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ORIGINAL ARTICLE

# Surfactant-free preparation of an ostrich carotid artery scaffold using liquefied dimethyl ether and DNase

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**Abstract** Instead of combining the surfactant sodium dodecyl sulphate and DNase, a combination of liquefied dimethyl ether (DME) and DNase was used to decellularise the scaffold of ostrich carotid artery tissue. Firstly, lipids were extracted from ostrich carotid artery tissue using liquefied DME at 25 °C and a pressure of 0.59 MPa. After DME extraction, the ostrich carotid artery tissue was collected from the extraction column, and the DME remaining in the tissue was evaporated at atmospheric pressure and temperature. DNA fragmentation by DNase was then carried out using a method almost identical to the conventional method. Finally, the tissue was washed to remove fragmented DNA. The DNA was completely fragmented to a size of less than 100 bp after 1 day of DNase treatment. The residual DNA had a concentration of 28 ng/mg dry weight after 7 days of treatment with DNase. Haematoxylin and eosin staining showed that most of the cell nuclei were removed from the aortic tissue. These results indicate that the combination of liquefied DME extraction and DNase treatment eliminates the need for surfactant treatment in ostrich carotid artery tissue decellularisation. Although previous decellularisation studies have focused on porcine tissue, we herein show the potential of ostrich tissue as an alternative to alleviate religious concerns.


## 1. Introduction

Organ transplantation is one of the available treatments for severe organ failure. However, organ transplantation has several complications, including ethical issues, lack of organs for transplantation, and host rejection. To solve these problems, organs that do not cause host rejection by culturing the cells of patients in a three-dimensional scaffold are being generated. Current issues are expected to be resolved by decellularising tissue to create a scaffold. Decellularised porcine tissue is being studied as a scaffold for regenerative medicine; however, because pigs are a religious taboo for Muslims, it is essential to provide a scaffold for regenerative medicine derived from sources other than porcine tissue. To this end, we focused on the largest bird species, the ostrich, as an alternative tissue source and attempted to decellularise its carotid

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artery. There are very few existing studies on the decellularisation of ostrich tissue. One study described the successful decellularisation of ostrich tendons using sodium dodecyl sulphate (SDS) (Hosseini et al. 2015).

Methods for decellularising porcine tissue can be classified as chemical, such as acid/alkali treatment and detergent and enzymatic digestion, or physical, such as quick freezing and mechanical compression (Badylak, 2004; McFetridge et al., 2004; Rieder et al., 2004; Ketchedjian et al.,2005; Sawada et al., 2008). The process is most commonly carried out using detergent, which has the advantage of simplicity. A typical detergent treatment involves three steps. The first step is lipid extraction using the surfactant SDS. An important aspect of successful decellularisation is the removal of lipids from the tissues; adding DNase without removing the lipids will not decellularise the cells. The next step is DNA fragmentation by DNase. The final step involves washing the tissue using water and ethanol to remove DNA fragments. SDS has a very strong degreasing effect on tissues; however, SDS also causes inflammation in the tissues it comes into contact with and has a very high affinity for proteins, leading to protein denaturation. This protein denaturation can severely damage the extracellular matrix. If SDS remains in the scaffold, there is a concern that the subsequent host cell culture will be inhibited or that later in vivo transplantation may be impaired. Therefore, SDS must be completely removed after the decellularisation process. In summary, long processing times, changes in mechanical properties, and toxicity are current issues associated with the use of SDS for decellularisation (Korossis et al., 2005; White et al., 2005; Gilbert et al., 2006; Prasertsung et al., 2008; Funamoto et al., 2010; Crapo et al., 2011; David et al., 2012; Timothy et al., 2015; Wu et al., 2015).

Acid- and base-containing protocols utilise agents such as peracetic acid and techniques such as reversible alkaline swelling (Gilpin and Yang, 2017). Thinner tissues, such as the small intestine submucosa (Syed et al., 2014) and urinary bladder tissue (Gilbert et al., 2008), have been decellularised with peracetic acid. These were shown to be biocompatible, but the cells were not completely removed (Syed et al., 2014). Furthermore, the mechanical properties of these tissues were altered, presenting with increased yield stress and elastic modulus (Gilbert et al., 2008). In other words, the production of a stiff extracellular matrix means that peracetic acid may not be suitable for tissues wherein elasticity is desired, including blood tube. Studies on base-containing protocols for the decellularisation of porcine carotid arteries (Chuang et al., 2009) and bovine pericardium (Mendoza-Novelo et al., 2011) have been conducted using alkaline solutions or mixtures with surfactants. Both tissues were successfully decellularised; however, swelling was reported in the case of bovine pericardium due to a negative charge on the collagen in the tissue, which could be reversed with ammonium sulphate. Despite this, the swelling reduced the glycosaminoglycan content and viscoelasticity of the tissue (Mendoza-Novelo et al., 2011).

