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Research Article

DECELLULARIZATION OF COW AORTA VIA SUPERCRITICAL CO_2

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ABSTRACT. Decellularized tissues have been extensively studied in recent years thanks to functioning as potential tissue scaffolds consisting of natural extracellular matrix components (ECM). The most critical issue to obtain a decellularized tissue is the removal of cells on the ECM without destroying the ECM components. Besides, while decellularizing a tissue, keeping the ultra-structure of the 3D composition of native ECM and thus, saving mechanical behavior is essential. In this study, the decellularization of cow aorta with SFE and with a hybrid method combining TritonX-100 / SFE was compared. The aorta was treated by SFE for 1h, 4h, 6h, and a combined method (SFE/Triton X-100) was performed as 2 hours Triton-X-100/1-hour SFE treatment and, overnight Triton-X-100 /1h SFE treatment. The complex structure of the ECM after decellularization via SFE was obtained fairly similar to native ECM compared to TritonX-100 treated cow aortic tissue samples according to the SEM images and H&E staining results. At the same time, tissue samples treated with SFE demonstrated an equal decellularization yield in a shorter time than those decellularized with chemical methods. Besides, mechanical strength and protein integrity of decellularized ECM by SFE was determined similar to the native tissue.

Keywords: *Decellularization, Supercritical carbon dioxide ($scCO_2$), Cow aorta tissue*

INTRODUCTION

Bypass grafting of coronary and peripheral vascular is carried out more than 600,000 times annually in the United States and Europe to cure cardiovascular diseases [1]. Nowadays; up to 40% of patients need bypass surgery and autologous vessels are mainly preferred as a graft material. However, these patients might not have a healthy artery or saphenous vein of the intended length for utilizing as an autograft. Although patients have an convenient venous tissue system for transplantation, occlusion or vascular narrowing of the vessels may be observed after a while [2].

The vascular grafts aim to replace or repair damaged tissue or organs in our bodies. Xenografting is a popular approach for vascular grafting that to acquire viable or similar tissue constructs from other animal species. Under this purpose, a decellularizing xenograft is a beneficial approach for tissue repair. In the decellularization process, a 3D ECM is obtained from an animal tissue that conserves its biomechanical, structural and biochemical properties while refining the cell debris. Decellularized matrices are produced by a multistep tissue-specific decellularization process including lysis of the cell membranes, separation of the cellular components from the ECM, solubilization of the cytoplasmic/nuclear components and removal of the cellular debris [3]. During the decellularization process of the tissue or organs, conservation of the native form of the ultrastructure and main composition of ECM is very important. The extracellular matrix

(ECM) is a noncellular component within all tissue and organs. It ensures physical scaffolding for the cells. Additionally; it initiates the significant biochemical and biomechanical behavior that are the main issue for tissue homeostasis, differentiation and morphogenesis [4]. Addition to these properties; decellularizing xenografts reduce the ratio of antigenicity, inflammation, and calcification [5]. Various types of tissue or organs were studied for decellularization such as tendon[6], heart valve [7], bladder [8] and artery[2].

The main goal of the decellularization process is to remove nuclear and cellular material with minimum damage to the ECM. According to this purpose, chemical, enzymatic, physical methods or combinations of the mentioned methods are preferred to remove cells and genetic materials from the tissue while conserving its structural and regulatory proteins. Especially, to perform the chemical decellularization procedure, hypertonic sodium chloride, sodium hydroxide, sodium dodecyl sulfate (SDS), Triton X-100 are widely used. Currently, SDS and Triton X-100 are widely chosen for decellularization studies. SDS is a highly ionic and amphipathic detergent, which can affect protein-protein interactions in cell membranes. Although SDS is widely preferred chemical detergent for the decellularization process, its usage is limited for the vascularized tissues. Enzymatic treatments should combine with other techniques including osmotic shock and freeze-thaw methods. On the other hand, decellularization may finalize in a long-time period because of the slow diffusion of detergents inside non- or poorly vascularized tissues. Ultimately, remaining detergents in the tissue may cause cytotoxic and adverse effects [9]. Trition X-100 is a member of the non-ionic detergent class and have the ability to disrupt the lipid–protein and lipid–lipid interactions on cellular membranes [10]. At the same time; in literature, only ethanol treatment is performed to be getting decellularized tissue for sterilization process. Results revealed nonignorable loss in proliferation, amount of collagen, cross-linking of peptide materials and reduction of cell attachment on the decellularized liver tissue. On the other side, although peracetic acid (PAA) is utilized for obtaining decellularized tissue, the amount of glycosaminoglycans (GAG) in ECM was considerably loss, only 56% of the original GAG was remained[11]. The physical methods such as freeze-thaw processing, hydrostatic pressure, and non-thermal irreversible electroporation are common techniques for decellularization[4].

Nowadays, supercritical carbon dioxide ($scCO_2$) is an alternative technique. A supercritical fluid is obtained when brought higher than its critical temperature and at a pressure higher than its critical pressure. When reached above its critical point, fluid demonstrate unique properties that intermediate between those of liquids and gases. The critical point is that 7.2 MPa/ 37°C for $scCO_2$ and the critical coordinates of CO_2 are Tc=31.06 °C and P=7.38 MPa[12]. Thus, supercritical fluids can penetrate the tissue and dissolve the cells thanks to their liquid-like densities and gas-like densities. Besides, it does not affect the cross-linked proteins and does not leave cytotoxic residual chemicals behind [13]. $scCO_2$ exhibits gas properties as it can easily diffuse through solid materials, and at the same time exhibits solvent properties as a liquid. The special behaviors owing to $scCO_2$ present its effect on decellularization period via increasing the diffusion kinetics [14]. This phase is utilized for various processes such as supercritical drying of tissues, extraction, cleaning, and sterilization of tissues [15]. Besides, $scCO_2$ is an apolar material. Therefore, polar materials such as ethanol are required as immersive during the decellularization procedure. It is generally used to eliminate charged molecules in cellular membranes, such as phospholipids [16]. Considering the above-mentioned important

features of $scCO_2$, the hypothesis of whether $scCO_2$ is effective for decellularization in dense and tight tissues was studied in this study.

