2017-01-01

Characterization & Extraction Of Extracellular Matrix From Porcine Adipose Tissue

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Dedication

This thesis is dedicated to my father who encourage me to pursue my dreams and finish my thesis and help me in great and small things. Throughout my life he always been there during bad and good times. I would not be who I am today without the love and support of my father. And I also like to dedicate this work to my best friend and my spirit “Payal” who always there for me when my spirit and courage needs extra boost. I cannot thank you enough for your support and time that we spent. You are truly and extraordinary gift. And lastly, I like to thanks my family to be with be during my study.

CHARACTERIZATION & EXTRACTION OF EXTRACELLULAR MATRIX FROM PORCINE ADIPOSE TISSUE

By

RAHULKUMAR KANTIBHAI BHOI, B.E. METALLURGY ENGINEERING

THESIS

Presented to the Faculty of the Graduate School of

The University of Texas at El Paso

in Partial Fulfillment

of the Requirements

for the Degree of

MASTER OF SCIENCE

Department of Metallurgical, Materials and Biomedical Engineering

THE UNIVERSITY OF TEXAS AT EL PASO

December 2017

Acknowledgements

Throughout my graduate research assistance position Dr. Namsoo Kim has been an excellent teacher and mentor. He truly understands what hard work and dedication can bring my life. The door to Dr. Kim's office was always open whenever I ran into trouble spot or had a question about my research. This thesis would not have happened without his support. I would like to express my gratitude toward Dr. Jongwha Chang and Dr. Guikaun Yue for your support and being a part of my thesis committee members. I will always owe a great deal of gratitude towards these professors. I would also like to acknowledgement to my lab mate Jae Seok eo and Diana Cho. And finally, I must express my gratitude to Dr. Michel Monica who helped me with lab chemicals, equipment and thesis writing.

Abstract

Tissue in particular extracellular matrix(ECM) plays a vital role in the growth and the function of each cell types and promote tissue regeneration and repair an impair organs. Many techniques have been developed so far to decellularized porcine tissue for the purpose to use as regenerative medicine and in tissue engineering. We aimed to optimized a method to decellularized porcine tissue while effectively eliminating xenogeneic epitopes, lipids and to retain necessary component of it intact without disrupting ECM. We have developed 2-week protocol to optimize well preserved ECM component that may keep xenogeneic biomaterial for tissue engineering and regenerative medicine. Porcine adipose tissue was first thaw, minced and then homogenized in ultrapure water followed by chemical, physical and enzymatic treatments. After the decellularization protocol, material was freeze-dried for further analysis. With a significant reduction, trifle amount of inherent immunogenic components such as cells and nucleic acid were recorded using Histology (H&E staining) and DNA quantification. However, significant ECM component such as collagen, glycosaminoglycan and elastin were determined and found well preserved. The entire analysis and research on porcine adipose tissue using 14-day protocol have suggested that the method to decellularized ECM derived from porcine adipose tissue could be useful one with little modification in the protocol to optimize the method. we assure that the method to obtain extracellular matrix is efficient for cell removal and preserved the collagen which may possessed the most favorable biocompatibility and most befitting material for tissue engineering and regenerative medicine.

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Chapter 1 : Introduction

1.1 TISSUE ENGINEERING

Tissue engineering is increasingly viewed as the future of medicine. The emerging field of tissue engineering has great promise for the replacement and repair of tissues and organs that have been lost or compromised by disease or trauma. Tissue engineering therapies are under active clinical evaluation and still more energetically under investigation in labs across the globe.[1] Fundamentals of tissue engineering examines the properties of stem cells, primary cells, growth factors, and extracellular matrix as well as their impact on the development of tissue engineered devices.[1] Traditional in vitro cell culture does not accurately mimic the biological functions and complexity found in vivo. In addition, there are some limitations such as cell to cell interactions, cell to matrix interactions, biomechanical effects, and the influence of the limitations of diffusion.[2]

There has been new method is widely studying called culture of cells in 3D, and this method more adequately mimic the complex microenvironments and phenotypes found in vivo. [3] Enabling technologies focused upon those strategies typically incorporated into tissue engineered devices or utilized in their development, including scaffolds, nanocomposites, bioreactors, drug delivery systems, and gene therapy techniques. Tissue engineering application presents synthetic tissues and organ that are currently under development for regenerative medicine, applications.

1.2 Extracellular Matrix

Tissue are not made up solely of cells. A substantial part of their volume is extracellular space, which is largely filled by and intricate network of macromolecules constituting the extracellular matrix. This matrix is composed of a variety of proteins and polysaccharides that are secreted locally and assembled into an organized meshwork in close association with the surface of the cell that produced them.

