Examination of conditions for optimized decellularized liver preparation

Jaeyong Cho[1*], Yukako Fukuda[1], Nana Shirakigawa[1], Hiroyuki Ijima[1]

[1]Department of Chemical Engineering, Faculty of Engineering, Graduate School, Kyushu University, 744 Motooka, Nishi-ku, Fukuoka 819-0395 Japan.

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ABSTRACT

Aims: The main aim of our study was to examine the concentration of surfactant that can cause significant disruption of the resulting decellularized liver structure. Furthermore, it is our goal to determine the suitable solvent that can boost the potential of each surfactant.
Methodology: The porcine liver disks of 8-mm diameter and 2-mm thickness were prepared. These were soaked in aqueous solution of either sodium dodecyl sulfate (SDS) or Triton X-100 (TX), and placed on a rotational shaking machine (100 rpm).
Results: TX was unable to completely remove the cellular components under any of our experimental conditions. The salt concentration did not affect the decellularization in TX. The pH buffer, however, was found to affect the decellularization. Also, in the solvent study, the conditions under which SDS effectively exerted power were not the salt concentration and pH, but the condition that was close to water. We also confirmed that the shrinkage of tissue occurred when decellularization with 0.1% SDS in CMF-PBS. However, 0.1% SDS in distilled water didn't cause the deformation of tissue. This is considered to be due to the low salt concentration of solvent.
Conclusion: This work establishes the concentration range of the surfactant that causes the collapse of the cellular structure during decellularization. In addition, the solvent suitable for each surfactant has also been established.

Keywords: decellularization, decellularized liver, detergent, solvent, optimization

1. INTRODUCTION

Liver is the largest organ in the body and is known to have more than 500 functions, including metabolism, detoxification, and emission. Even when the liver is damaged by 85% due to the illness or accidents [1], regeneration is possible. Liver damage often goes unnoticed by the patient and, consequently, liver disease is usually only noticed when it becomes a severe problem. Liver transplantation is the only radical therapy available for severe liver diseases such as hepatitis and cirrhosis. The patient is given a portion of the liver from a living donor or is given the entire liver obtained from a brain-dead donor. However, several problems including those associated with immune rejection and lack of donors for transplantation exist [2]. Recently, in order to solve these problems, studies have been conducted to construct a transplantable liver using tissue engineering. However, it is difficult to maintain a detailed structure and construct a liver that can carry out as many functions.

E-mail address: ijima@chem-eng.kyushu-u.ac.jp

In the recent years, decellularized organs have attracted much attention as functional scaffolds worldwide. A decellularized liver (DCL) can be obtained by removing cellular components from the liver. In addition, the DCL has a blood vessel structure similar to that of the original liver, which is expected to supply sufficient oxygen to the restructured liver [3-5]. Besides, the DCL has been reported to suppress the rejection by immune antigens [6-7].

Although many decellularized liver preparation techniques have been described till date, the destruction of the vascular structure has been reported under some conditions [8]. In addition, the concentration at which the vascular structure is disrupted remains unclear. Furthermore, since the solvent of surfactant solution was different in different experiments, it becomes necessary to study the solvent. Given these, in the current study, we have examined the concentration of surfactant that destroys the structure of the decellularized liver as well as the solvent that brings out the effects of each surfactant.

2. MATERIAL AND METHODS

2.1 Preparation of porcine liver disks

Porcine livers (2.0 kg) were bought from Fukuoka shokuniku hanbai (Fukuoka, Japan). The blood in the porcine whole liver was removed by flushing Calcium-Magnesium Free Phosphate-buffered saline (CMF-PBS) containing 0.19 mg / ml GEDTA from the portal vein of the porcine liver. Subsequently, such porcine liver was cut into blocks of 6 cm × 10 cm × 6 cm and stored in -80 ℃ frozen condition. The frozen porcine liver was sliced in to liver disks with a thickness of 2 mm. Then, by using a puncher, liver disks with a diameter of 8 mm and a thickness of 2 mm were prepared. Liver disks weighing 80–100 mg were selected for further analysis. The experimental protocol mentioned in this study was reviewed and approved by the Ethics Committee on Animal Experiments of Kyushu University (Fukuoka, Japan).

