). The wavelength for the microplate reader was set at 555 nm to obtain the absorbance and calculated concentration of collagen content. For GAGs content, the Blyscan Glycosaminoglycan Assay Kit (Biocolor Ltd., UK) was used. The samples were incubated in papain extraction reagent and assayed following the standard protocol. This principle of this assay kit was based on the binding of blue 1, 9-dimethylmethylene to the GAGs components and the absorbance was recorded using microplate reader at wavelength of 650 nm.

2.7 Statistical analysis

The data for DNA quantification, GAGs and collagen content were analyzed using the Student t-test. The *p-value* for each analysis between samples was set at 0.05.

3. Results

3.1 Histological evaluation for cellular components removal

The photographs in Figure 1 showed the van Gieson staining from the surface and middle parts of the tissues at 40x magnifications with yellow arrow showing the presence of blue-black nuclei. Based on Figure 1C and 1F, there was absence of nucleus in sonication decellularized scaffolds compared to native (1B, 1E) and immersion decellularized scaffolds (1A,AD). It can be monitored that the immersion treatment did not manage to remove all of the cellular components within the tissues as there were some of the nucleus stained detected as represented by the yellow arrow.

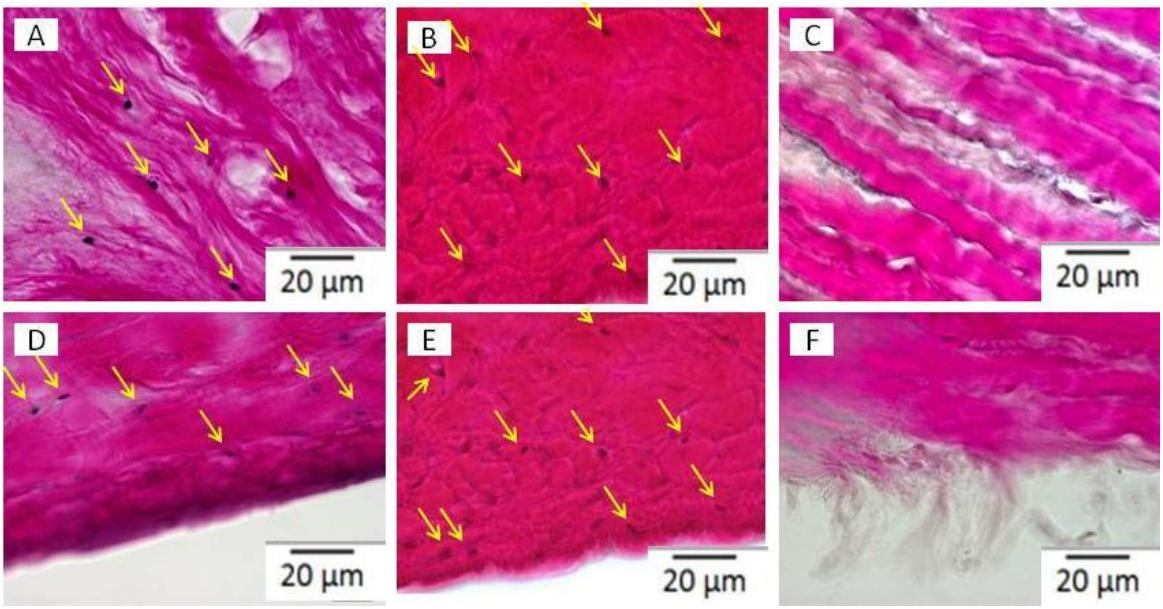

Fig. 1. The images of van Gieson staining showed the middle and surface parts of immersion decellularized scaffolds (A,D), native tissues (B,E) and sonication decellularized scaffolds (C,F) at 40x magnification.

3.2 DNA quantification and Fragments analysis

As for quantitative analysis, DNA content in native, immersion and sonication decellularized scaffolds were showed respectively in Figure 2. This quantification portrayed significant difference ($p < 0.05$) in the DNA content between native tissues with decellularized scaffolds. There was 8% and 32% of the residual DNA detected using DNA quantification in the sonication and immersion decellularized scaffolds respectively.