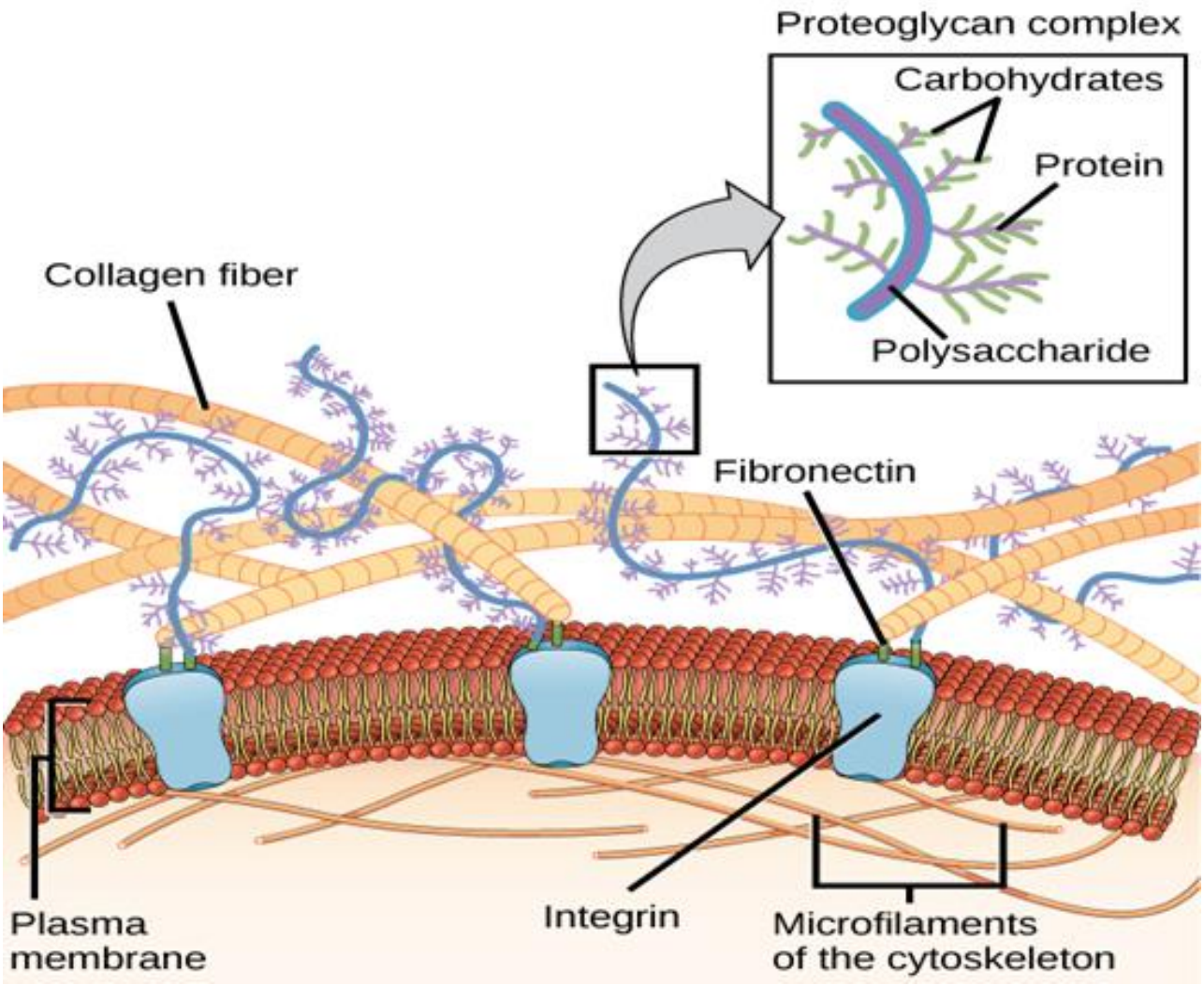

Figure 1 Extracellular Matrix and its components.[4]

Fig 1 shows complex meshwork of the extracellular matrix components like collagen fibers, polysaccharides and fibronectin.

1.2.1 Functions and role of ECM

Cells sense and respond to variety of signals such as growth factors, differentiation factors, cytokines, and ion gradients. Moreover, cell behavior and phenotype is controlled by responses to other types of signals that include mechanical forces, electrical stimuli and various physical actions.[5] ECM formation is important in the process like growth, wound healing and fibrosis. An understanding in ECM structure and its compositions helps in complex dynamic nature of tumor and cancer.

1.2.2 Molecular components of ECM

Collagen

Collagen is the most abundant proteins in the ECM make up the one-third of all the protein in the body and model the framework of connective tissues.[6] collagen is the functional aggregates which form the macrostructures including fibrils, basement membrane, filaments, canals and sheets. Collagen can be divided into several families according to type of structure they form. Collagen proteins are composed of a right-handed bundle of three parallel left-handed polypeptide where all the bonds are in the trans conformation and two hydrogen bonds in triple as shown in Fig 2.

1. Fibrillar (Type I, II, III, V, XI)
2. Facit (Type IX, XII, XIV)
3. Short chain (Type VIII, X)
4. Basement membrane (Type IV)
5. Other (Type VI, VII, XIII)

Collagen presence in the human body

1. Collagen I- found in bones, tendons, organs
2. Collagen II- found mainly in cartilage
3. Collagen III- found mainly in reticular fibers
4. Collagen IV- found in the basement membrane of cell membranes
5. Collagen V- found in hair, nails