2.2 Decellularization of porcine liver disks

The prepared porcine liver disks were inoculated into a 12-well plate. Then, 2 ml of surfactant solution was added into each well to decellularize liver disks and the 12-well plate was put on a rotational shaking machine (100 rpm). Sodium dodecyl sulfate (SDS) (Wako Pure Chemical Industries, Osaka, Japan) and Triton X-100 (TX) (Sigma, St Louis, MO, USA) were used as detergents. The following were used as solvents: distilled water, CMF-PBS (75 mM, 150 mM, 300 mM), NaCl (77 mM, 154 mM, 308 mM), $MgSO_4$ (77 mM, 154 mM, 308 mM), $MgCl_2$ (77 mM, 154 mM, 308 mM), $CaCl_2$ (77 mM, 154 mM, 308 mM), 154 mM KCl, and pH buffer. The pH buffer for pH 5.0, 7.0, and 8.5, was prepared using $NaH_2PO_4 \cdot 2H_2O$ and $Na_2HPO_4 \cdot 12H_2O$.

2.3 Histological analysis

Decellularized porcine liver disks (8mm diameter) were prepared using biopsy punch and fixed by 10% neutral buffed formalin. Tissue samples were embedded in paraffin and sectioned. Hematoxylin and eosin (H&E) staining was performed to evaluate the tissue sections. Also, frozen sections (8 μm in thickness) were prepared for Hoechst staining and were observed using fluorescence microscopy (Tokyo Rikakikai co, Tokyo, Japan).

2.4 DNA analysis

DNA was obtained from each 20 mg of tissue by using QuickGene SP Kit DNA Tissue (Kurabo Industries, Osaka, Japan). Extracted DNA was stained using Hoechst 33258 and measured intensity was quantified.

3. RESULTS

3.1 Detergent concentration

Porcine liver disks were decellularized using various concentrations of SDS in CMF-PBS for 48 h. After 48 h in 1 - 6 % SDS, the decellularized disks turned white (Fig. 1); in 0.1 % SDS, the disks were almost white. The remnant cells were seen at the central part of the disks. Additionally, in 1 - 6 % SDS, the disks shrunk to about 50 % of their original sizewhereas in 0.1 % SDS, the disks shrunk to about 30 % of their original size. Additionally, after 48 h in 1 - 6 % TX solution, the disks turned white (Fig. 1).

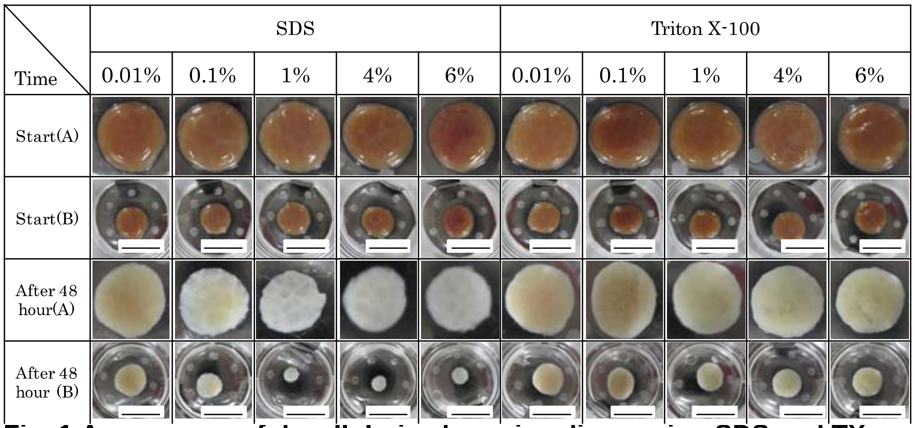

Fig. 1 Appearance of decellularized porcine discs using SDS and TX and CMF-PBS. Scale bars = 1cm.

3.2 Effect of salt on decellularization

Decellularization was performed using 4 % TX and 0.1 % SDS in NaCl and $MgCl_2$ aqueous solution (Fig. 2A); 0.1 % SDS in NaCl, $MgCl_2$, and $MgSO_4$ aqueous solution (Fig. 2B); and 4 % TX in distilled water, NaCl, $MgSO_4$, $CaCl_2$, KCl, and $MgCl_2$ aqueous solution (Fig. 2C). After decellularization with 0.1 % SDS in NaCl aqueous solution, the porcine liver disks became harder, and crystals were observed in 0.1 % SDS in $MgCl_2$ aqueous solution (Figs.2A and 2B). However, decellularization did not progress in 0.1 % SDS supplemented with $MgCl_2$ and $MgSO_4$ (Fig.2B). When any of the salts was added in 4% TX, decellularization was not observed (Fig.2C). In other words, decellularization was observed in 0.1% SDS, and only when in NaCl was used as a salt.