In other studies, a high hydrostatic pressure method was successfully employed to decellularise carotid artery tissue, and SDS was not used. However, this high hydrostatic pressure technology requires special processing equipment that can handle ultra-high pressures of 980 MPa (Mahara et al., 2015; Mahara et al., 2020).

In this study, we used dimethyl ether (DME) in order to address the issues associated with SDS, acid- and base-treatments, and the high hydrostatic pressure method. DME is a gas at ambient temperature and pressure; if DME can be transformed into a liquid at a higher temperature and pressure than the standard boiling point and used as a solvent to extract lipids, this would present a solution to the issues presented by SDS. DME is the simplest ether and has unique properties compared to other ethers, such as diethyl ether. The standard boiling point of DME is −24.8 °C (Wu et al., 2011). Furthermore, because DME is weakly polar, it can be partially mixed with water (Holldorff and Knapp, 1988; Tallon and Fenton, 2010). The European Food Safety Authority recognises the safe residue limits of DME to be 3 mg/kg for non-fat protein products and 9 μg/kg for gelatin, and considers DME to have no safety concerns when used as a solvent for food processing (European Food Safety Authority, 2009). In the United States, chocolate is mixed with liquefied DME and sprayed to coat the surface of sweets (Varlet et al., 2014). Unlike other ethers, DME is a safe substance that hardly generates peroxides (Naito et al., 2005). Moreover, liquefied DME has already been extensively studied as a lipid extraction solvent for plant cells. For example, it has been shown that similar amounts of lipids can be extracted from wet plant cells, algae, labyrinthulea, and wastewater sludge by using DME than by using conventional toxic organic solvents (Afraz et al., 2019a,b; Boonnoun et al., 2019; Kanda et al., 2015; Owen et al., 2003; Hoshino et al., 2016; Hoshino et al., 2017; Kanda et al., 2020a; Li et al., 2014). Furthermore, DME does not remain in the extraction residues of samples (Hoshino et al., 2017; Kanda et al., 2020b). In the case of the plant microalga *Euglena gracilis*, which does not have a cell wall, lipids extracted by conventional organic solvents and liquefied DME have the same molecular weight distribution and elemental proportions, and all lipids can be completely extracted (Kanda et al. 2015). This indicates that liquefied DME can also effectively remove lipids from animal cells, and SDS could be replaced by liquefied DME for removing lipids during scaffold preparation.

In this study, using the carotid artery as a model, we focused on ostrich tissue decellularisation in order to address religious concerns regarding the use of porcine tissue and the SDS residues in the decellularised tissue. The ostrich was chosen as the model organism because they have long carotid arteries, which could therefore be used as a scaffold for transplantation to various parts of the human body.

In general, the criteria required to verify a tissue as decellularised are as follows: the cell nucleus should not be visible by haematoxylin–eosin (H-E) staining; the amount of residual DNA should be less than 50 ng/mg dry weight; and the fragment of residual DNA should be smaller than 200 bp (Rana et al., 2017; Crapo et al., 2011; Brown et al., 2009; Manfredi et al., 2009; Nagata et al., 2010; Zhang et al., 2010). In this study, we examined whether DME can be used to prepare decellularised tissues that meet these criteria.

## 2. Materials and methods

### 2.1. Materials

Fresh ostrich carotid arteries were obtained from a livestock farmer (Oyama Ostrich Farm, Oyama City, Japan). The ostrich carotid arteries used in this study were sourced from

ostriches slaughtered for food. The carotid arteries were excised and cut into 1.5 cm lengths. The water contents of ostrich carotid arteries were 36.4–38.9 wt%, which were measured from the weight difference before and after heating the ostrich carotid artery at 107 °C until the weight did not change. The lipids of ostrich carotid arteries were cut with a knife and immersed in phosphate-buffered saline free of $Ca^{2+}$ and $Mg^{2+}$ and stored immediately at 4 °C.

### 2.2. Preparation of decellularised ostrich carotid artery tissue

The decellularisation protocol consisted of three steps: lipid extraction by liquefied DME; DNA fragmentation by DNase; and washing for removal of DNA fragments. The main difference from the conventional method of decellularisation by SDS is the use of liquefied DME instead of SDS, while the other two steps remained the same. To check the reproducibility of this method, a series of experiments were carried out in triplicate.