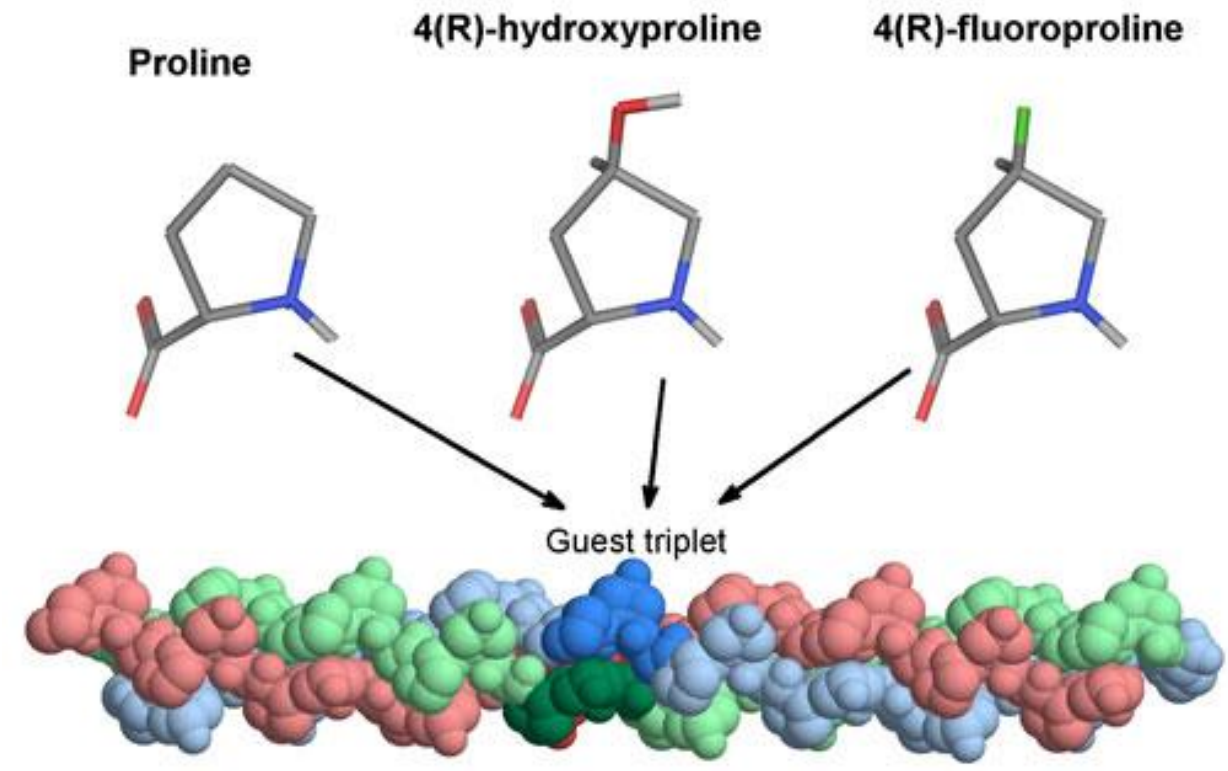

Figure 2 Triple helix structure of collagen. [7]

Elastin

In contrast to collagen, elastin gives elasticity to tissue, they can allow to stretch them when needed and return to its original shape. This is the one of the useful properties for blood vessels, the lungs and in the skin as it has high amount of elastin in it. Elastin are synthesized by fibroblast and muscle cells and they are highly insoluble. In normal canine arteries elastin ranges from 58% to 75% of the weight of the dry defatted artery.[8] this fibers can stretch up to 1.5 times to their length, and return to their original shape.

Fibronectin

It is a glycoprotein found in soluble forms in plasma and in insoluble form in loose connective tissue and basement membranes. Fibronectin binds cell surfaces as well as various other ECM molecules like collagen, heparan sulfate proteoglycans, and fibrin. It allows cells to move through the ECM. It's very important functions is help at the site of tissue injury by binding to platelets during the blood clotting and facilitate cell movement to the affected area during wound healing.[9]

Laminin

It is a common ECM component found in basement membranes. It is used as a substratum for cell migration by many cell types. Laminin form network of web-like structures that resist tensile forces in the basal lamina. Laminin helps in cell adhesion and binds other ECM components.

Proteoglycans and glycosaminoglycans

Proteoglycans are essentials for diverse functions and embryogenesis. The major role of proteoglycans depends upon the glycosaminoglycans which allow connective tissue of ECM to be able to withstand compressional forces. One of the type of proteoglycan the Heparin sulfate chain comprise of repeating disaccharide structure which forms glycosaminoglycan which is regionally modified by various enzymes that produce epimerization, vary sulfating patterns, and alter chain length.[10] The GAG chain create polycationic binding sites for attachment of proteins. GAG is carbohydrate polymer which is attached to extracellular matrix proteins to form proteoglycans. There are four different types of proteoglycans.

1. Heparan sulfate

2. Chondroitin sulfate
3. Keratan sulfate
4. Hyaluronic acid

1.2.3 Mechanical properties of ECM

Extracellular matrix in all tissue possesses distinct properties that allow tissue cohesion and gives biochemical environment to the surrounding cells. ECM components like proteins and carbohydrate can self-associate in highly order and predictable fashion which is tissue and cells specific. Self-assembly of ECM is the one of the most salient feature. It forms either two-dimensional structure or three-dimensional structures in space such as that of the territorial matrix. ECM possesses varying degree of properties like elasticity, stiffness from tissue to tissue. Elasticity property generally dependent on fibrous ECM network of collagen and elastin and it has essential role in cell functions of cells.[11] within this environment of ECM cells have great sense of mechanical properties by application of forces. [12] It is very important properties as it helps to govern various cellular actions like cell migration, cell proliferation and cell differentiation. [13–15] stiffness and elasticity also guide cell migration; this process is calls durotaxis.

1.2.4 Cell adhesion in ECM

Various component of ECM binds the cells in ECM. There are two ways how it binds the cells, by focal adhesions and hemidesmosomes, where focal adhesions connect the ECM to actin filaments of the cell, while hemidesmosomes connect the ECM intermediate filaments such as keratin. This all cell adhesion process can be regulated by cellular adhesion molecules called integrins, which is cell surface proteins that bind cell to ECM structures, such as fibronectin and laminin. Fibronectin has very important role in binding ECM macromolecules and facilitate their

binding to transmembrane integrins. Properties of connective tissue is determining by the components of ECM and there are many cells that contribute development of type of ECM. Most common cell type in connective tissue is fibroblast. Fibroblast provide cell structural framework synthesize ECM and it is ground substance. Chondrocytes are responsible in the formation of cartilage where osteoblasts are for bone formation.