The effect of CMF-PBS and NaCl on decellularization was studied using 4 % TX and 0.1 % SDS (Fig.3). In the case of SDS, decellularization was found to be progressing with time, and liver disks in were found to have decellularized well by 3 days under all conditions. However, no difference between CMF-PBS and NaCl in terms of their effect on the decellularization process was observed. On the other hand, disks changed to gray in pure water and NaCl when decellularization was carried out using TX; only a few changes were observed after 12 hours.

Influence of the salt concentration on decellularization was studied using 0.1 % SDS (Fig.4). Decellularization was inhibited with increase in the salt concentration. This phenomenon was seen when using CMF-PBS and NaCl. On the other hand, decellularization with 0.1 % SDS in pure water seemed to be have completed within 7 hours of incubation. Additionally, shrinkage of the disks could not be confirmed during the decellularization.

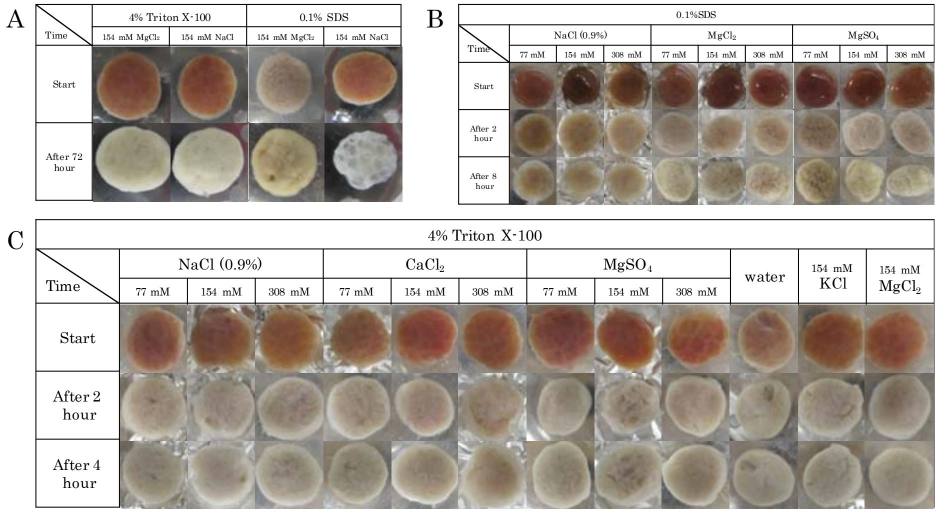

Fig. 2 Appearance of decellularized porcine discs in 4% TX and 0.1% SDS in NaCl and MgCl2 aqueous solution (A) and 0.1% SDS in NaCl, MgCl2 and MgSO4 aqueous solution (B), and in distilled water, NaCl, MgSO4, CaCl2, KCl, and MgCl2 aqueous solution (C).

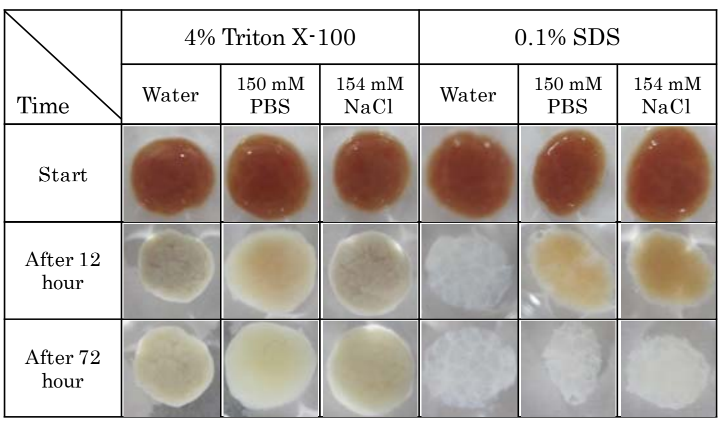

Fig. 3 Images of decellularization of a surfactant-supplemented CMF-PBS and NaCl.