#### 2.2.1. Lipid extraction by liquefied DME

Lipid extraction using liquefied DME was conducted according to previous studies of lipid extraction from plants and algae (Kanda et al., 2015; Hoshino et al., 2017; Kanda et al., 2020a; Li et al., 2014). A schematic diagram of the experimental setup is shown in Fig. 1. Liquefied DME (Spray Work Air Can 420D; Tamiya, Shizuoka, Japan) was placed into the extraction column from a storage vessel filled with liquefied DME (TVS-1–500, volume: 500 mL; Taiatsu Techno Corp., Saitama, Japan). The vapour pressure of the saturated DME in the storage vessel was increased by heating the storage vessel to 35 °C. Pressurised liquefied DME was pushed from the storage vessel by its vapour pressure and rapidly cooled in a connected 1/16-inch SUS tube (1 m length) into the extraction column. At the inlet of the extraction column, the temperature and pressure were 25 °C and 0.59 MPa. The flow rate of DME was controlled to be 5 ± 1 mL/min by a manual flow control valve (1315G4Y, Swagelok, Hyogo, Japan) connected at the outlet of the extraction column. Wet carotid artery tissue (2.44–2.45 g) was loaded into a 96 $cm^3$ glass pressure vessel used as an extraction column (cylindrical shape with a narrow lower end; customised HPG-96–3, Taiatsu Techno Corp.). Since DME flows from the bottom of the extraction column to the top, the dead space around the ostrich carotid artery tissue in the column was filled with glass beads and cotton to fix the tissue. This fixation ensured that the tissue was not shaken in the extraction column by the flow of DME. In the extraction column, lipids were extracted using liquefied DME. The outlet of the extraction column was connected to an empty 96 $cm^3$ pressure vessel (HPG-96–3, Taiatsu Techno Corp.) via an SUS connection tube. The used liquefied DME flowed into the empty pressure vessel. The empty pressure vessel was made of transparent glass and had a volume memory printed on it like a measuring cylinder. The DME flow rate was obtained using the volume memory. When an appropriate amount (around 30 mL) of DME was stored in the vessel, the manual flow control valve was stopped and the vessel was quickly replaced with a new vessel. The pressure reducing valve of the old vessel was opened to reduce the pressure inside the vessel. DME was evaporated by decompression, and the extracted lipid was retained in the vessel. Finally, the sample was obtained.

#### 2.2.2. DNase treatment

DNA fragmentation was performed by adding 30 mL DNase saline to 1.0 g ostrich carotid artery tissue with lipid extracted using DME. NaCl (0.9%) and 1% penicillin and streptomycin deionised water (Thermo Fisher Scientific, Kanagawa, Japan) were prepared and used as saline. DNase saline contained 0.2% DNase (Roche Diagnostic, Tokyo, Japan) and 0.05 mol/L $MgCl_2$ $6H_2O$ (Wako, Osaka, Japan). The prepared DNase saline was handled on a clean bench to avoid contact with the outside air. After DME extraction, the ostrich carotid artery tissue immersed in DNase saline was shaken at 4 °C for 1–7 days.