1.3 PROBLEM STATEMENT IDENTIFICATION

The proper use of chemicals and treatment is essential for of ECM extraction from native porcine adipose tissue. In order to get success a well-studied protocol for ECM extraction was prepared in which I used 1% triton x-100 as a surfactant for cell lysis and 0.25% trypsin to break down the protein bond to cell membrane. Moreover, DNase was used to remove DNA content from the native tissue.

Tissue engineering is emerging field in medical science which has a great promise the replacement and repair of tissue organs. The most abundant and easily accessible source of ECM is the pig bladder. However, it comes with great challenge which is unwanted immunogenic components that need to be eliminate using physical, chemical and enzymatic treatment such that it doesn't affect the necessary compositions and structure of ECM.

1.4 OBJECTIVE OF THIS RESEARCH

The main objective of this research is to obtain ECM by distinct protocol to decellularize adipose tissue which may be the most effective method in tissue engineering and regenerative medicine. Removal of Cells, DNA and other immunogenic component with retaining ECM structure intact was the primary focus of this work. Analysis of Developed ECM and its components such as histological analysis, scanning electron

microscopy, DNA quantification, lyophilization and to quantify necessary components like collagen, GAG and elastin.

1.5 THESIS OUTLINE

Generally, the entire thesis revolving around extraction of ECM from native porcine adipose tissue and analysis of its components. The thesis has been divided into 5 chapters.

Chapter 1 “introduction” which describe the tissue engineering and ECM with its current scenario of the research as well as I also discussed ECM, its properties and its various components. At last I have briefly discussed the problem statement identification and objective of this research.

Chapter 2 “Literature Review” here I have described the scope of this research with the brief discussion of background and current research. I have also rendered source of ECM and its medical and tissue engineering application. And finally, I demonstrate use of polymers in tissue regenerative medicine.

Chapter 3 “Materials and Methods” here in this chapter I have explain the process and method use to extract the ECM from the native porcine adipose tissue. Furthermore, I have discussed experiment use to evaluate the final product in details.

Chapter 4 “Results” illustrate the outcome of the current research

Chapter 5 “Discussion” comprehensive discussion on the entire thesis

Chapter 6 “Conclusion” stated the essence of the current research

Chapter 2 Literature review

2.1 SCOPE OF THIS RESEARCH

There has been many methods and materials found for surgical operation to mitigate and fulfillment of different trauma and human body organs replacement. However, most of the time we need to rely upon the autologous tissue transplantation.[16] Which in fact not successful in all the cases because of the distinct functionality and of both tissue. This genre of operation cause morbidity and necrosis inside the tissue due to avascular response would be experience by organs that has been replaced. [17] There are various methods by which impair organs or bones can be treated. However, due to introduction of the foreign biological materials insertion degree of immune response become decrease and in addition volume integrity mismatch that causes abnormal condition and discomfort or dysfunction. Therefore, regenerative medicine become the interest in the tissue engineering to mitigate and eliminate all the disadvantages come from the conventional method.

Research in the field of Extracellular Matrix(ECM) attract tissue engineers due to its affirmative characteristics and abundant source in all the mammal. As cell embryo grow it secrete a non-living substance called ECM which is found in connective tissue, epithelium tissue, bones and blood vessels. It provides structural and biochemical support to the surrounding cells.[18] In turn, within the matrix and cells it provides signaling phenotype and characteristic among the other cells and promote healing in a number of tissues, especially the skin and tendons.[19] Various research has been conducted to harvest mammalian tissue organ to extract ECM which possess distinct structures and chemical composition for various biological purposes. However, it is unknown whether the tissue-specific composition and architecture of ECM scaffolds derived from

individual organs are necessary to maintain the phenotype and three-dimensional arrangement of cells native to those same tissues compared with ECM materials derived from nonhomologous sources.[20]

However various study suggest that adipose tissue is the abundant source of the ECM which is ideal material for the phenotype, functional behavior, recellularization and differentiation of the cells.[21] Nevertheless, it is quite difficult to say which method is befitting for specific regenerative medicine application since every method yield distinct structure and composition. Normally, most of the method till now created to extract ECM is comprises of combination of multiple methods like chemical, physical and enzymatic treatment.[22] However, after decellularization some of the characteristics of the original tissue remains intact, although all the mammalian species have similar compositions of extracellular matrix but may be differ in microstructure, physical properties, size and shape of the cells. [23] There are methods that is solely made to remove the cellular components and xenogeneic epitopes while preserving necessary ECM composition, however trace amount of cellular content such as DNA and a-gal epitope may be responsible for inflammation and poor result after implantation.[24]

2.2 SOURCE OF ECM AND MEDICAL APPLICATION FOR TISSUE ENGINEERING

ECM has been found to cause regrowth and healing of tissue. The ECM has two main purpose in terms of injury repair and tissue engineering, first, it prevents the immune system from triggering from the injury and responding with inflammation and scar tissue. Moreover it facilitated the surrounding cells to repair the tissue instead of forming scar tissue.[25] It is very hard to find a material that will support cell adhesion, and also ensure cell survival growth and appropriate cell differentiation following adhesion. There are alternative available for tissue engineering including commercial mixed matrices based upon the composition of basement

membrane for instance Matrigel which is available in both growth factor replete and depleted forms. Pig bladder is the most abundant and cheap source of ECM for medical uses for its relatively easily accessible and unused source.

