

# Examination of conditions for optimized decellularized liver preparation

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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

The 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 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.

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

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

### 46 **2.1 Preparation of porcine liver disks**

47 Porcine livers (2.0 kg) were bought from Fukuoka shokuniku hanbai (Fukuoka, Japan). The  
48 blood in the porcine whole liver was removed by flushing Calcium-Magnesium Free  
49 Phosphate-buffered saline (CMF-PBS) containing 0.19 mg/ml GEDTA from the portal vein of  
50 the porcine liver. Subsequently, such porcine liver was cut into blocks of 6 cm × 10 cm × 6  
51 cm and stored in -80 °C frozen condition. The frozen porcine liver was sliced into liver disks  
52 with a thickness of 2 mm. Then, by using a puncher, liver disks with a diameter of 8 mm and  
53 a thickness of 2 mm were prepared. Liver disks weighing 80–100 mg were selected for  
54 further analysis. The experimental protocol mentioned in this study was reviewed and  
55 approved by the Ethics Committee on Animal Experiments of Kyushu University (Fukuoka,  
56 Japan).  
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### 58 **2.2 Decellularization of porcine liver disks**

59 The prepared porcine liver disks were inoculated into a 12-well plate. Then, 2 ml of  
60 surfactant solution was added into each well to decellularize liver disks and the 12-well plate  
61 was put on a rotational shaking machine (100 rpm). Sodium dodecyl sulfate (SDS) (Wako  
62 Pure Chemical Industries, Osaka, Japan) and Triton X-100 (TX) (Sigma, St Louis, MO, USA)  
63 were used as detergents. The following were used as solvents: distilled water, CMF-PBS (75  
64 mM, 150 mM, 300 mM), NaCl (77 mM, 154 mM, 308 mM), MgSO<sub>4</sub> (77 mM, 154 mM, 308  
65 mM), MgCl<sub>2</sub> (77 mM, 154 mM, 308 mM), CaCl<sub>2</sub> (77 mM, 154 mM, 308 mM), 154 mM KCl,  
66 and pH buffer. The pH buffer for pH 5.0, 7.0, and 8.5, was prepared using NaH<sub>2</sub>PO<sub>4</sub>·2H<sub>2</sub>O  
67 and Na<sub>2</sub>HPO<sub>4</sub>·12H<sub>2</sub>O.  
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### 70 **2.3 Histological analysis**

71 Decellularized porcine liver disks (8mm diameter) were prepared using biopsy punch and  
72 fixed by 10% neutral buffered formalin. Tissue samples were embedded in paraffin and  
73 sectioned. Hematoxylin and eosin (H&E) staining was performed to evaluate the tissue  
74 sections. Also, frozen sections (8 μm in thickness) were prepared for Hoechst staining and  
75 were observed using fluorescence microscopy (Tokyo Rikakikai co, Tokyo, Japan).  
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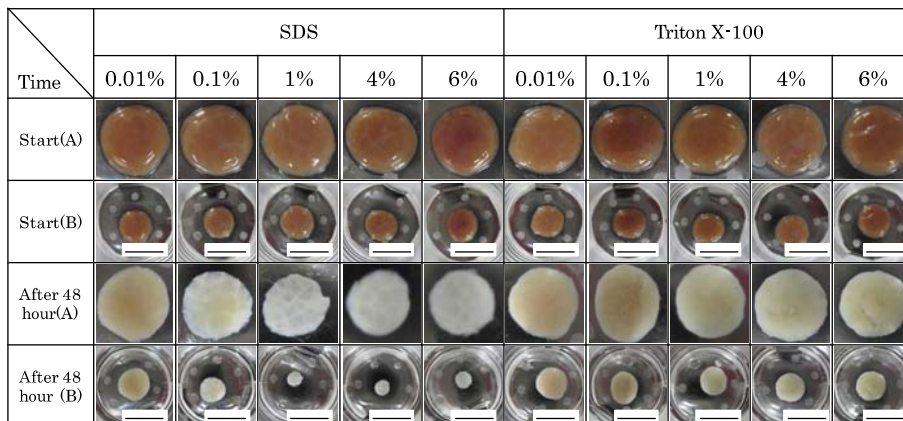
### 77 **2.4 DNA analysis**

78 DNA was obtained from each 20 mg of tissue by using QuickGene SP Kit DNA Tissue  
79 (Kurabo Industries, Osaka, Japan). Extracted DNA was stained using Hoechst 33258 and  
80 measured intensity was quantified.  
81