MATERIALS AND METHODS

Materials

Supercritical fluid extraction system (Applied Separations-Speed SFE), cow aorta is supplied from a local slaughterhouse, DNA purification kit (Zymo Research/ Quick DNA Miniprep Plus Kit), Nanodrop (Thermo Scientific), Coomassie Brilliant Blue G-250 for Bradford Assay, pure ethanol Elabscience Collagen ELISA Kit, ELISA Reader (Infinite 200 PRO), Scanning Electron Microscopy(SEM) (Zeiss Evo LS10), H&E staining (Vector Laboratories) and Perkin Elmer DMA 8000 Dynamic Mechanical Analyzer.

Preparation of Cow Aorta Tissue Sample

Freshly harvested cow hearts were randomly obtained from a local slaughterhouse that moved on dry ice to prevent distortion and dryness. The aorta was carefully separated from the heart using a scalpel so as to form a cylindrical shape. The surrounding fatty tissues and pericardial fragments were completely removed. All aortic samples were cut into pieces approximately 2 cm thickness and washed several times with distilled water that incubated for 5 minutes at 4°C in phosphate-buffered saline (PBS) so as to be sterilized. Each tissue sample was dried at room temperature for 5 minutes using whatman filter paper in incubator at 37°C which then placed in the extraction cell neatly. Firstly, three of the samples were treated with SFE for 1 hour (1H), 4 hours (4H), 6 hours (6H) and for the hybrid protocol; samples were treated with 0.1% prepared Triton X-100 detergent for 2 hours before SFE (2H-T) incubation and treated with Triton X-100 overnight before 1-hour SFE (1H-O) incubation.

Super Fluid Extraction Method with Supercritical Carbon Dioxide (SFE)

High-pressure extraction apparatus was utilized for the decellularization procedure of cow aorta tissue samples as shown in **Fig.1** that consists of liquid CO_2 cylinder, pressurize pump, thermostat, extraction cell (100 cm^3 and a diameter of 3cm), pump inlet and temperature setter. The cow aortic specimens were carefully placed on the extraction cell and extraction cell was filled with 60 mL of ethanol as a scavenger, which is a co-solvent that dissolves phospholipids in the cell membrane and nucleus membrane. The operating temperature was stabilized at 37 ^{O}C during the treatment time. Cow aortic tissue samples were surrounded by a mixture of supercritical carbon dioxide and ethanol fluid during the experiment. Carbon dioxide is compressed from cylinder to the liquid form by the helping of pressurize pump to into the extraction cell as desired flow rate. Extraction cell is made up of stainless steel and surrounded by two handcuffs which are connecting with a temperature setter so as to form a balance at the external temperature of the extraction cell at desired rate. When carbon dioxide reaches the required pressure after entering the extraction cell, it shows diffusion property like gas and also fluidity like liquid. This process reached until 32MPa and the temperature was set at 37^{o}C. Samples called 1H group were incubated in the extraction cell for 1 hour under 32 MPa and 37^{o}C conditions. At the end of 1H, the mixed fluid of supercritical carbon dioxide and ethanol were quickly discarded from the extraction cell by pump outlet at 1 mL/min flow rate so as to be depressurization that was until the system reached ambient temperature. In this study, the

pressure was strictly controlled to protect cow tissue samples from drying. Finally, CO_2 passes to the gaseous phase and easily diffuse outside from the tissue samples. As a result, undesired chemical remaining does not occur within the tissue samples and decellularized tissue samples will be suitable for transplantation to the recipient.

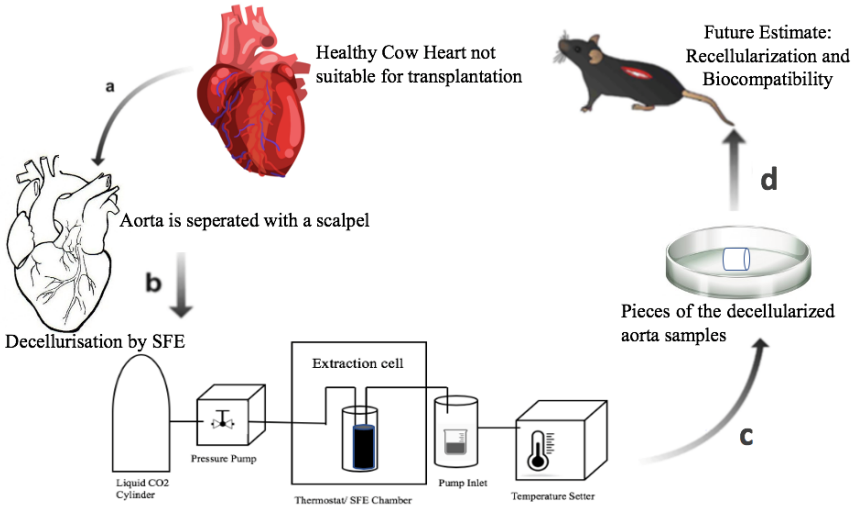

Fig.1. *Schematic diagram of experiment*

Surface Morphology Analysis of Decellularized Tissues

The surface morphology of both native and decellularized cow aorta tissue samples was analyzed with thermionic emission scanning electron microscopy (Zeiss Evo LS10) (SEM) that is applying for comparing ultrastructure of the cow aorta tissue samples before and after the decellularization treatment. Tissue samples were prepared as 5 mm long strips from the decellularized and intact aorta. Before the surface morphology analyses, cow aorta tissue samples were lyophilized overnight at 65°C. Au-Pd coating (Quorum SC-7620) was performed on samples for 1 minute. Low energies were used at (7.00kV) to inhibit the problem of charging on samples. At the same time, surface morphology analyses were maintained at 10.00K X magnification.