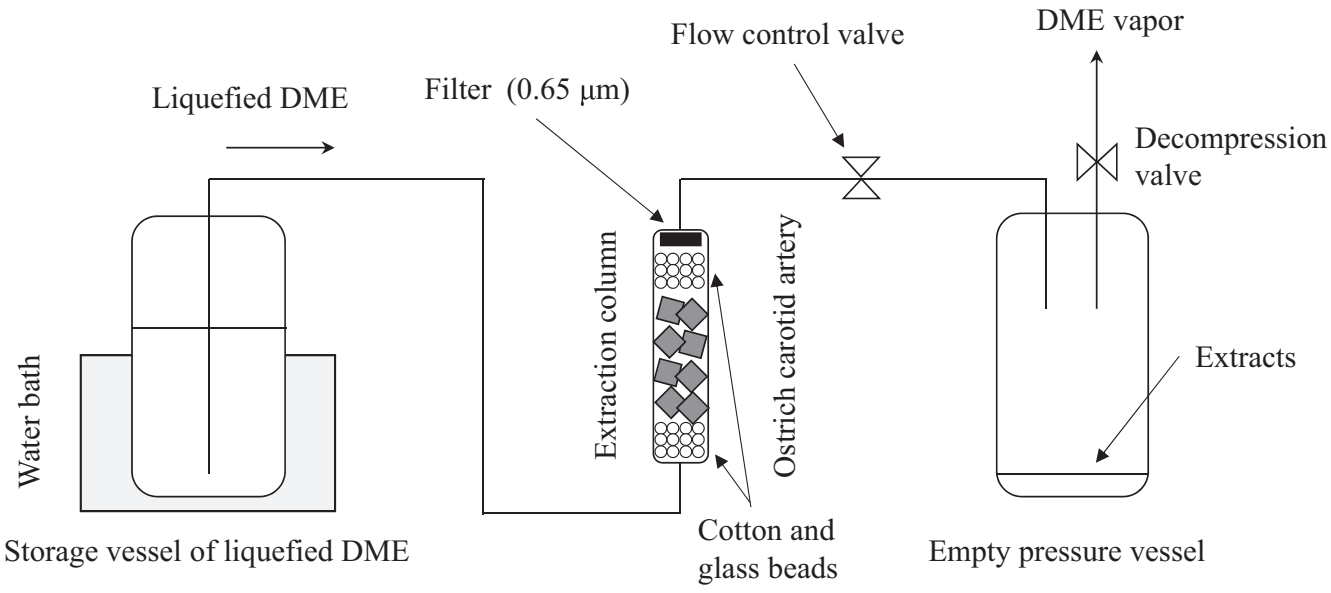


**Fig. 1** Schematic diagram of lipid extraction using liquefied dimethyl ether (DME).

#### 2.2.3. Washing process

After DNA fragmentation by DNase, the ostrich carotid artery tissue was washed by 80/20 (v/v) ethanol/saline for 1 h. Then, the tissue was immersed in fresh 80/20 (v/v) ethanol/saline containing antibiotics and stored at 4 °C. This was repeated 1, 2, and 3 days later, respectively. Subsequently, the samples were immersed in saline containing antibiotics at 4 °C for 1 day. This process uses ethanol, which raises religious concerns; however, this study focuses on the conventional lipid removal process.

### 2.3. Evaluation of residual DME in decellularised tissue

The amount of DME remaining in the ostrich carotid artery tissue was detected using a GC/MS head space system according to an existing protocol (Hoshino et al. 2017). In brief, 1 day after DME extraction, 0.50 g ostrich carotid artery tissue was placed in 10 mL headspace vials. GC/MS analysis was carried out using an Agilent 7890B GC system connected to an Agilent 5977A mass spectrometer on a cyanopropyl capillary column (VF0624MS; 60 m × 0.32 mm (i.d.) × 1.8 µm, Agilent Technologies Tokyo Ltd., Hachioji, Japan). An Agilent 7697A headspace sampler was also connected. The heating temperature of the head space was 50 °C. The vial equilibration time was set at 10 min. The GC oven temperature was initially set at 40 °C for 5 min; the temperature was subsequently increased to 260 °C at a rate of 5 °C $min^{-1}$. The mass range was 29–450 $m/z$.

### 2.4. Evaluation of decellularised tissue

#### 2.4.1. Optical observation by H-E staining

The decellularised ostrich carotid artery tissue was stained with 1% H-E. The tissue was then sliced and observed using an optical microscope.

#### 2.4.2. Amount of residual DNA

The remaining DNA was obtained from the DME-extracted ostrich carotid artery tissue and purified as follows. Five milligrams of ostrich carotid artery tissue was mixed with 200 µL proteinase K solution (1 M Tris-HCl aqueous solution [pH 7.8]: 0.5 M ethylenediaminetetraacetic acid [EDTA; pH 8.0] aqueous solution: proteinase K: water: SDS = 1:2:2:95:0.5 v/v/v/v/w) in a microtube at 55 ℃ for 1 day and converted to a solution. Tris-HCl and EDTA were purchased from Nippon Gene Co., LTD (Tokyo, Japan). Proteinase K was purchased from Takara Bio Inc. (Kusatsu, Japan). DNA was extracted from the tissues by phenol/chloroform extraction and purified by ethanol precipitation (Negishi et al. 2015).

For DNA quantification, 1 µL TE Buffer aqueous solution with dissolved DNA was measured by ultraviolet–visible spectrophotometry (NanoDrop Microvolume Spectrophotometers and Fluorometer, Thermo Fisher Scientific, Kanagawa, Japan). The detection wavelength was 260 nm for DNA. Phenol peaks, which indicate impurities, were observed at 265 nm. Furthermore, the ratio of intensities at 260–280 nm was approximately 2:1, confirming that protein contamination could be almost completely removed.

#### 2.4.3. DNA fragment distribution

The distribution of the fragmented DNA contained in the solution obtained in Section 2.4.2 was measured by an agarose gel electrophoresis system (WSE-1150 PageRunAce, Atto Corporation, Tokyo, Japan). The molecular weight range of fragmented DNA was 70–1,800 bp. Precast polyacrylamide gels (12.5%, EHR-R12.5L e-PAGEL HR, Atto Corporation) were used with a polyacrylamide/tris-HCl buffer. The DNA solution was mixed with loading dye buffer (WSE-7040 EzApply DNA, Atto Corporation) and tris–glycine buffer (WSE-7055 EzRunTG, Atto Corporation) as electrode buffer at a ratio of 1:1:1 (v/v/v). Subsequently, the DNA fragments were dyed using fluorescent stain reagent (WSE-7130 EzFluoroStain DNA, Atto Corporation) and molecular weight marker (WSE-7030 EzDNA Ladder, Atto Corporation).