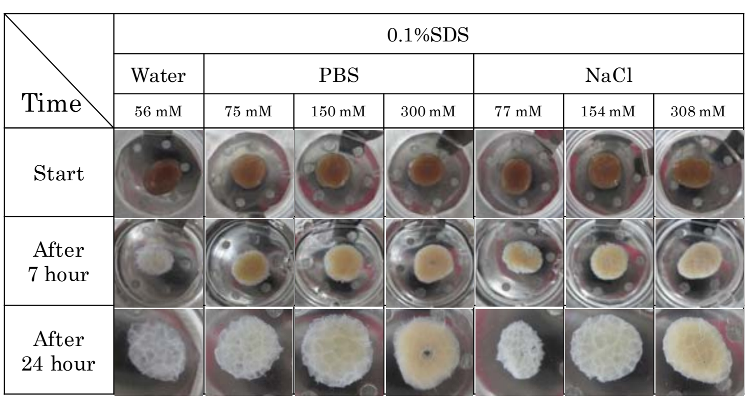

Fig. 4 Influence of salt concentration on decellularization.

3.3 Effect of pH on decellularization

When we used 4 % TX, the disks became gray at pH 5, and further decellularization was difficult (Fig.5A). However, decellularization continued slowly at pH 7 and 8.5. The influence of pH on 0.1 % SDS was similar to that observed for TX (Fig.5). However, decellularization in 0.1 % SDS was considerably faster than in TX (Fig.5). Based on these results, it was revealed that decellularization should be performed in the pH range of 7 to 8.5.

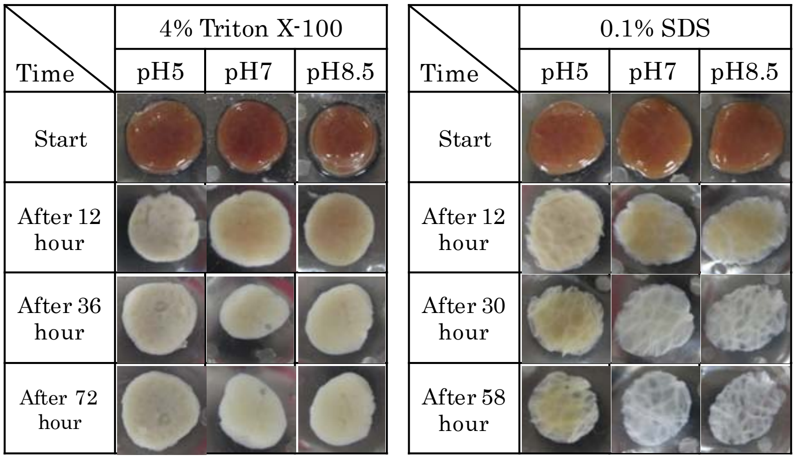

Fig. 5 Influence of pH in decellularization.

3.4 Decellularization with distilled water as a solvent

Influence of SDS concentration in pure water as a solvent on decellularization was studied. Decellularization was not observed in 0.01 % SDS. On the other hand, decellularization within 12 hours was confirmed for 1 - 6 % of SDS(Fig.6). However, in 1 - 6 % of SDS, a remarkable shrinkage of disks was also seen. Interestingly, the shrinkage of disk was confirmed in the 0.1 % SDS along with the accomplishment of fastest decellularization. To be more specific, decellularization was accomplished within four hours in 0.1 % SDS.