Total DNA Assay

The total DNA concentrations of each sample were analyzed by Zymo Research Quick DNA Plus Purification Kit. 30 mg sample was used for the lysis protocol. The samples were incubated overnight in lysis buffer containing 10µL proteases K at 37°C at shaking water bath. DNA purification from lysates was accomplished by using DNA purification kit based on the manufacturer's instructions. Ultimately, DNA concentration of each tissue samples were determined by absorbance at 260 and 280 nm with nanodrop (Nanodrop One, Thermo scientific).

Total Protein Assay

The Bradford Test is a fast and simple protein quantification method that is based on coomassie dye binding to proteins under acidic conditions, resulting in brown to blue discoloration. The presence of basic amino acid residues such as histidine, lysine, and arginine lead to the formation of a blue color. 50 mg coomassie bright blue G-250 was

dissolved in 50 ml methanol. Then, 100 ml 85% (w/v) phosphoric acid (H_3PO_4) was added slowly. The resulting acid solution was carefully transferred in 850 ml H_2O. Whatman paper was used for filtration to remove the precipitates of Bradford reagent. At the same time, cow aortic tissue samples were measured at 100 mg for the lysis protocol that transferred to microcentrifuge tubes and 900µl cold PBS was added at each tube. All tissue pieces were digested by the homogenization and centrifugated at 5,000 G at room temperature. The supernatant of each sample was taken into new microcentrifugation tubes. 60 µl of each sample was pipetted in new test tubes and 940µl ultrapure water was added. The bovine serum albumin (BSA) was used as a protein standard. 75mg BSA was dissolved in 7,5 mL H_2O. The standard concentrations were 10, 5, 2,5, 1,25 µg/µL and 30 µl ultrapure water was used as blank. Bradford reagent (1,5 mL) was added into both sample and standard solution tubes. The absorbance was measured at 595 nm for each sample in ELISA reader. Unknown sample concentrations were calculated based on the standard curve formula given below

Hematoxylin &Eosin (H&E) Staining

The cow aorta tissue samples were stained with H&E that is a highly common staining method for observing the nucleus of cells in mammalian tissues on a light microscope, directly. The RNA and nuclei rich regions are stained with hematoxylin as blue, on the contrast; cytoplasm and collagen part of the cell is stained with Eosin as purple/pink, remarkably. The tissue samples were embedded into the paraffin, different blocks were used for each sample and cooled at room temperature. Paraffin blocks were sliced approximately 4-5micron sections for each cow aorta tissue sample so as to be deparaffinization process. Then, paraffin blocks were placed into the incubator for 30 minutes and treated with xylene at 15 minutes, washed with alcohol 5 minutes, finally washed with distilled water. All these cleaning steps were repeated twice the time. The deparaffinated tissue samples were kept in Hematoxylin (Mayer's) for 5 minutes and then placed in distilled water at 2 minutes. Then, the Eosin Y solution was used to stain samples for 5 minutes. Finally; samples were washed with alcohol a few times and transferred to a coverslip to visualize at 100X magnification in the light microscope.

Dynamic Mechanical Analysis (DMA)

Dynamic Mechanical Analyzer, Perkin Elmer DMA 8000, was utilized to compare the mechanical properties samples. The cow aorta tissue samples were cut strip like structure for putting in the rectangular-shaped measurement pockets. All DMA analysis were carried out at room temperature (25°C) at a constant frequency of 1Hz by a deformation mode of single cantilever bending for 60 minutes.

RESULTS

Surface Morphology Analysis of Decellularized Aorta Tissues

SEM images were examined to compare the ultrastructure behavior of the tissue before and after the decellularization treatment via $scCO_2$. This morphology and structure analysis were applied on the transversal section of the circumferential direction of the intact and decellularized aortas. As seen in **Fig.2**, the ultrastructure of the cow aortic tissue was changed following the decellularization. According to the SEM results of the

cow tissue samples, density of the ECM was considerably decreased in decellularized tissue samples. Additionally, native ECM of the aortic tissue (**Fig.2-A**: A) demonstrated more dense structure than the hybrid and SFE applied samples. The native form presented a smooth image based on the smooth muscle cells (SMCs) embedded in the cross-linked ECM network of collagen and elastin fibers. The hybrid method applied cow aorta tissue samples (**Fig**.2-A: C, F) were treated with TritonX-100 detergent that non-ionic detergent has disrupted the ECM structure and ultimately caused the breakage of cross-links between fibers within the ECM. The most porous construction belongs to the samples treated via a hybrid method with TritonX-100 detergent. TritonX-100 treated tissue samples were observed in a more transparent form than others as seen in **Fig.**2-B (E) and (F). Probably, these results have proceeded from the ECM disruption and loss of the protein content of ECM.