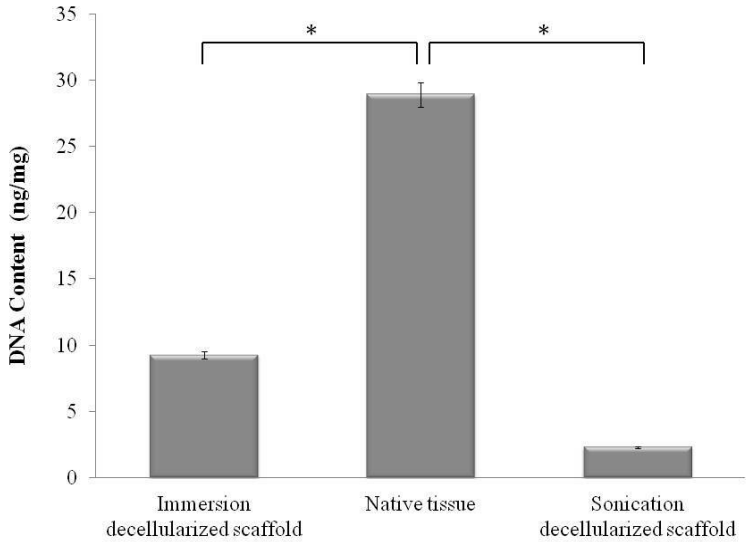

Fig. 2. The graph showing the mean DNA content with standard deviation for immersion decellularized scaffold, native tissues and sonication decellularized scaffolds. The significance difference was set at *$p < 0.05$.

The gel in Figure 3 portrayed the absence of DNA bands for sonication decellularized scaffolds. Native tissue and immersion decellularized scaffolds demonstrated the presence of the bands. However, by comparing to native tissues, the bands obtained in immersion decellularized scaffolds were brighter and had lower intensity.

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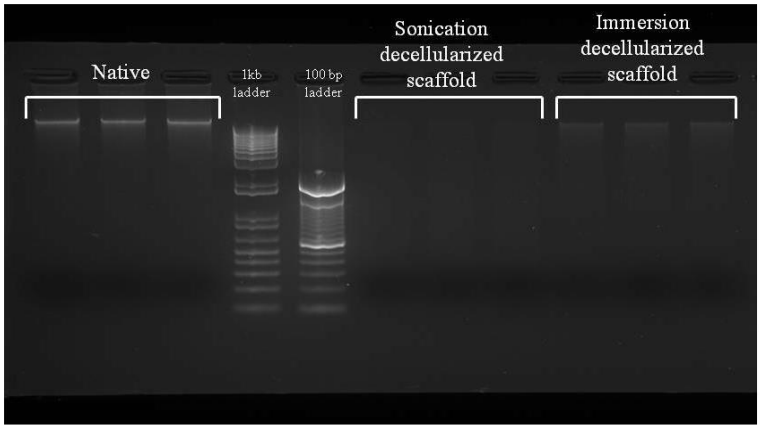

Fig. 3. Gel electrophoresis of purified DNA extracted from the native, sonication and immersion decellularized scaffolds.

3.3 Histological evaluations for collagen and GAGs networks distribution

The picrosirius red staining slides were observed using polarized light microscope at 4x magnification as shown in Figure 4A-C. The yellow and green color stained represented the Type I and Type III collagen. This staining was completed to evaluate the distribution of collagen networks after decellularization process. A very dense and packed network with high Type I and Type III collagen distribution can be observed in Figure 4B. By referring to Figure 4A, it had porous networks distribution and lack of type III distribution where the collagen might become loosely packed due to immersion treatment. The sonication decellularized scaffolds portrayed preservation of collagen Type I and Type III represented by yellow and green color structure respectively which was still intact and resembles the collagen networks distribution in native tissues.