2.3 POLYMERIC MATERIAL AND ECM.

ECM has shown great properties like mechanical and biochemical and it has been used as model for tissue engineering constructs for the production of tissue such a s cartilage, bone, nerve and skin and also continued efforts toward the production of more complicated organs. Tissue engineering application relied upon readily available polymeric materials, both naturally derived and chemically synthesized. These materials for specific application are chosen on the basis of their aggregate mechanical properties, ease of processing, degradation profiles, and biochemical activity. Among those most common materials are natural ECM based polymers such as collagen, fibrin glues, hyaluronic acid, and alginate. However the biological activities and biocompatibility of these materials are useful, the lack of control over desired mechanical, degradation, and processing properties has motivated use of synthetic polymers such as poly glycolic acid, poly lactic acid, ploy glycolic co lactic acid, poly ethylene glycol hydrogels.[26–28]

Chapter 3 **:** Materials and method

2.1 EXPERIMENTAL DESIGN

2.1.1 Preparation of adipose tissue ECM

Porcine adipose tissue was brought from the local market weighing about 1.76Lbs. Tissue was kept frozen at -80˚C before thawing. Then tissue was thaw overnight, red fat was cut off and removed, tissue part then minced and grind to a paste. Tissue was then placed in a beaker containing ultrapure water until it gets immersed and homogenized for two days. Every step of this protocol was performed onto orbital shaker at 100RPM. After two days elapsed tissue was placed into 0.5M NaCl for four hours followed by 1M NaCl for same time. After completion of this step tissue was washed into ultrapure water for overnight for two days. Tissue was then transferred to 0.25% Trypsin and rinsed for 2 hours followed by 1 hour washed in distilled water. Tissue was then rinsed in 1% Triton X-100 a nonionic surfactant for 5 days, changed the solution every day. Tissue was rinsed with DNase for 3 hours followed by 2 days washed in ultrapure water. Resulting material then washed in phosphate- buffer saline (PBS, pH 7.4) for 1 day and stored at 4˚C. Entire protocol to decellularize porcine adipose tissue is described in table 1.

Table 1 Decellularization protocol.

Ultrapure water	0.5 M NaCl	1.0 M Nacl	Ultrapure water	0.25% trypsin	Distilled water	1% triton x-100	DNase 37°C	Ultrapure water	PBS
48 h	4 h	4 h	48 h	2 h	1 h	5 days	3 h	48 h	24 h

2.2 CHARACTERIZATION AND CONFIRMATION OF DECELLULARIZATION.

Sample material resulting from the decellularization protocol was analyzed using different biological technique to determine microstructure, DNA content, morphology and other chemical species such as collagen, GAG and elastin content.

2.3 SCANNING ELECTRON MICROSCOPY ANALYSIS

Scanning Electron Microscopy uses electron beams that scan samples and reveals vital information about sample's surface topography and morphology. Decellularized ECM Samples were first fixed in paraformaldehyde for 60 minutes and dehydrated. After dehydration samples were washed with graded Ethanol (50%, 60%, 70%, 80%, 90% and 100%). Samples were sliced off using blade and coated with thin gold layer using sputter coating method. sample were then put onto scanning Electron Microscope sample holder and imagine at 5.0 kV in Hitachi S-4800(Fig. 3). However, SEM imaging for native porcine tissue was done using freeze fracture method using different dehydration trials followed by critical point drying (CPD).

1. FF → 100% Acetone (extracts fat) overnight → N_2(l) → CPD → Pd coat → view in the SEM

Figure 3 Scanning electron microscope (Hitachi S-4800) was used to analyze structure of ECM

2.4 HISTOLOGY

After the successful decellularization, tissue specimens were fixed in 10% (v/v) neutral buffered formalin, followed by dehydration with a graded ethanol (50%, 60%, 70%, 80%, 90% and 100%) and then embedded in paraffin wax. 5.0 µm thick section were cut off by using a cryostat microtome system and fixed on a glass slide. Representative sections were stained with hematoxylin and eosin to study the structure native porcine tissue and decellularized ECM.

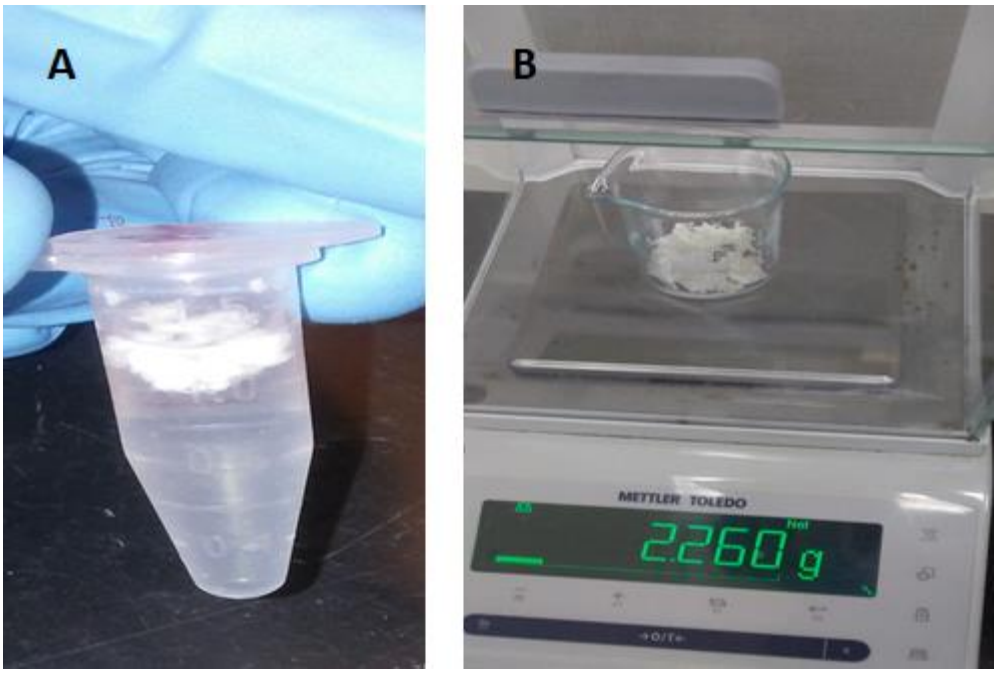

Figure 4 Acid-pepsin sample digestion for collagen quantification (1.5 ml microcentrifuge tube) (A) and balance was used to weigh the samples in entire project.