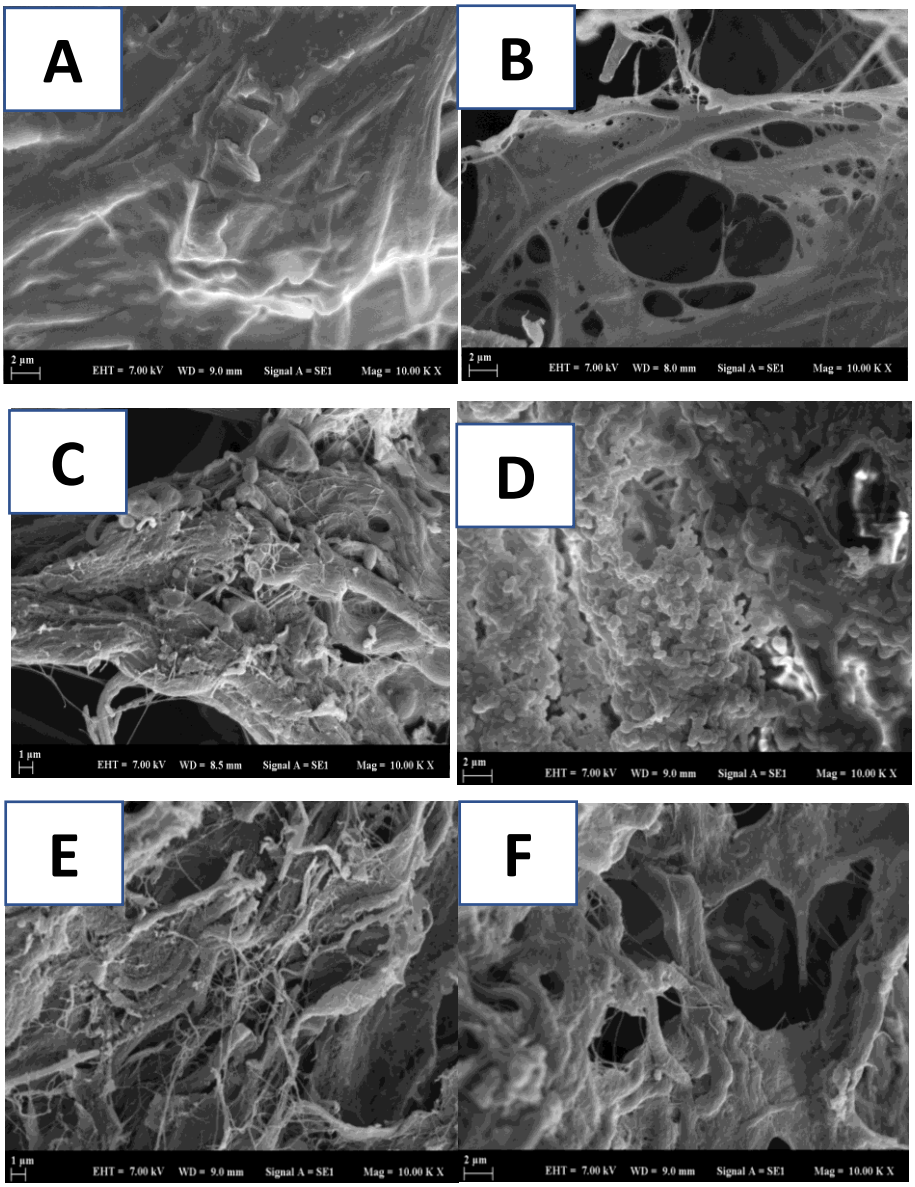

Fig.2-A*. Native (A), 1H (B), 2H-T (C), 4H (D), 6H(E), 1H-O (F)*

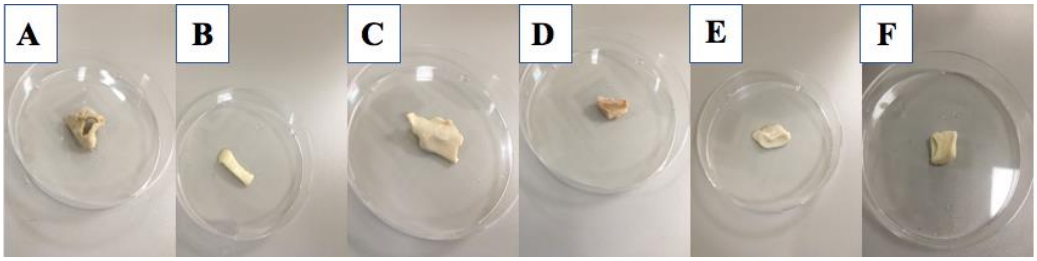

Fig. 2-B. *Native (A), 4H (B), 6H (C), 1H (D), 1H-O (E), 2H-T (F)*

Histological Analysis with Hematoxylin &Eosin (H&E) Staining

The assessment of the amount of removal of genetic material and the extent of removal of phospholipid is a significant factor for evaluating the decellularization of tissues. Because of these reasons, remained genetic materials and nuclei of the cells were examined by the histochemical staining methods in this study. The collagen and the nucleus are seen as pale pink and blue, respectively. The decellularized cow aorta tissue samples presented an intact extracellular matrix. Additionally, genetic materials were discarded compared to native form of the tissue sample. H&E staining of the cow aorta tissue samples were given in **Fig.3**. We confirmed the H&E staining results by spectrophotometric analysis of the total DNA. When these results compared with the non-ionic detergent system (**Fig.3** C, F), the high diffusion capacity and low viscosity behavior of the supercritical CO_2 fluid exhibited the quick access of the fluid into the extracellular matrix and the cell nuclei were dissolved under the supercritical condition easily. On the other hand, TritonX-100 detergent (**Fig.3** C, F) affects the cell membrane. Therefore, the morphological differences are significant when $scCO_2$ and chemical decellularized aorta tissue samples are compared. There are significant differences in tissue thickness, stiffness, elasticity, and transparency. The thickness and stiffness of samples were conserved with the samples treated with the only $scCO_2$. On the other hand, you can easily observe the loss of ECM's original thickness at the vessel wall.