## 82 **3. RESULTS**

83 **3.1 Detergent concentration**

84 Porcine liver disks were decellularized using various concentrations of SDS in CMF-PBS  
 85 for 48 h. After 48 h in 1 - 6 % SDS, the decellularized disks turned white (Fig. 1); in 0.1 %  
 86 SDS, the disks were almost white. The remnant cells were seen at the central part of the  
 87 disks. Additionally, in 1 - 6 % SDS, the disks shrunk to about 50 % of their original size  
 88 whereas in 0.1 % SDS, the disks shrunk to about 30 % of their original size. Additionally,  
 89 after 48 h in 1 - 6 % TX solution, the disks turned white (Fig. 1).  
 90



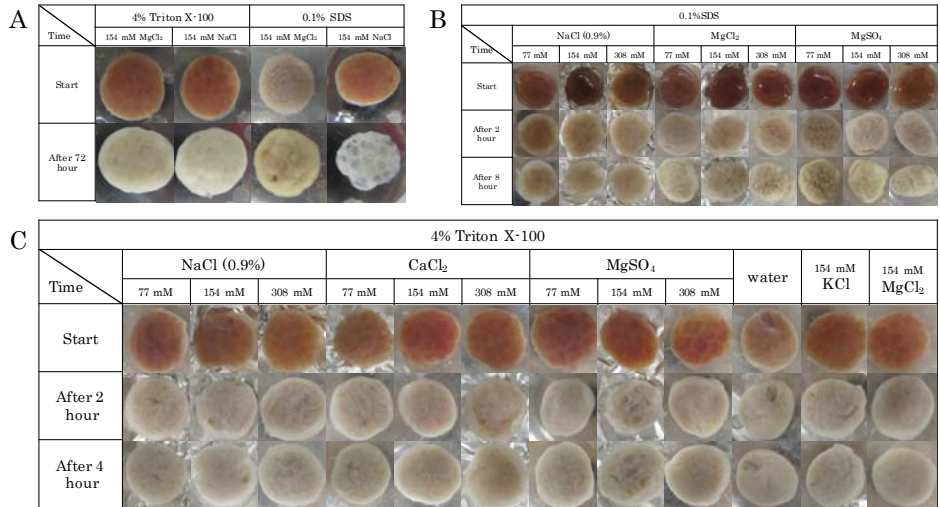
**Fig. 1 Appearance of decellularized porcine discs using SDS and TX and CMF-PBS. Scale bars = 1cm.**

91  
 92 **3.2 Effect of salt on the decellularization**

93 Decellularization was performed using 4 % TX and 0.1 % SDS in NaCl and MgCl<sub>2</sub> aqueous  
 94 solution (Fig. 2A); 0.1 % SDS in NaCl, MgCl<sub>2</sub>, and MgSO<sub>4</sub> aqueous solution (Fig. 2B); and 4  
 95 % TX in distilled water, NaCl, MgSO<sub>4</sub>, CaCl<sub>2</sub>, KCl, and MgCl<sub>2</sub> aqueous solution (Fig. 2C).  
 96 After decellularization with 0.1 % SDS in NaCl aqueous solution, the porcine liver disks  
 97 became harder, and crystals were observed in 0.1 % SDS in MgCl<sub>2</sub> aqueous solution  
 98 (Figs.2A and 2B). However, decellularization did not progress in 0.1 % SDS supplemented  
 99 with MgCl<sub>2</sub> and MgSO<sub>4</sub> (Fig.2B). When any of the salts were added in 4% TX,  
 100 decellularization was not observed (Fig.2C). In other words, decellularization was observed  
 101 in 0.1% SDS, and only when in NaCl was used as a salt.  
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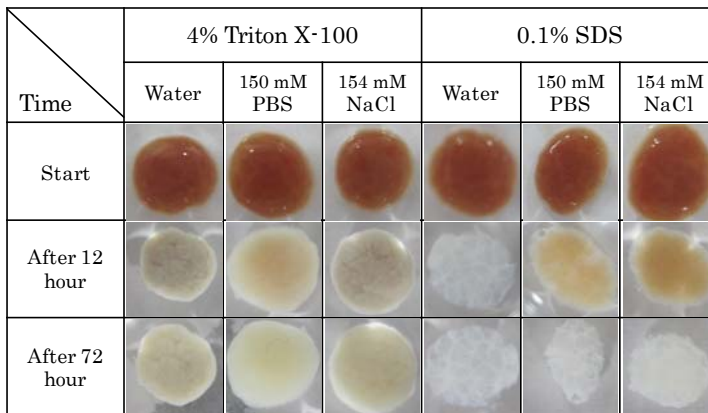
103 The effect of CMF-PBS and NaCl on decellularization was studied using 4 % TX and 0.1 %  
 104 SDS (Fig.3). In the case of SDS, decellularization was found to be progressing with time,  
 105 and liver disks were found to have decellularized well by 3 days under all conditions.  
 106 However, no difference between CMF-PBS and NaCl in terms of their effect on the  
 107 decellularization process was observed. On the other hand, disks changed to grey in pure  
 108 water and NaCl when decellularization was carried out using TX; only a few changes were  
 109 observed after 12 hours.  
 110