#### 2.4.4. Fourier transform infrared (FTIR) spectra

Liquefied DME partially mixes with water; therefore, water is also extracted from the ostrich carotid artery tissue during lipid extraction, which may cause chemical changes in the tissue. To investigate the chemical changes in the structure of the carotid artery tissue extracted by DME, we obtained FTIR

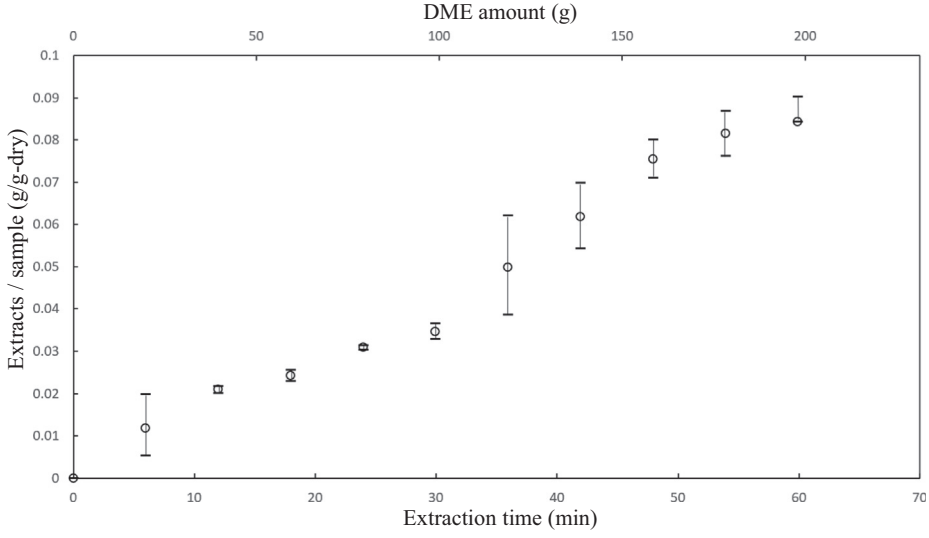


**Fig. 2** Amount of lipid extracted from ostrich carotid artery tissue by liquefied dimethyl ether (DME) extraction.

spectra of the original carotid artery tissue and the carotid artery tissue extracted with DME using ATR-FTIR (Spectrum Two, PerkinElmer Japan K.K., Yokohama, Japan). The DME-treated carotid artery tissue was also measured after rewetting with DNase solution.

## 3. Results and discussion

### *3.1. Relationship between the amount of DME and lipid extraction*

Fig. 2 shows the efficiency of liquefied DME for extracting lipids from ostrich carotid artery tissue. Liquefied DME extraction for 1 h yielded 0.84 wt% lipids from the ostrich carotid artery tissue. As the density of liquefied DME at 25 °C is 0.661 $cm^3/g$ (Wu et al., 2011), the weight of the liquefied DME during the 1 h extraction was approximately 200 g (Fig. 2). The ostrich carotid artery tissue was obtained from the extraction column after extraction. The results of headspace GC/MS analysis showed that residual DME was not detected in the ostrich carotid artery tissue, because DME was completely evaporated by its depression to atmospheric pressure. Lipids were removed from ostrich carotid artery tissue without using SDS as the extraction solvent; instead DME was used, which has a low boiling point. If the DNA is fragmented and removed by subsequent DNase treatment and washing, then decellularisation can be successfully achieved without any concerns regarding SDS residues.

### *3.2. Optical observation by H-E staining*

Decellularised tissues treated for various periods using DNase and subjected to washing were observed by H-E staining (Fig. 3). Compared to the untreated ostrich carotid artery tissue, that treated with liquefied DME showed similar cell nuclei