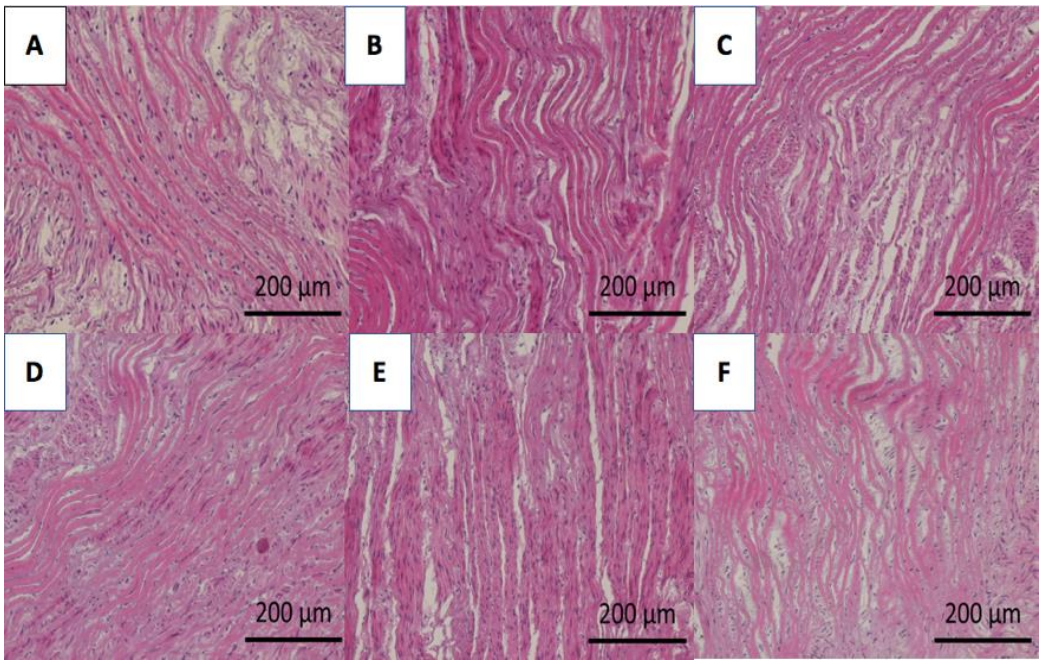

Fig.3. *Native (A), 1H (B), 2H-T (C), 4H (D), 6H(E), 1H-O (F)*

Total Protein Assay

Histologic studies were supported with Bradford Assay as seen in **Fig.4**. H&E staining results of intact aorta exhibited characteristic organization of ECM. Thus, H&E staining images approved the removal of the cells from all aorta tissue samples. In our study, native, TritonX-100 /SFE treated and only SFE treated samples were compared. The standard curve was performed by different concentrations of bovine serum albumin (BSA) proteins. The total protein concentrations were determined as 29,53 mg/mL for N, 27,08 mg/mL for 1H, 29,48 mg/mL for 2H-T, 27, 27 mg/mL for 4H, 27,50 mg/mL for 6H and 28,30 mg/mL for 1H-O based on statistical analysis of the t-test analysis. All the decellularized aorta samples were compared with the native form of the cow aorta tissue. P value was calculated for 1H and 4H as P<0,01 by the means that there is a significant difference when compared with the native form of the cow aorta tissue sample. P>0,05 is obtained when the samples 2H-T, 6H and 1H-O were calculated which means there is no significant difference.

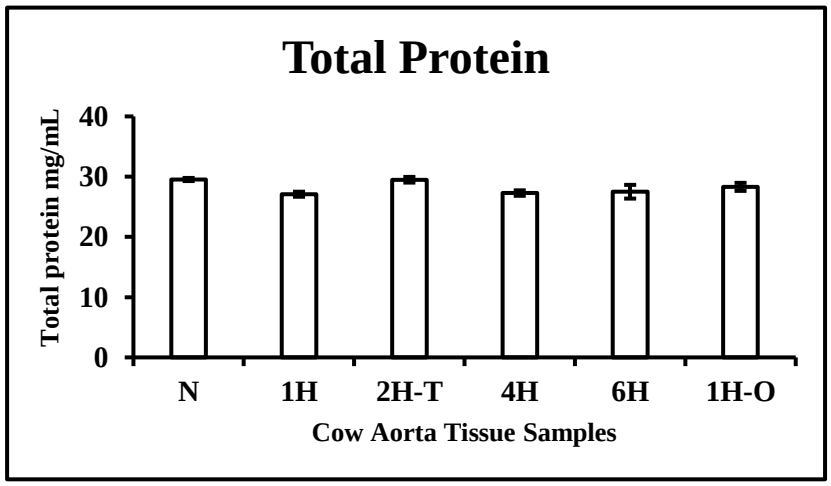

Fig.4. *Native (A), 1H (B), 2H-T (C), 4H (D), 6H(E), 1H-O (F)*

Total DNA Analysis

The decellularization of tissues and organs to produce ECM bio-scaffold requires the preservation of the natural ECM structure as well as the elimination of all cellular structures such as DNA, mitochondria/cytoplasm membrane, fats and cytosolic content. If these cellular residues and components are not removed sufficiently, they may have an inflammatory effect on the recipient. Cell density, matrix density, thickness and morphology of tissue can affect the success of tissue and organ decellularization, and thus alter the integrity and physical properties of the latest ECM scaffold structure. According to literature, the minimum criteria for a successful decellularization process are; I. <50 ng dsDNA / ECM dry weight (mg), II. <200 bp DNA fragment length, III. 4 ', 6-diamidine-2-phenyl indole (DAPI) or hematoxylin and eosin staining on histopathological tissue sections.[17][18]. The remaining DNA concentrations of native and decellularized aorta tissue were calculated as N:168,6 ng/mg±1,03, 1H:30 ng/mg±0,71, 2H-T:33 ng/mg±1,14, 4H:59ng/mg±,0, 6H:73 ng/mg±1,14 and O:31 ng/mg±1,0 in this study (**Fig.5**). The value obtained for N was assumed as %100 and then, all decellularized tissues were compared with native form of the aorta. Remaining DNA