111 Influence of the salt concentration on decellularization was studied using 0.1 % SDS  
 112 (Fig.4). Decellularization was inhibited with an increase in the salt concentration. This  
 113 phenomenon was seen when using CMF-PBS and NaCl. On the other hand,  
 114 decellularization with 0.1 % SDS in pure water seemed to be have completed within 7 hours  
 115 of incubation. Additionally, shrinkage of the disks could not be confirmed during the  
 116 decellularization.  
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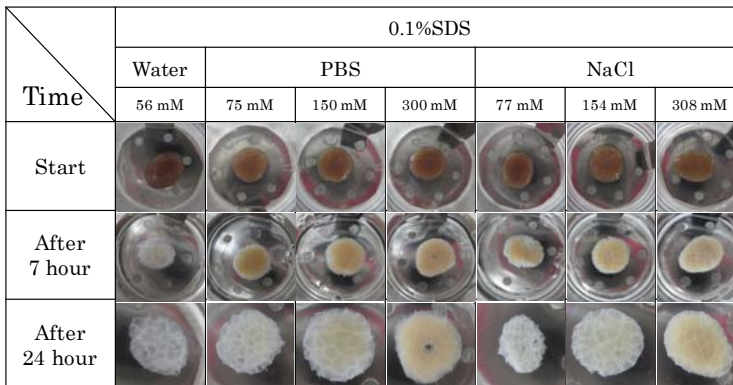
**Fig. 2** Appearance of decellularized porcine discs in 4% TX and 0.1% SDS in NaCl and MgCl<sub>2</sub> aqueous solution (A) and 0.1% SDS in NaCl, MgCl<sub>2</sub> and MgSO<sub>4</sub> aqueous solution (B), and in distilled water, NaCl, MgSO<sub>4</sub>, CaCl<sub>2</sub>, KCl, and MgCl<sub>2</sub> aqueous solution (C).

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

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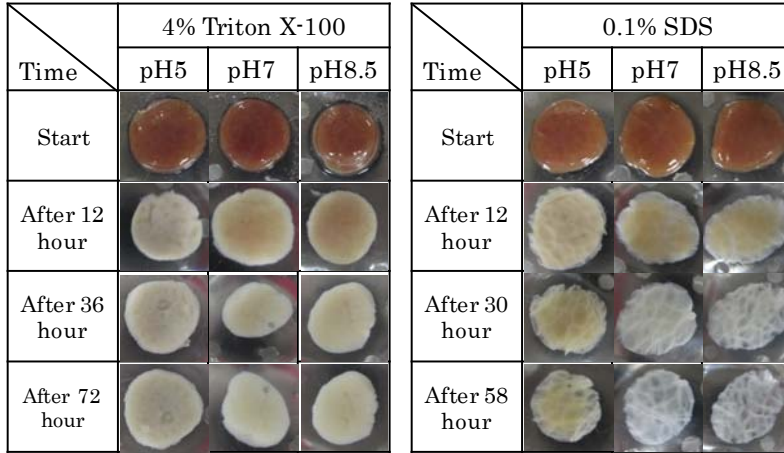
**Fig. 4** Influence of salt concentration on decellularization.