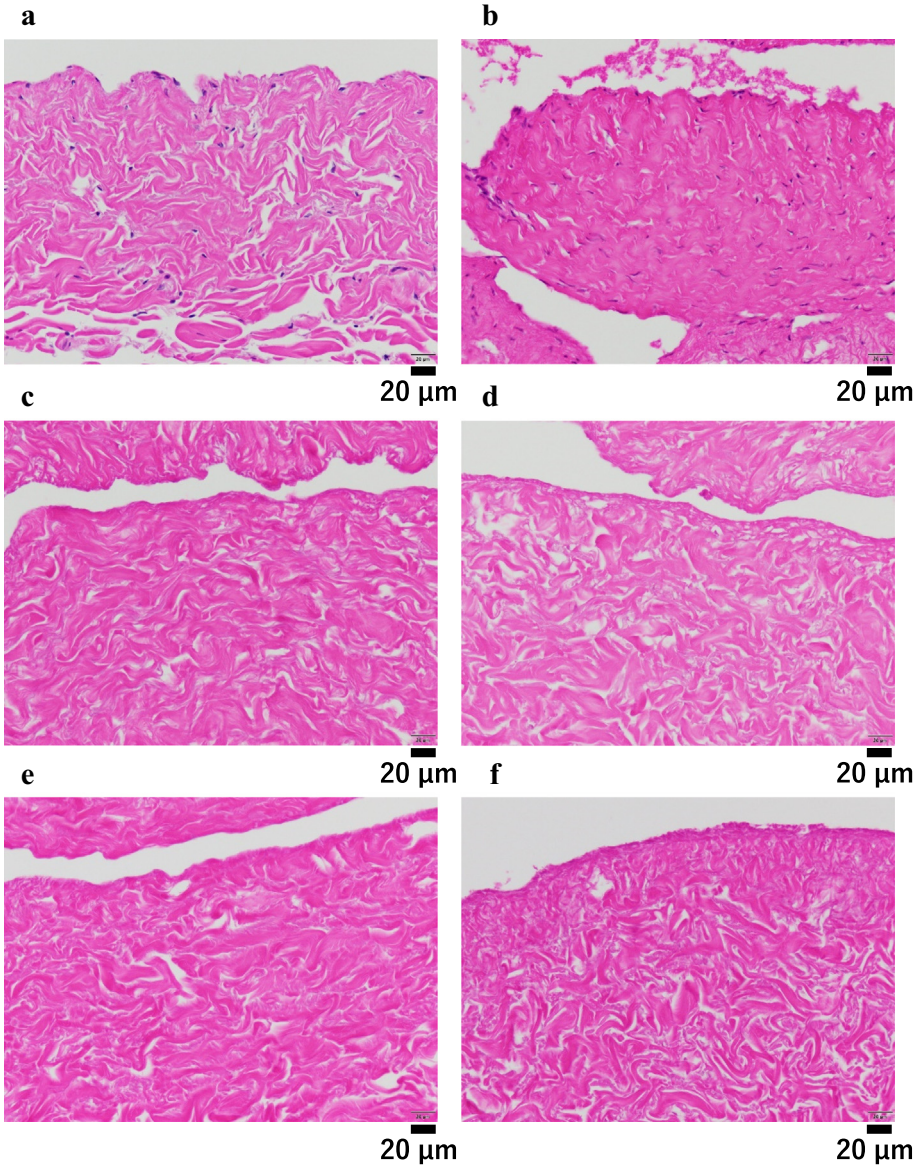


**Fig. 3** Haematoxylin-eosin (H-E) staining. (a) Untreated. (b) Dimethyl ether (DME) extraction only (c–f) DNase treatment for 1 (c), 3 (d), 5 (e), and 7 (f) days following DME extraction.

(Fig. 3 (a, b)). In the samples treated with DME followed by DNase for 1–7 days, cell nuclei were not observed and were completely removed (Fig. 3 (c–f)). These results indicate that liquefied DME is ineffective at removing cell nuclei; however, cell nuclei could be removed without using SDS by combining DNase treatment with washing.

### 3.3. Amount of residual DNA

The amount of DNA remaining in the ostrich carotid artery tissue treated by DME and then DNase for various periods is shown in Fig. 4. The amount of residual DNA was 110 ng/mg dry weight when only DME extraction was performed, which is above the target value of 50 ng/mg dry weight (Brown et al., 2009; Manfredi et al., 2009; Nagata et al., 2010; Zhang et al., 2010). The amount of residual DNA after 1 day of DNase treatment was 96 ng/mg dry weight, indicating that the fragmentation by DNase had not yet progressed. The amount of residual DNA after 3 days of DNase treatment was an average of 58 ng/mg dry weight. After 5 days of DNase treatment, the amount of residual DNA averaged 41 ng/mg dry weight, and was not below the target for one of the three trials. The amount of residual DNA after 7 days of DNase treatment was 28 ng/mg dry weight, which was below the target for all trials. This indicates that 7 days of DNase treatment is necessary to produce ostrich carotid artery tissue scaffolds.