content of 1H is %17,8, 2H-T %19,6, 4H %35, 6H %43,3 and O %18,4 based on the native form of the aorta. In addition to all these calculations, statistical analyses were performed with t-test analysis system. The decellularized aorta tissue samples were confronted with native form. P-value is calculated $p<0,01$ for all decellularized aorta tissue samples which means there is a significant difference when native form and treated groups were compared with each other. According to our results, treated only with SFE group demonstrated more efficient decellularized construction than the hybrid group. 1H group was exhibited more effective result with appropriate ECM construction.

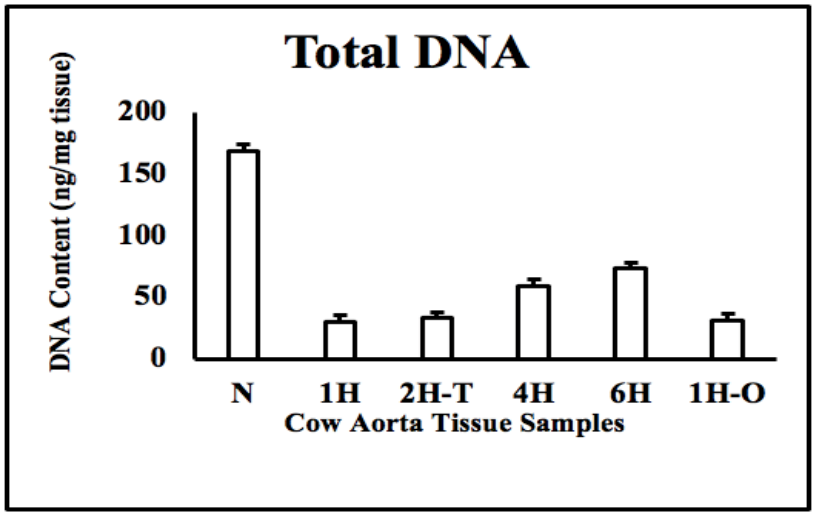

Fig. 5. *Native (A), 1H (B), 2H-T (C), 4H (D), 6H(E), 1H-O (F)*

Mechanical Behavior Analysis

In this study, mechanical strength of the cow aorta tissue samples was determined with DMA analysis that examined by the Emod as the slope of the stress/strain curve in a linear part (Young's modulus). Thus, static stress/strain graph was calculated. According to the graph, sample of 1H was exhibited the largest peak stresses, while 6H was demonstrated lowest peak stress based on the least amount of energy dissipation(**Fig.6**).

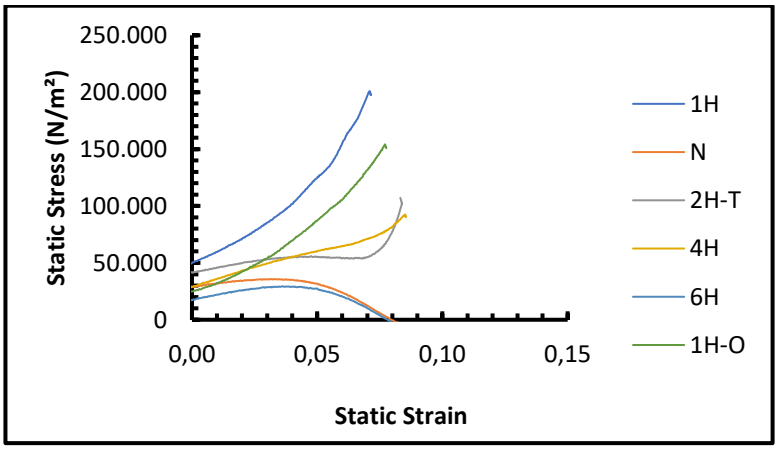

Fig. 6. *Mechanical behavior of each cow tissue sample; Native, 1H, 2H-T, 4H, 6H, 1H-O*