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### 3.3 Effect of pH on decellularization

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

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### 3.4 Decellularization with distilled water as a solvent

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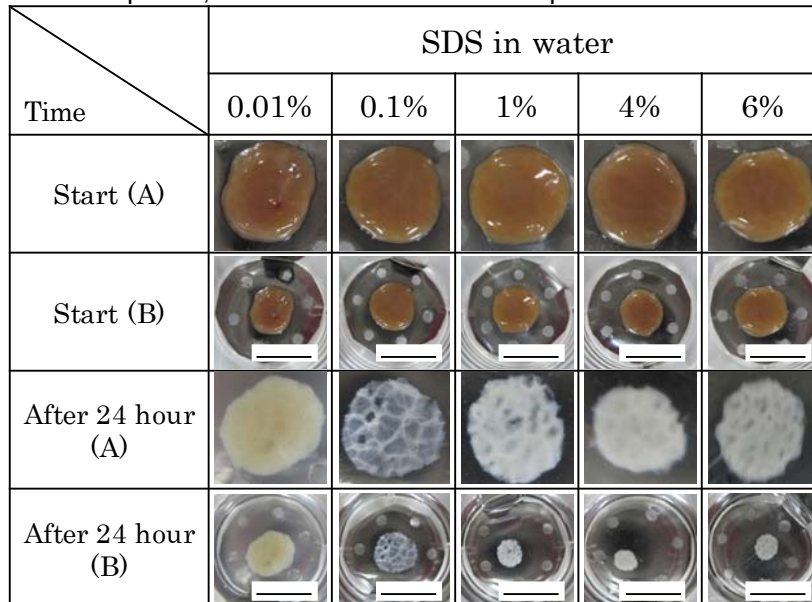
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Influence of SDS concentration in pure water as a solvent on decellularization was studied. Decellularization was not observed in 0.01 % SDS. On the other hand, decellularization within 12 hours was confirmed for 1 - 6 % of SDS(Fig.6). However, in 1 - 6 % of SDS, a remarkable shrinkage of disks was also seen. Interestingly, the shrinkage of the 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.



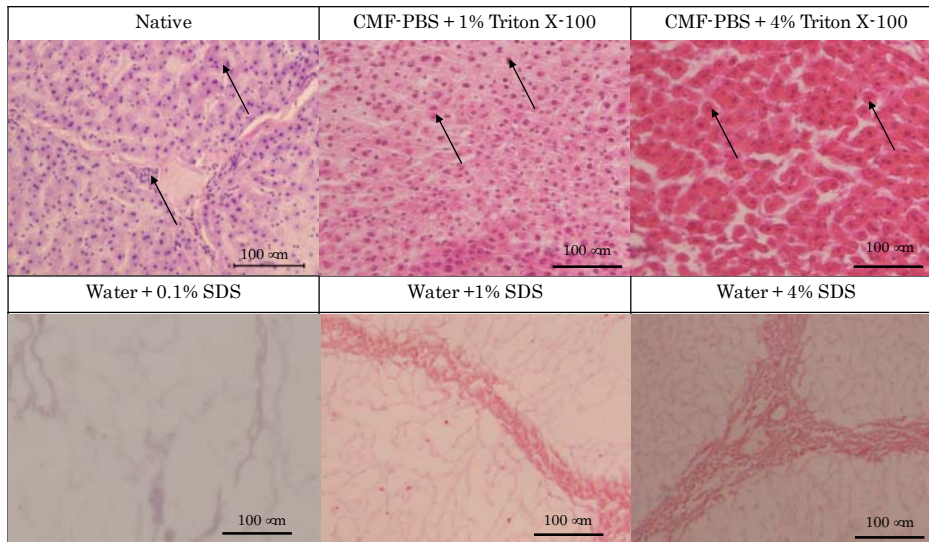
**Fig. 6 Influence of SDS concentration in pure water for decellularization. Scale bars = 1 cm.**

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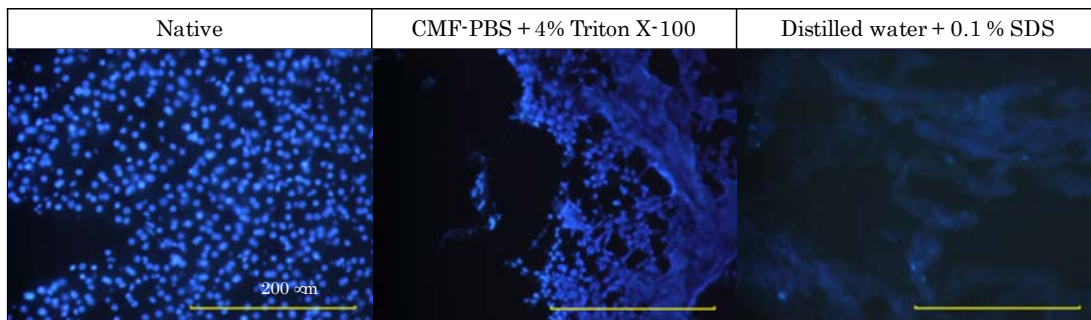
### 3.5 Confirm decellularization of liver disc