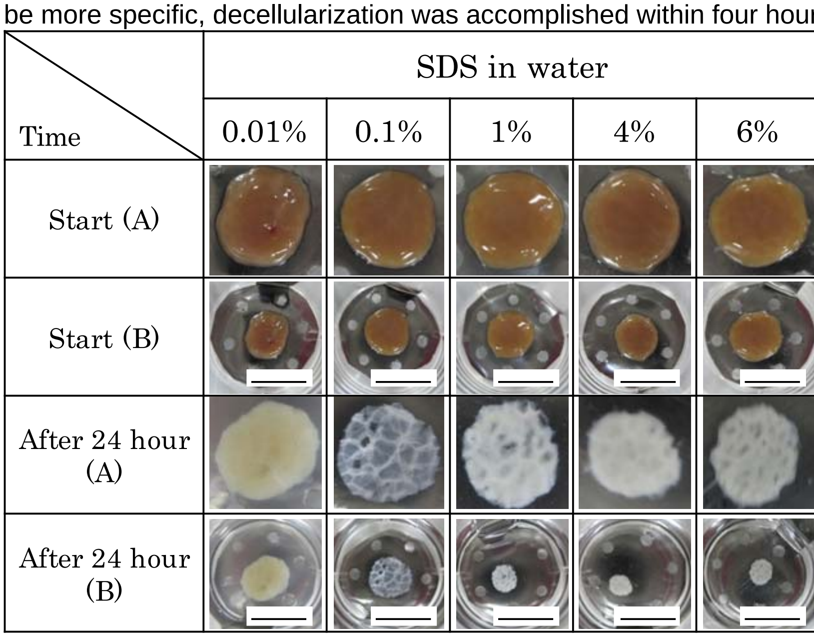

Fig. 6 Influence of SDS concentration in pure water for decellularization.
Scale bars = 1 cm.

3.5 Confirm decellularization of liver disc

Fig.7 shows Histological analysis of decellularized sections. Decellularization using CMF-PBS as a solvent in 1-4% TX solution, the sections appeared to be similar to native tissue. On the other hand, decellularization with 0.1-4% SDS solution using distilled water as a solvent, complete removal of cellular contents can be seen.

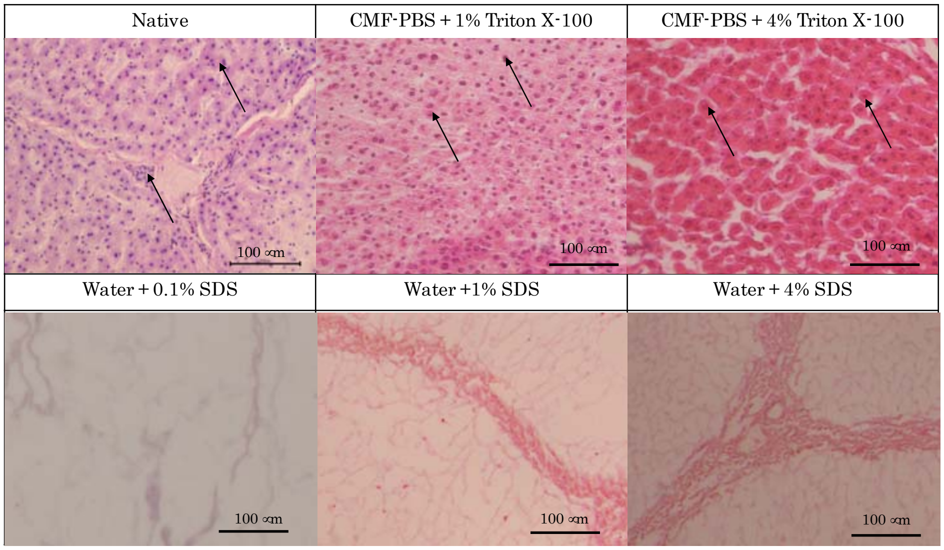

Fig. 7 Influence of SDS concentration in distilled water and Triton X-100 concentration in CMF-PBS for decellularization.

Scale bars = 100μm. Arrows indicate hepatocyte cells.

Hoechst staining (Fig. 8) clearly indicates effective removal of nuclear content when 0.1-4% SDS solution in distilled water was used as a solvent for decellularization. Also DNA quantification (Fig. 9) clearly indicated 99% removal of DNA content with 0.1-4% SDS solution in distilled water as solvent for decellularization.

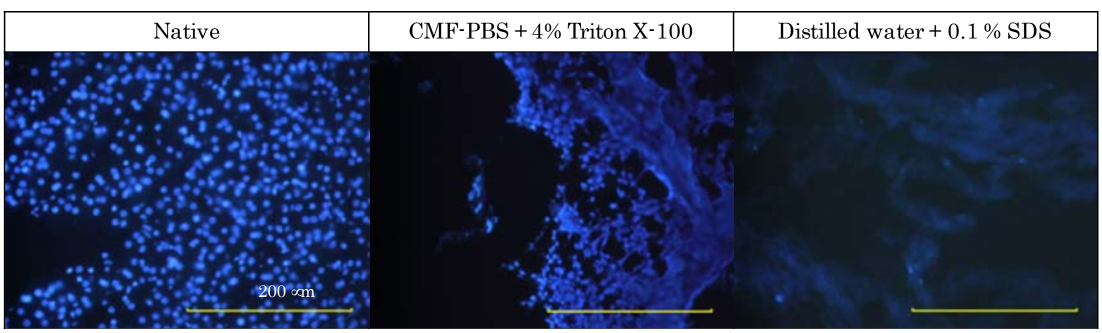

Fig.8 Nucleus staining of decellularized tissue by Hoechst.