### 3.4. DNA fragment distribution

The distribution of the DNA fragments remaining in the samples was evaluated by agarose gel electrophoresis, as shown in Fig. 5. In the original and DME-treated samples, the size of the remaining DNA was large (>1,000 bp), indicating that the DNA had not been fragmented (Fig. 5 (a, b)). After DME extraction and DNase treatment for 1 day, the DNA was completely degraded to less than 100 bp and was not detected (Fig. 5(c)). Furthermore, after a longer period of DNase treatment, DNA was not detected (Fig. 5 (d–f)). The

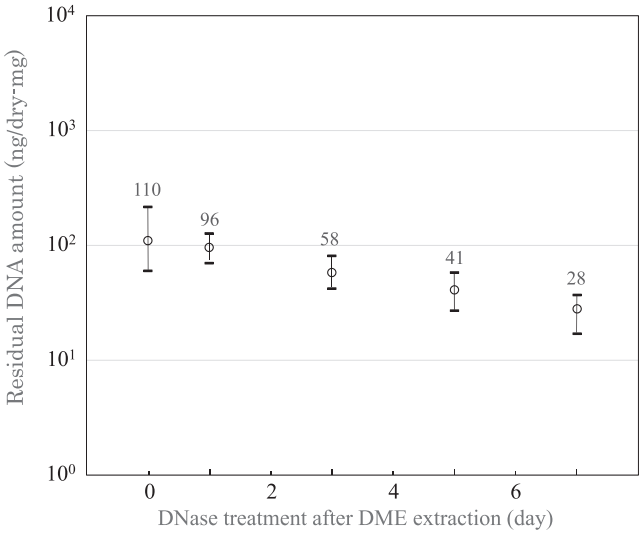


**Fig. 4** Amounts of residual DNA in the ostrich carotid artery tissue.

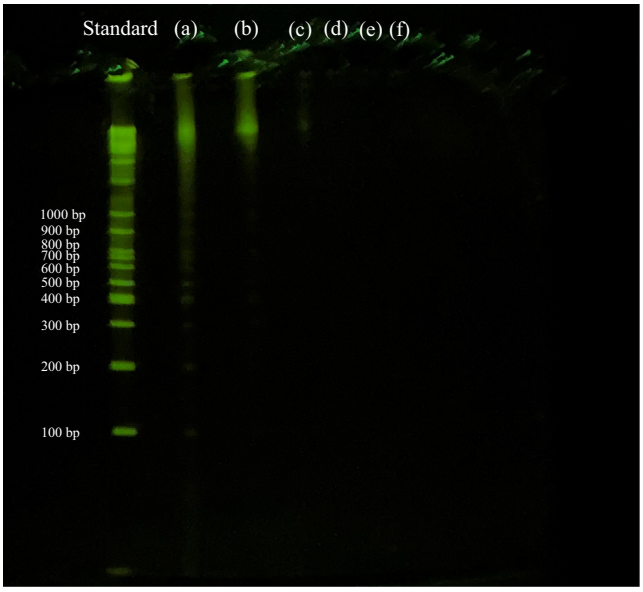


**Fig. 5** Fragments of residual DNA in the samples detected by agarose gel electrophoresis. (a) Untreated. (b) Dimethyl ether (DME) extraction only. (c–f) DNase treatment for 1 (c), 3 (d), 5 (e), and 7 (f) days following DME extraction.

results of agarose gel electrophoresis and the amount of residual DNA in the sample shown in Fig. 4 suggest that simply fragmenting the DNA to less than 100 bp with DNase is insufficient for removing the fragmented DNA during the subsequent washing process. Thus, further fragmentation by prolonged DNase treatment is necessary.

The results of DNA fragmentation are in agreement with the results of H-E staining and the quantitative analysis of the remaining DNA. Furthermore, all results indicate that the target was achieved. These findings show that ostrich carotid artery tissue can be decellularised without SDS by extracting lipids with liquefied DME, followed by DNA fragmentation with DNase. In the future, it will be necessary to find an alternative medium to ethanol, which is used in the final washing process, in order to produce scaffolds that can be used without any religious concerns.

### 3.5. FTIR spectra

FTIR spectra of the original (black curve) and DME-treated (red, before rewetting; green, after rewetting) carotid arteries are shown in Fig. 6. All carotid arteries show the typical two amide band of porcine gelatin (Pradini et al., 2018). The amide band at 1,538–1,551 $cm^{-1}$ implies N-H bending and C-N stretching vibrations, while the amide band at 1,631–1,633 $cm^{-1}$ implies C = O stretching vibrations (Pradini et al., 2018). The original carotid artery is characterised by a large OH group peak at 3,280–3,286 $cm^{-1}$ because of its high water content. In the carotid artery after DME treatment, this large peak disappeared due to the removal of water, The DME-treated carotid artery tissue showed small peaks at 2,923 $cm^{-1}$, which implies C-H vibration of the protein typically observed in gelatin (Pradini et al., 2018). In general, when proteins are dehydrated, a cross-linked structure may occur due to a Schiff base reaction between the amino and carbonyl