138 Fig.7 shows the Histological analysis of decellularized sections. Decellularization using  
 139 CMF-PBS as a solvent in 1-4% TX solution, the sections appeared to be similar to native  
 140 tissue. On the other hand, decellularization with 0.1-4% SDS solution using distilled water as  
 141 a solvent, complete removal of cellular contents can be seen.  
 142



**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.**

143  
 144 Hoechst staining (Fig. 8) clearly indicates effective removal of nuclear content when 0.1-4%  
 145 SDS solution in distilled water was used as a solvent for decellularization. Also, DNA  
 146 quantification (Fig. 9) clearly indicated 99% removal of DNA content with 0.1-4% SDS  
 147 solution in distilled water as a solvent for decellularization.  
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**Fig.8 Nucleus staining of decellularized tissue by Hoechst.**

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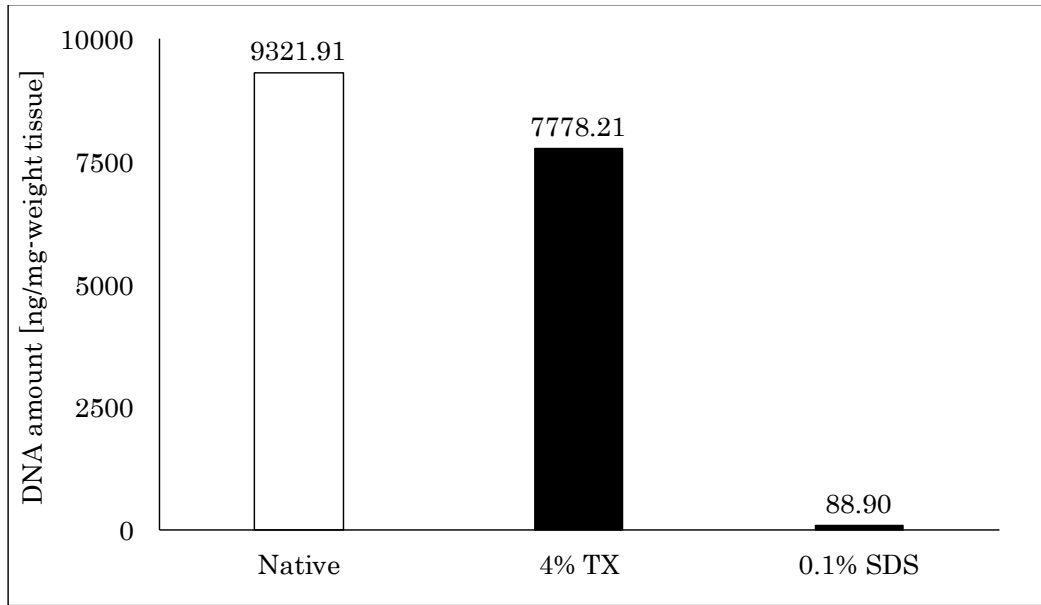


Fig.9 DNA quantification in native and decellularized liver tissue prepared with various methods.

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#### 4. DISCUSSION

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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 colour 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 don't contain many cells.

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

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

## 193 194 **5. CONCLUSION**

195  
196 In this current study, we established the concentration condition of surfactant that causes  
197 the collapse of the structure during decellularization. In addition, since the solvent suitable  
198 for each surfactant has been established, more effective preparation of the decellularized  
199 liver can be expected even with the same concentration of the surfactant. These findings  
200 would prove to be useful in the preparation of not only the decellularized liver but also other  
201 decellularized organs.

### 202 203 **Ethical Approval**

204  
205 The experimental protocol mentioned in this study was reviewed and approved by the Ethics  
206 Committee on Animal Experiments of Kyushu University (Fukuoka, Japan).

### 207 208 **Patient's Consent**

209 NA

## 210 211 **ACKNOWLEDGEMENTS**

212  
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215 language editing.

## 216 217 **COMPETING INTERESTS**

218  
219 The authors declare no conflict of interest.

## 220 221 **AUTHORS' CONTRIBUTIONS**

222  
223 Jaeyong Cho, Hiroyuki Ijima. conceived and designed the experiments; Jaeyong Cho.  
224 performed the experiments; Jaeyong Cho. and Nana Shirakigawa. analyzed the data; and  
225 Jaeyong Cho. and Yukako Fukuda. wrote the paper.

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