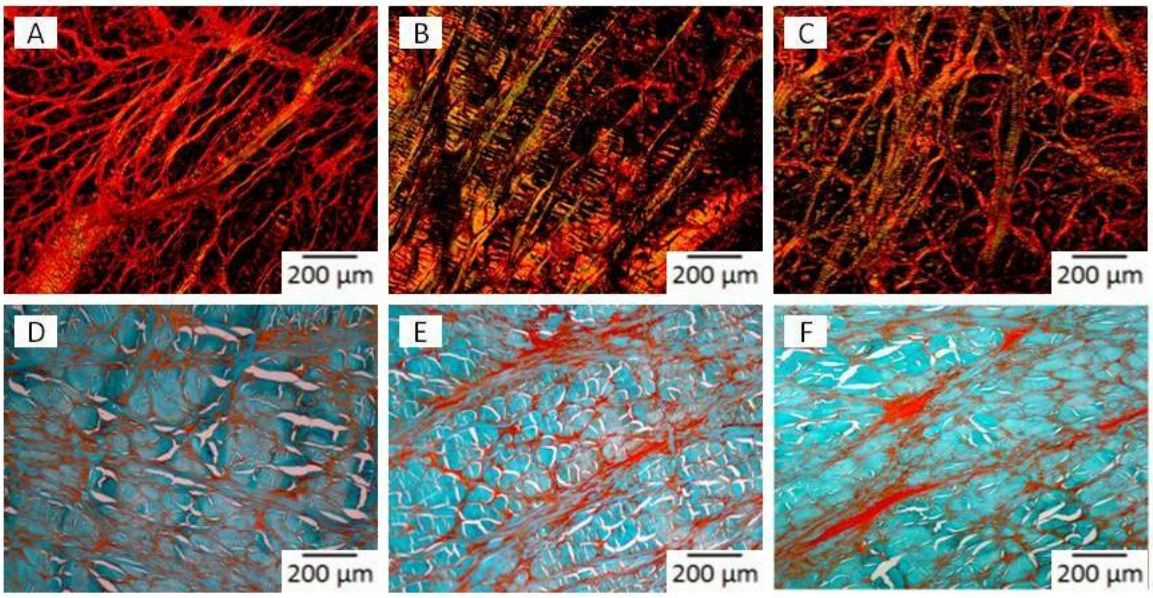

Fig. 4. The images of picrosirius red (A,B,C) and Safranin O/Fast Green staining (D,E,F) showed the surface parts of immersion decellularized scaffolds (A,D), native tissues (B, E) and sonication decellularized scaffolds (C,F) at 4x magnification.

The red and green coloration in Safranin O/Fast Green demonstrated the distribution of GAGs and non-collagen networks as shown in Figure 4. Based on Figure 4D and 4F, both decellularized scaffolds showed lack of red colour GAGs networks stained compared to native tissues in Figure 4E.

3.4 Biochemical evaluations

The results in Table 1 summarized the collagen and GAGs content for native tissues and decellularized scaffolds. For collagen, the decellularized scaffolds significantly had a higher content compared to native tissues. As for GAGs content, native tissues had 149.83 ± 13.75 ng/mg which is slightly higher than immersion decellularized scaffolds and sonication decellularized scaffolds. There was a reduction of approximately 60% and 40% GAGs within the sonication and immersion decellularized scaffolds. This showed that after decellularization, the GAGs content significantly reduced than native tissues.

Table 1: Collagen ad GAGs content of native tissue, immersion and sonication decellularized scaffolds. Data are expressed as mean ± standard deviation. *Significance difference: $p<0.05$

Samples	Components (ng/mg dry weight)	
	Collagen content	GAGs content
Native tissues	613.36 ± 5.88	149.83 ± 12.75
Immersion decellularized scaffolds	680.58± 7.75*	88.92 ± 6.21*
Sonication decellularized scaffolds	770.89 ± 7.99*	57.82 ± 4.09*

4. Discussions

In the application of tissue engineering for the reconstruction of new tissues, biological scaffold materials composed of extracellular matrix components derived from decellularization treatment are widely used. Scaffolds are crucial components for tissue regeneration as it act as a structural support that should mimic the native tissue microenvironment for the cells to attach and proliferate.The quality of decellularized scaffolds are determined through high elimination of cells and genetic material, retention of ECM compositions architecture and biomechanical properties [19]. The decellularized bioscaffolds are crucial in the regenerative medicine field as it promotes important remodeling host response once it is applied as therapeutic bioscaffolds. Currently, the development for optimal decellularization protocol had been a vital concern to ensure complete decellularization with no potential inflammations.

In this research, the decellularization process was achieved using our developed sonication decellularization treatment system which consisted of 40 kHz in 0.1% SDS. Based on the van gieson staining and genomic DNA quantification, the sonication decellularization treatment system successfully decellularized meniscus tissues with absence of blue-black nucleus stained and significantly low residual DNA content compared to native tissues. Further confirmation of decellularization process was completed through gel electrophoresis of purified DNA as previously done as shown in Figure 3 that portrayed the DNA fragments and bands[**20**]. For sonication decellularized scaffolds, there was absence of any DNA fragments or bands. According to the published study, the characteristics of an excellant prepared decellularized scaffolds should completely clear from cellular or nuclear components through histology, relatively very low residual DNA amounts and <200 bp fragments detected through gel electrophoresis [**21**].

Based on the results in Figure 1-3 for immersion decellularized treatment, the usage of 0.1% SDS as the only decellularization agent was proved to be inadequate to achieve an effective decellularization process to remove whole cellular components and genetic material. The SDS which recognized as ionic detergent is extremely effective in solubilized the membrane during decellularization. However, it has a deleterious effect towards the extracellular matrix structure and compositions such as removal of growth factors and GAGs to some extents depending on the concentrations [**22**]. It also has toxicity effects that required an extensive washing process using PBS to ensure complete removal of residual SDS for bioscaffolds preparation. The cell lysis process was triggered by the presence of SDS solution through the formation of micelles that facilitates the disruption of hydrophobic proteins, solubilized nuclear and cells membrane [**23**]. The solubilization of the membrane can be described in three stages. Stage 1 involves the initiation of interactions between detergents and membrane that start to partitioning into the intact lipid bilayer membrane. For stage 2, the disintegration of bilayer occurs and the detergent-lipid saturated bilayer starts to convert

into mixed micelles. For the last stage, the size of the mixed micelles reduces due to the interactions with more detergent molecules in aqueous solution that consequently disrupt the membrane [**24**].