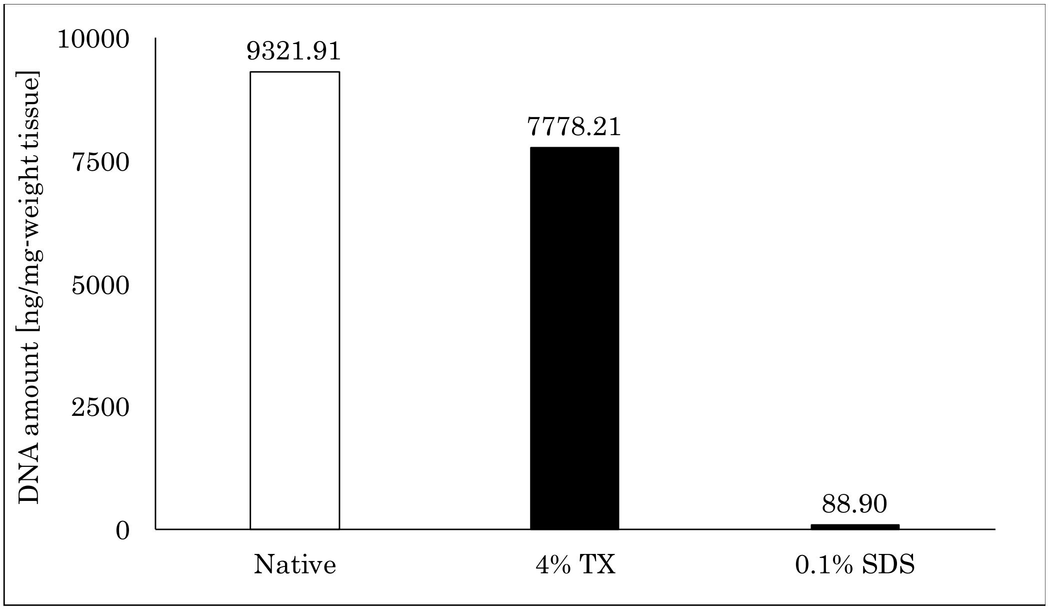

Fig.9 DNA quantification in native and decellularized liver tissue prepared with various methods.

4. DISCUSSION

In recent years, in order to solve the problem of liver donor shortage, researches on the creation of an artificial organ for transplantation have attracted attention. Under such circumstances, studies aiming at reconstructing the liver by using DCL as a scaffold have been performed [9-10]. When preparing a decellularized liver, it is important to remove the cellular components while maintaining the structure of the liver [11]. Until now, although many decellularized liver preparation techniques have been reported, the disruption of the vascular structure of the liver has been reported under some conditions [8]. In order to prepare a decellularized liver which can be used as a good scaffold for cells, it is necessary to determine the conditions under which the vascular structure collapses (deformation of structure causes). We confirmed the effects of concentration of surfactant, salt concentration and pH buffer on the decellularization of the liver disc. In addition, we observed the changes of color and size of decellularized liver discs and reveal the effect of surfactant and solvent on decellularization.

Triton X-100 was unable to completely remove cellular components under any conditions of concentration. Furthermore, salt concentration did not affect decellularization with Triton X-100, while the pH buffer affected decellularization. However, in the case of acidic solvent (pH 5), the surface of liver discs appeared to be damaged by the acid, and it seemed that the acid predominantly removed the cell components rather than the surfactant. The neutral or alkaline solution was able to confirm the removal of the cellular component as compared to the native liver disc, but the removal of cellular components could be confirmed more in neutral condition than in alkaline. From the above, Triton X-100 can be expected to remove cell components although it takes longer time. In addition, TritonX-100 can instigate milder decellularization and can cause less damage to tissue structure compared to SDS. Therefore, Triton X-100 is expected to be a suitable solvent not only for the liver but also for soft organs which doesn’t contain many cells.