DISCUSSION

The properties of the ECM obtained after decellularization are critical and several key features should be reviewed to assess the quality of the ECM. There are four main aspects; extraction of genetic material, discard of cells, conservation of protein content and preservation of the mechanical behavior. Removal of genetic material has the critical role for tissues or organs to prevent immune rejection[19]. The decellularized ECM must have contain less than 50 ng double-stranded DNA (dsDNA) per mg ECM dry weight, 200 bp DNA fragment length and no visible genetic material not to cause immune rejection[20]. In the present study, we aimed to examine the decellularization of cow aortic tissue samples via two different methods: SFE and TritonX-100/SFE hybrid method. The ratio of cell removal results was determined by examining the total amount of DNA analysis. Therefore, we can define the 1H group as a completely cell-free tissue, taking into account the data. Given the total amount of DNA determined in the 2HT-1H and OT-1H groups, samples can be defined as partially cell-free tissues. Additionally, we observed that to obtain a cell-free ECM, 1 H SFE treatment is adequate, and longer treatments had no effect on DNA removal, besides reduced mechanical strength of ECM. We have concluded that optimization of the ethanol content in the ScCO2 system may be critical for more efficient DNA removal with SFE treatment. In the literature, Ren et al. have treated rat liver with sodium dodecyl sulfate (SDS) and Triton X-100 chemicals. They have performed, immunofluorescence assays, protein assays, SEM imaging, and DNA analysis. The quantitative DNA data obtained were similar to the results obtained from our study [10]. On the other hand, Casali et al. have reported that a totally decellularization was achieved via a 48-hour hybrid method utilized by $scCO_2$ washing. The entirely decellularization of the tissue were determined by DNA quantification (<0.04 μg DNA/mg tissue) analyses [21]. Besides, the supercritical carbon dioxide system allows rapid flow through the extracellular matrix compared to detergent methods because of the high diffusion coefficient and low viscosity of the supercritical fluid. All these unique properties of the supercritical fluid confirmed by SEM analysis which demonstrated the conservation of ultra-infrastructure of cow aorta tissue samples via $scCO_2$. In hybrid tissue samples SEM images were more scattered that is based on the decreasing mass of the collagen network. Similar results were given by Antons et al. with cartilage tissue. However, they have not detected a visible density change for tendons and skin tissue[14]. On the other hand, Zou & Zhang were studied on porcine aorta by using enzymatic and chemical combination method. They have presented SEM images of the treated samples and the form of the decellularized fibers observed by SEM were similar to our images. [1]. The results were confirmed by histological staining. The aorta has a significant role within the body to resist the high blood pressure. This role is carried out with dense and thick vessel walls of the aorta and consisting of three layers including inner monolayer composed of endothelial cells, medial layer with predominantly smooth muscle cells and elastic fibers, and finally, outer layer with collagen fibers increase its mechanical strength [22]. Thus, the residual cell nuclei can lead to critical immune rejection and remaining phospholipids can cause the calcification of the tissues. The integrity of the ECM obtained after decellularization via SFE was found fairly robust compared to TritonX-100 treated cow aortic tissue samples based on the SEM images and H&E staining results. The loss of thickness causes a decrease in the mechanical strength of the aorta tissue. In the literature, similar results were given by S.Guler et al.[23]. They have studied on the bovine aorta and bovine cornea. According to their results, the 2500 psi pressure treated aorta group with supercritical carbon dioxide was

successful for decellularization. They have also examined high pressure effect on decellularization, by the regulation of pressure rate to increase the solvent effect of supercritical fluid. However, they have reported that high pressure upon 4500 psi does not appropriate for the ECM components. Also, they have supported their results with H&E staining. At the same time, Sawada et al. have studied the porcine aorta and they have applied a mixture fluid composed of a small amount of ethanol as entrainer and supercritical carbon dioxide at 15MPa, 37◦C. Results were supported by H&E staining. They have reported that cell nuclei were discarded from tissue within 1H successfully[12] . In the literature, Casali et al. have studied the porcine aorta tissue. They have performed H&E and Masson's Trichrome Staining. According to the Masson's Trichrome staining, elastin is stained with red color, collagen is stained blue color and cell nuclei are stained black. Their results were similar to our total DNA results [24]. On the other hand; Syazwani et al. were focused on improving a sonication decellularization system for obtaining a complete decellularized artery. [25]. Throughout their study, treated with sonication of decellularized tissue samples did not demonstrate a significant difference in stiffness compared to the native form of the tissue sample. Especially, collagen is responsible for tensile stiffness for resistance and elastin is relevant in compliance and elasticity[26] [27].

In the light of many preferred protocols for removing cellular content from ex-vivo tissues in the literature, in the present study, optimization of popular chemical and SFE techniques has been studied to demonstrate which decellularization method is the most effective in maintaining the mechanical behavior and integrity of natural tissue. Mechanical behavior of the decellularized tissues or organs are critical for the recellularization procedures. The composition and physical structure of the ECM affect cell fate significantly. The ECM is significant for the 3-dimensional structure of the tissue that preserves the native organ geometry. ECM's proteins are effective in the recellularization of the tissue. Additionally, the mechanical behavior of tissue and fate of cells on the tissue are set by ECM composition. ECM proteins including collagen, elastin, laminins specify the above circumstances. Triton X-100 is effective in removing cell residues from the thicker tissues such as valve conduits. One of the non-ionic detergent, TritonX-100 has the ability to solubilize cell membranes and separate DNA from proteins, however, it causes dissociation of proteins in the ECM. In literature, Lumpkins et al. have studied on porcine tissue treatment that were treated with different chemicals; TritonX-100, SDS, acetone/ETOH. Respectively, the acetone/ethanol and Triton X-100 samples were exhibited the largest and smallest degrees of hysteresis and peak stress [28]. On the other side, Williams et al. have focused on the decellularization of rabbit carotid arteries. They have examined the mechanical properties and opening angles for native and decellularized carotid arteries. The decellularized arteries were remarkably stiffer and less extensible than native arteries according to their results. They have calculated tensile modules for native sample as 2.29 ± 0.24 and decellularized tissue as 3.48 ± 0.72b [27]. They have also reported that SFE-treated samples reach to the same decellularization yield in a much shorter time than those decellularized by chemical methods. On the other hand, protein integrity and mechanical behavior of decellularized ECM via SFE was found almost as close to the protein integrity of native tissue.

REFERENCES

[1] Zou, Y, Zhang, Y. (2012): Mechanical evaluation of decellularized porcine thoracic aorta. J Surg Res 175, 359–368.

[2] Fitzpatrick, J. C., Clark, P. M., Capaldi, F. M. (2010): Effect of Decellularization Protocol on the Mechanical Behavior of Porcine Descending Aorta. Int J Biomater.