For sonication decellularized scaffolds, both SDS and ultrasound plays a crucial role during the treatment. The involvement of 40 kHz frequency that emits the vibration into the solution enhances the permeation of the detergent thoroughly within the compact tissue structure. The application of ultrasound also initiates the formation of cavitation bubbles along with the cavitation process in the fluid that resulted in increased membrane permeability. The cavitation bubbles usually retained its spherical shape in a uniform environment. However, the membrane that functions as a physical boundary triggers the collapsed of the cavitation bubbles asymmetrically once it hits the solid surface. The collapsion of the bubble was reported to create high-speed liquid jet driving onto the membrane of the tissue [**25**]. This occurrence consequently leads to membrane disruption and allows easy permeation of detergent solution to completely decellularize the tissue.

Proteoglycan with glycosaminoglycans chain is one of the prominent constituents in the meniscus. The GAGs contribute to increasing tissue viscosity, whereas in the middle and inner meniscus, where GAGs are most abundant, these molecules also enhance the tissue's ability to withstand compressive loads [**22**]. The GAGs depletion in decellularized scaffolds was verified through safranin o/fast green staining and GAGs quantification. There was lack of red GAGs stained observed in decellularized scaffolds. The decellularized scaffolds demonstrated high GAGs depletion compared to native tissues which was consistent with reported study [**26**]. Glycosaminoglycans have a highly water-soluble characteristic that allows attraction of water into tissue for hydration. Due to this characteristic, it triggers the reduction of GAGs during decellularization as it was conducted in aqueous 0.1% SDS solution that easily washed away the denatured GAGs. The frequency of 40 kHz in sonication treatment facilitated the permeation of the detergent thoroughly within the tissue structure that lead to higher GAGs depletion compared to immersion treatment.

The collagen in the extracellular matrix is responsible to provide high tensile properties in the tissues. In meniscus tissues, the collagen is the most abundant components distributed in all regions. Based on the results obtained, the scaffolds prepared using the sonication decellularization treatment system managed to preserve the collagen. The collagen networks distribution based on yellow and green coloration in picrosirius red staining portrayed that the sonication decellularized scaffolds managed to mimic native tissues. Compared to immersion decellularized scaffolds, it had lack of green stained that represented Type III with porous collagen networks revealed. This occurrence was due to the limitation of SDS solution to permeate completely through the fibrous tissue structure. The agitation of 70 rpm during immersion treatment was inadequate to ensure the simultaneous decellularization of the cells in the exterior and interior parts of the tissues that cause the porous networks distribution obtained as demonstrated in Figure 4. The releasing of protease enzyme triggered by the membrane disruption during decellularization process has the capability to destroy the extracellular matrix and other proteins components such as glycosaminoglycans and collagen [**27-28**]. This activity might cause disruption in the networks distribution and loosened the arrangement of collagen. In contradict, the sonication decellularized scaffolds managed to retain the collagen networks to native tissues. This is because the presence of 40 kHz frequency caused the decellularization of the exterior and interior cells to occur simultaneously which subsequently reduce the probability of the protease enzyme to be released.

The collagen content of sonication decellularized scaffolds was recorded as significantly higher than immersion decellularized scaffolds and native tissues. The decellularization process caused a significant elimination of cellular components including glycosaminoglycans that subsequently generated a higher collagen fraction per dry weight in decellularized scaffolds compared to native [29-31]. Previous study that decellularized porcine meniscus using 1% SDS also reported the similar result [32]. The collagen fibers were well preserved following decellularization due to non-water soluble character of fibers that was not affected by chemical SDS solution [31]. These decellularization agents only cause collagen networks to be loosely arranged and not removal of the content. The collagen networks distribution and content in sonication decellularized scaffolds was assumed to be well preserved derived from the combination results of picrosirus red staining and collagen quantification. The ECM retention in the scaffolds is essential for the imitation of native tissues that provide biochemical signals that facilitate cell adhesion, proliferation, migration and differentiation [19]. However, this research has certain limitations concerning the study of biocompatibility of prepared scaffolds. Further study in decellularized scaffolds based on surface tissue ultrastructure, biomechanical properties, *in vitro* and *in vivo* research must be completed.

Conclusion

The sonication decellularization treatment system has succeeded in effectively decellularized bovine meniscus tissues. High cellular components and residual DNA removal with preserved extracellular matrix components concluded from histology, gel electrophoresis and biochemical assessments have revealed that sonication scaffolds have a great

potential as an outstanding tissue engineering scaffolds. The sonication treatment system is one of the effective decellularization techniques.

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