2.5 COLLAGEN QUANTIFICATION

Decellularized ECM was first weigh (Fig. 4 A and B) and digested using Acid-Pepsin solution for overnight using Sircol Soluble Collagen Assay Kit (Biocolor, U.K.). Samples were extracted with 0.5M acetic acid containing 1%(w/v) pepsin (P7012; Sigma) for overnight. Briefly the collagen isolation and concentration protocol were performed adding 100 µl Acid Neutralizing reagent to 1.0 ml acid extract and cold 200 µl Isolation and concentration reagent to 1.0ml acid extract. The soluble collagen then incubated with 1.0 ml Sircol dye reagent for 30 minutes at room temperature (Fig. 5 A). The collagen dye complex was precipitated after 30 minutes in a pellet form by centrifugation (Fig. 6 D) at 12,000 rpm for 10 minutes, and unbound dye removed carefully. Gently later on 750 µl Acid salt wash reagent to remove unbound dye from the surface of the pellet, centrifuge at 12,000 rpm for 10 minutes and drain the tube carefully. The pellets were dissolved in 250 µl alkali reagent, and the relative absorbance was measured in 96-well plate at 550 nm using microplate reader (Fig. 6 B).

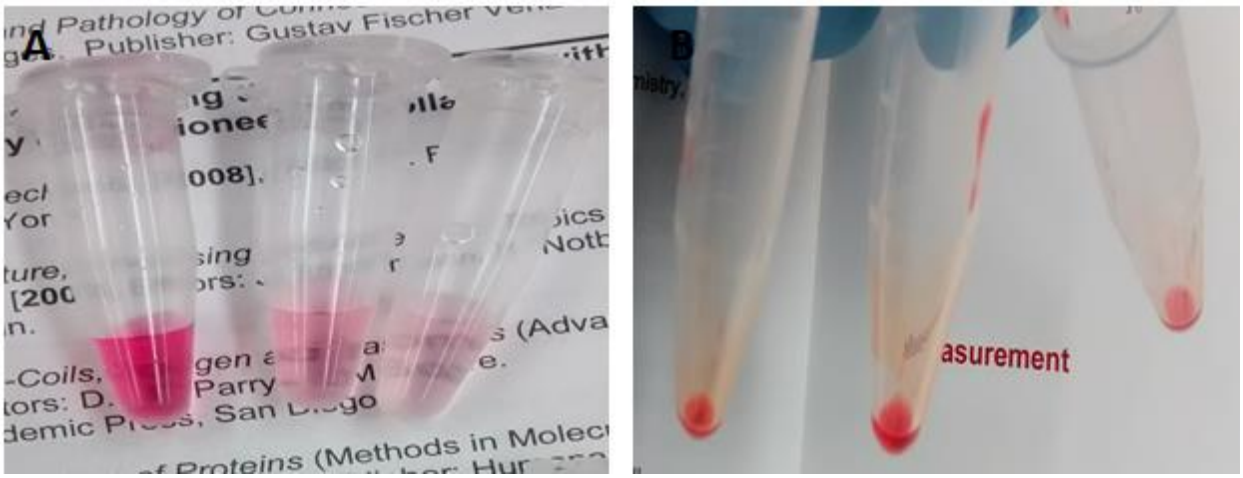

Figure 5 Collagen dissolve after adding Sircol dye reagent (1.5 ml microcentrifuge tube) (A) and elastin pellets after centrifugation (B) (1.5 ml microcentrifuge tube)

2.6 GLYCOSAMINOGLYCANS QUANTIFICATION

The sulfated Glycosaminoglycans content of the decellularized ECM was quantified using a Blyscan sulfated GAG assay kit (Biocolor, U.K.). Prior to measurement of sulfated GAG, sGAG were extracted using Papain extraction method. Samples were digested with a 0.2M Sodium Phosphate buffer, adding sodium acetate, EDTA, cysteine HCL and introduce 250 µl of a papain (P3125, Sigma) suspension, containing 5 mg of the enzyme for three hours at 65˚C in hot plate (Fig. 6 A). Solution then centrifuge (Fig. 6 C) at 10,000 rpm for 10 minutes, decant off the supernatant for use with blyscan GAG assay protocol. The extracted sulfated GAG was then mixed with 1.0 ml Blyscan dye and shaken for 30 minutes to allow sulfated GAG-dye complex to be formed and precipitate out from the soluble unbound dye. Precipitate were collected by centrifugation at 12,000 rpm for 10 minutes and then pellets were dissolved in 0.5 ml dissociation reagent. The absorbance was measured in 96-well plate at 656 nm using microplate reader (Fig. 6 D).