[3] Aubin, H., Kranz, A., Hülsmann, J. et al (2013): Decellularized Whole Heart for Bioartificial Heart. In: Kao R. (eds) Cellular Cardiomyoplasty. Methods in Molecular Biology (Methods and Protocols), vol 1036. Humana Press, Totowa, pp 163-178.

[4] Crapo, P. M., Gilbert, T. W., Badylak, S. F. (2011): An overview of tissue and whole organ decellularization processes. Biomaterials. 32, 3233–3243.

[5] Hennessy, R. S., Jana, S., Tefft, B. J. et al (2017): Supercritical Carbon Dioxide–Based Sterilization of Decellularized Heart Valves. JACC Basic to Transl Sci. *2*(1), 71-84.

[6] Nyland, J., Larsen, N., Burden, R., et al (2009): Biomechanical and tissue handling property comparison of decellularized and cryopreserved tibialis anterior tendons following extreme incubation and rehydration. Knee Surgery, Sport Traumatol Arthrosc. *17*(1), 83-91.

[7] Meyer, S. R., Chiu, B., Churchill, T. A., et al (2006): Comparison of aortic valve allograft decellularization. J Biomed Mater Res Part A. *79*(2), 254-262.

[8] Atala, A., Bauer, S. B., Soker, S., et al (2006): Tissue-engineered autologous bladders for patients needing cystoplasty. Lancet. *367*(9518), 1241-1246.

[9] Ott, H. C., Matthiesen, T. S., Goh, S. K., et al (2008): Perfusion-decellularized matrix: Using nature's platform to engineer a bioartificial heart. Nat Med. *14*(2), 213-221.

[10] Ren, H., Shi, X., Tao, L., et al (2013): Evaluation of two decellularization methods in the development of a whole-organ decellularized rat liver scaffold. Liver Int. *33*(3), 448-458.

[11] Hussein, K. H., Park, K. M., Teotia, P. K. et al (2013): Sterilization using electrolyzed water highly retains the biological properties in tissue-engineered porcine liver scaffold. Int J Artif Organs. *36*(11), 781-792.

[12] Sawada, K., Terada, D., Yamaoka, T., et al (2008): Cell removal with supercritical carbon dioxide for acellular artificial tissue. J Chem Technol Biotechnol. *83*(6), 943-949.

[13] Eckert, C. A., Knutson, B. L., Debenedetti, P. G. (1996): Supercritical fluids as solvents for chemical and materials processing. Nature. *383*(6598), 313-318.

[14] Antons, J., Marascio, M. G. M., Aeberhard, P. et al (2018): Decellularised tissues obtained by a CO2-philic detergent and supercritical CO2. Eur Cells Mater. 36, 81–95.

[15] García-González, C. A., Concheiro, A., Alvarez-Lorenzo, C. (2015): Processing of Materials for Regenerative Medicine Using Supercritical Fluid Technology. Bioconjug. Chem. *26*(7), 1159-1171.

[16] Sun, M., Temelli, F. (2006): Supercritical carbon dioxide extraction of carotenoids from carrot using canola oil as a continuous co-solvent. J Supercrit Fluids. *37*(3), 397-408.

[17] Nagata, S., Hanayama, R., Kawane, K. (2010): Autoimmunity and the Clearance of Dead Cells. Cell. *140*(5), 619-630.

[18] Gouk, S. S., Lim, T. M., Teoh, S. H., Sun, W. Q. (2008): Alterations of human acellular tissue matrix by gamma irradiation: Histology, biomechanical property, stability, in vitro cell repopulation, and remodeling. J Biomed Mater Res - Part B Appl Biomater. *84*(1), 205-217.

[19] Mason, C., Dunnill, P. (2008): A brief definition of regenerative medicine. Regen. Med. 3(1), 1-5.

[20] Uematsu, K., Hattori, K., Ishimoto, Y., et al (2005): Cartilage regeneration using mesenchymal stem cells and a three-dimensional poly-lactic-glycolic acid (PLGA) scaffold. Biomaterials. *26*(20), 4273-4279.

[21] Casali, D. M., Handleton, R. M., Shazly, T., Matthews, M. A. (2018): A novel supercritical CO2-based decellularization method for maintaining scaffold hydration and mechanical properties. J Supercrit Fluids. *131*, 72-81.

[22] Kumar, V. A., Brewster, L. P., Caves, J. M., Chaikof, E. L. (2011): Tissue engineering of blood vessels: functional requirements, progress, and future challenges. Cardiovasc Eng Technol 2, 137–148

[23] Guler, S., Aslan, B., Hosseinian, P., Aydin, H. M. (2017): Supercritical Carbon Dioxide-Assisted Decellularization of Aorta and Cornea. Tissue Eng - Part C Methods. *23*(9), 540-547.

[24] Casali, D. M., Handleton, R. M., Shazly, T., Matthews, M. A. (2018): A novel supercritical CO2-based decellularization method for maintaining scaffold hydration and mechanical properties. J Supercrit Fluids 131, 72–81.

[25] Syazwani, N., Azhim, A., Morimoto, Y. et al (2015): Decellularization of aorta tissue using sonication treatment as potential scaffold for vascular tissue engineering. J Med Biol Eng. *35*(2), 258-269.

[26] Dobrin, P. B. (1978) Mechanical properties of arteries. Physiol Rev. 58(2), 397-460.

[27] Williams, C., Liao, J., Joyce, E. M. et al (2009): Altered structural and mechanical properties in decellularized rabbit carotid arteries. Acta Biomater 5, 993–1005.

[28] Lumpkins, S. B., Pierre, N., McFetridge, P. S. (2008) A mechanical evaluation of three decellularization methods in the design of a xenogeneic scaffold for tissue engineering the temporomandibular joint disc. Acta Biomater. 4(4), 808-816.