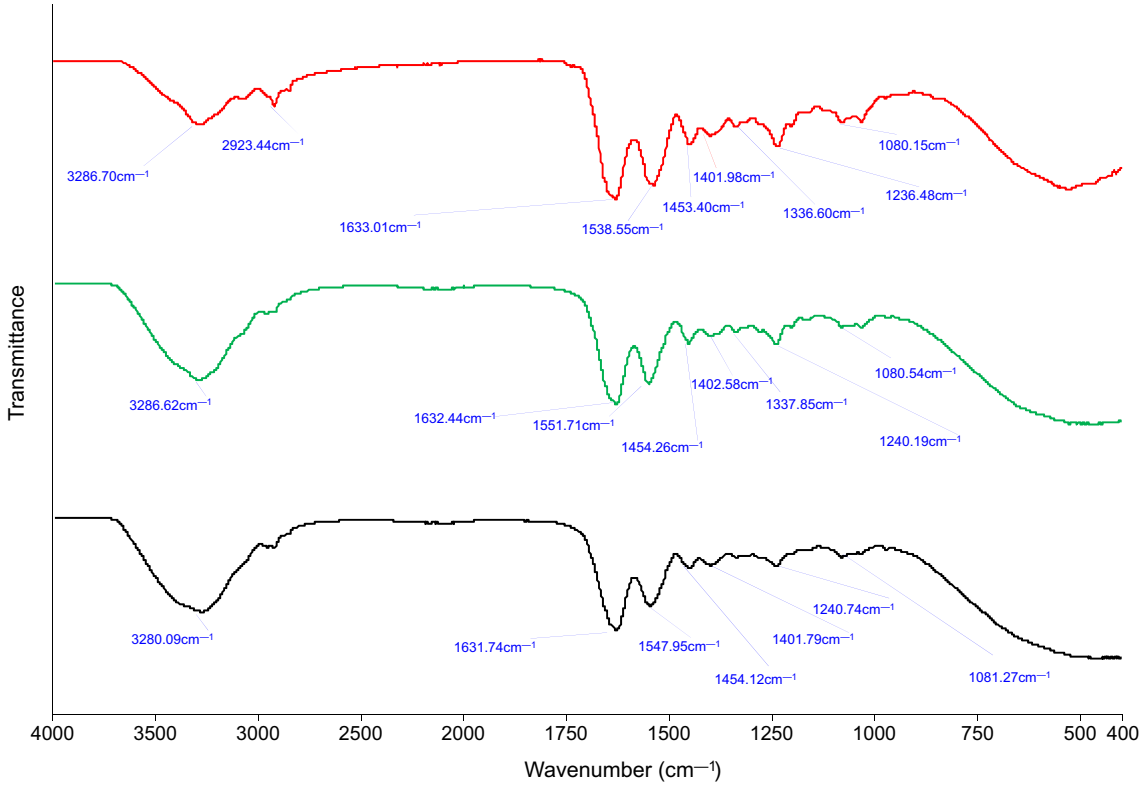


**Fig. 6** Fourier transform infrared (FTIR) spectra of ostrich carotid artery tissue. Black, original; red, after dimethyl ether (DME) treatment; and green, rewetted after DME treatment.

groups, resulting in a C = N bond (Kim et al., 2012). The C = N stretching vibration should appear at 1,736 $cm^{-1}$ (Kim et al., 2012), but the peak associated with this C = N stretching vibration was not observed in the DME-treated ostrich carotid artery tissue. The FTIR spectrum of the ostrich carotid artery tissue that was rewetted with DNase solution after DME treatment showed a very similar spectrum as the original carotid artery tissue. This finding indicates that dehydration by DME treatment has almost no effect on the chemical structure of the ostrich carotid artery tissue.

In the future, whether cells can safely grow in scaffolds created with subcritical DME should also be examined. Furthermore, the mechanical properties of the decellularised tissue may have been altered, so its mechanical properties should also be investigated.

## 4. Conclusions

As a first step in the decellularization process, lipids were extracted from ostrich carotid artery tissue using liquefied DME, which evaporated at −24.8 °C due to its low boiling point. Furthermore, liquefied DME did not remain in the ostrich carotid artery tissue. Liquefied DME failed to fragment DNA, while DNase completely fragmented DNA to less than 100 bp after treatment for 1 day. After treatment with DME followed by DNase for 7 days, the amount of DNA remaining in the ostrich carotid artery tissue was 28 ng/mg dry weight, which was less than the target value of 50 ng/mg dry weight. Furthermore, H-E staining showed that most of the cell nuclei were removed from the ostrich carotid artery tissue. In short, this study shows that ostrich carotid artery tissue could be used as an alternative to porcine scaffolds to alleviate religious concerns. Furthermore, the introduction of liquefied DME into the conventional decellularisation method eliminates the need for the use of surfactants.

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## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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