SDS has been reported to disrupt the vascular structure of the decellularized liver [8]. In this study, we also confirmed that the shrinkage of liver tissue occurred when decellularization with SDS in CMF-PBS. In fact, reports indicated that SDS disrupts protein-protein interactions and causes protein denaturation. In other words, it can be deduced that the shrinkage of tissue occurred when micelles in high concentrated SDS bound not only to the cell membrane but also to proteins contained in the decellularized liver and denatured the proteins. However, 0.1% SDS in distilled water didn't cause the deformation of tissue that can be attributed to the low salt concentration of solvent.

On the other hand, in high salt concentration, more micelles in surfactant are produced than in low salt concentration. In distilled water, it seemed that the salt concentration was low and less micelles bounded to the protein of tissue than in CMF-PBS. This is may be the reason why 0.1 % SDS in distilled water didn’t cause damage to the tissue.

5. CONCLUSION

In this current study, we established the concentration condition of surfactant that causes the collapse of the structure during decellularization. In addition, since the solvent suitable for each surfactant has been established, more effective preparation of the decellularized liver can be expected even with the same concentration of the surfactant. These findings would prove to be useful in the preparation of not only the decellularized liver but also other decellularized organs.

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COMPETING INTERESTS

The authors declare no conflict of interest.

AUTHORS’ CONTRIBUTIONS

Jaeyong Cho, Hiroyuki Ijima. conceived and designed the experiments; Jaeyong Cho. performed the experiments; Jaeyong Cho. and Nana Shirakigawa. analyzed the data; and Jaeyong Cho. and Yukako Fukuda. wrote the paper.

References

1. Michalopoulos GK. Liver Regeneration. J Cel Physiol. 2007;213(2):286-300.

2. Genda T, Ichida T, Sakisaka S, Sata M, Tanaka E, Ii A, Egawa H, et al. Waiting list mortality of patients with primary biliary cirrhosis in the Japanese transplant al-location system. J Gastroenterol. 2014;49(2):324-331.

3. Ning LJ, Zhang Y, Chen XH, Luo JC, Li XQ, Yang ZM, et al. Preparation and characterization of decellularized tendon slices for tendon tissue engineering. J Biomed Mater Res A. 2012;100(6):1448-1456.

4. Barkan D, Green JE, Chambers AF. Extracellular matrix: a gatekeeper in the transition from dormancy to metastatic growth. Eur J Cancer.2010;46(7):1181-1188.

5. Hoganson DM, Owens GE, O'Doherty EM, Bowley CM, Goldman SM, Harilal DO, et al. Preserved extracellular matrix components and retained biological activity in decellularized porcine mesothelium. Biomaterials. 2010;31(27):6934-6940.

6. Ott HC, Clippinger B, Conrad C, Schuetz C, Pomerantseva I, Ikonomou L, et al. Re-generation and orthotopic transplantation of a bioartificial lung. Nat Med. 2010:927-933.

7. Barakat O, Abbasi S, Rodriguez G, Rios J, Wood RP, Ozaki C, et al. Use of decel-lularized porcine liver for engineering humanized liver organ. J Surg Res.2012;173(1):11-25.

8. Shirakigawa N, Takei T, Ijima H. Base structure consisting of an endothelialized vascular-tree network and hepatocytes for whole liver engineering. J Biosci Bioeng. 2013;116(6):740-745.

9. Jaramillo M, Yeh H, Yarmush ML, Uygun BE. Decellularized human liver extracellular matrix (hDLM)-mediated hepatic differentiation of human induced pluripotent stem cells (hIPSCs). J Tissue Eng Regen Med. 2018;12(4):1962-1973.

10. Mazza G, Rombouts K, Rennie Hall A, Urbani L, Vinh Luong T, Al-Akkad W, Longato L, Brown D, Maghsoudlou P, Dhillon AP, Fuller B, Davidson B, Moore K, Dhar D, De Coppi P, Malago M, Pinzani M. Decellularized human liver as a natural 3D-scaffold for liver bioengineering and transplantation. Sci Rep. 2015; 5.

11. Song JJ, Ott HC. Organ engineering based on decellularized matrix scaffolds. Trends Mol Med. 2011;17(8):424-432.