Figure 6 instruments used throughout the component analysis of Decellularized ECM. hot plate for sample digestion (A), vortex mixer to homogenize the content (B), centrifuge equipment (C) and Microplate reader to measure the absorbance at various wavelength (D)

2.7 ELASTIN PROTEIN QUANTIFICATION

The soluble elastin content in the decellularized ECM was measured using a Fastin elastin assay kit (Biocolor, U.K.). Conversion of insoluble elastin to soluble elastin was done by placing weighed samples into 750 µl of 0.25 M oxalic acid (Sigma) at 100˚C for 60 minutes in hot plate (Fig. 6 A). Insoluble residues were separated and add further 750 µl of 0.25 oxalic acid and heat again while supernatant was collected. To extracted supernatant 50 µl of Elastin Precipitation reagent was added and leave the samples for 15 minutes and centrifuge (Fig. 6 C) at 10,000 g for 10 minutes. The extracted soluble elastin was mixed with Fastin dye reagent and mixed content, allow reaction for 90 minutes and centrifuge at 10,000 rpm for 10 minutes to collect the precipitate. Pellets then were dissolved using 250 µl of dye dissociation reagent. The absorbance was measured in a 96-well plate at 513 nm using microplate reader (Fig. 6 D).

2.8 DNA quantification

DNA from decellularized ECM and native porcine adipose tissue was extracted using PureLink Genomic DNA mini kit (K182000, ThermoFisher Scientific). Samples were digested using authors manual, mammalian tissue lysate protocol was used to digest the decellularized freeze dried ECM and native porcine adipose tissue and proceed immediately to purification protocol which is design to purify genomic DNA using column base purification procedure. The total DNA content was measured at UV absorbance at 260 nm in NanoDrop 1000 Spectrophotometer (Fig. 7). Comparison were made between native porcine tissue and decellularized ECM.

Figure 7 Nanodrop 1000 to quantify purified genomic DNA content of native porcine adipose tissue and decellularized ECM

Chapter 4 : Results

3.1 EXTRACTION OF ECM FROM PORCINE ADIPOSE TISSUE

Goal of this research was to decellularized the discarded Porcine adipose tissue and extract ECM using combination of chemical, mechanical and enzymatic treatments. An adult discarded pig tissue was collected from the local market from El Paso, Texas. The fresh pig adipose tissue in the beginning had lipids, cells, blood and other genetical and unwanted composition (Fig. 9 A) which had given a tissue light pinkish and oily texture, after each subsequent process mention in the table 1 cluster of lipids (Fig. 9 B) and cell debris were observed and effectively removed from the tissue samples (Fig. 9 C), after the completion of entire protocol tissue turned brighter and fibrous matrix shown in (Fig. 9 D), was stored at 4°C. Quantitively, 85% to 90% of initial weight was reduced during entire protocol and ECM matrix reproducibly demonstrate between 10% to 15% of the original porcine adipose tissue mass. However, it may completely depend on type of specific tissue source and health of the animal. Whole protocol was carried out at room temperature except trypsin 0.25% and DNase which was performed at 37˚C.

Decellularization efficiency was evaluated using histology (H & E staining) which confirm successful removal of most of the cells and retains necessary component of decellularized ECM. The presence of DNA in samples were measured using DNA quantification, both native tissue and ECM compared, while DNA content of decellularized ECM significantly reduced. The result demonstrates that decellularization significantly reduced the potential immunogenic components in the ECM, while preserve the chemical integrity and proteins of original tissue.

3.2 LYOPHILIZATION OF ECM

For each assay like collagen, Glycosaminoglycans, Elastin and DNA quantification decellularized ECM samples were weighed and freeze dried using Freeze dryer (Fig. 8) for 24 hours. Prior to lyophilization samples were freeze for 1 hour and put them in freeze dryer chamber. Significant weight reduction was observed as most of the water content of samples evaporated from the ECM samples can be seen in (Fig. 10) at certain pressure and temperature conditions. Motive of the lyophilization was to allow maximum exposure of tissue component rather than unwanted water quantity included throughout the assay.

Figure 8 Lyophilizer to freeze-dry the decellularized ECM samples

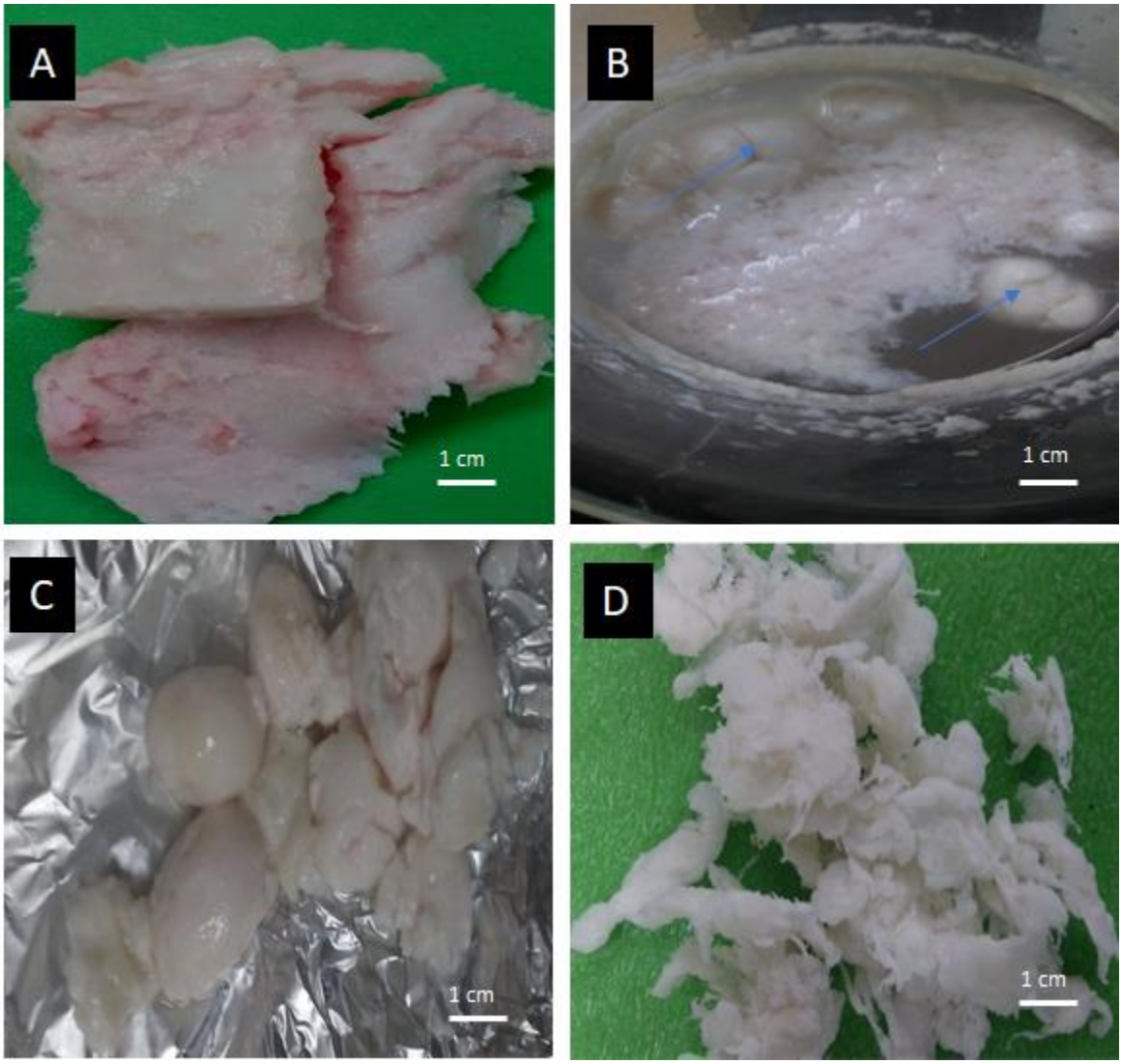

Figure 9 Macroscopic images of native porcine adipose tissue and decellularization. Native porcine tissue (A), clusters of lipids (B), lipids removed (C) and final ECM after freeze dry (D). scale bar represents 1 cm.

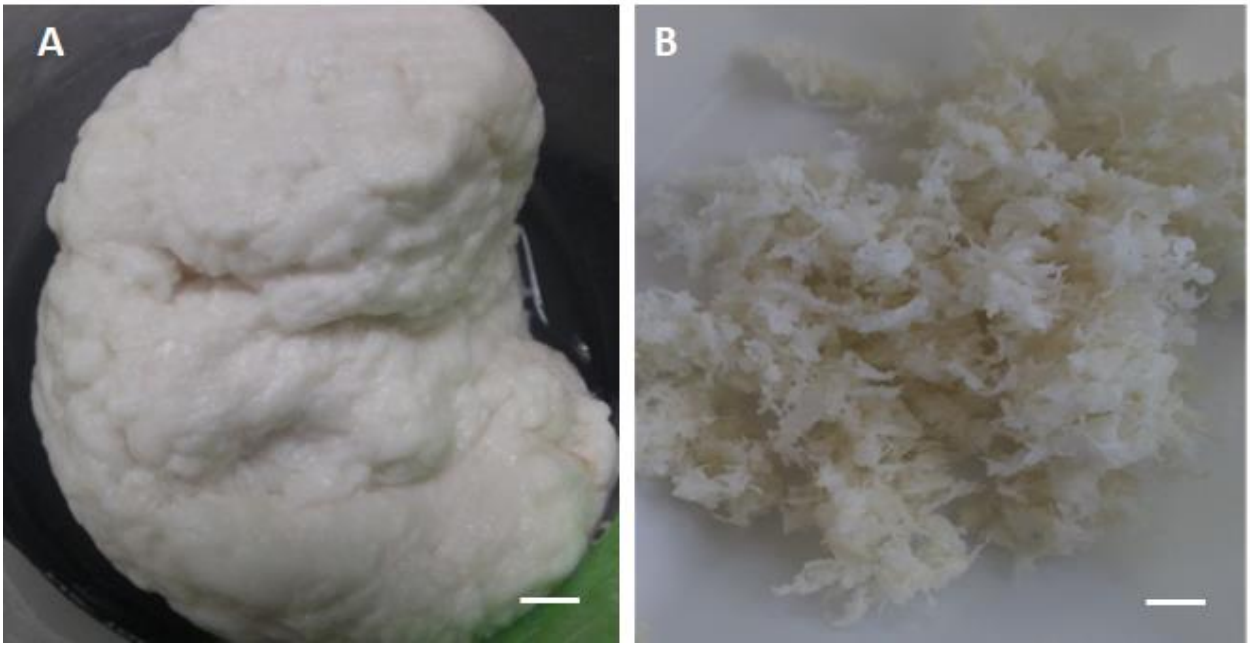

Figure 10 Freeze drying of ECM. Decellularized ECM (A) and freeze-dried ECM (B). Scale bar represent 1.0 cm

3.3 SCANNING ELECTRON MICROSCOPY

SEM images revealed the morphology and structure of native porcine tissue and decellularized ECM. Fig 11 shows scanning electron microscopy at different magnification for Native porcine tissue (Fig. A) and decellularized ECM (Fig. B, C and D). Surface morphology was found absolutely distinct for decellularized ECM than native porcine adipose tissue. However, decellularized ECM revealed consistency in fiber diameter, while fiber bundles were distorted, but the cellular component was effectively eliminated. Within the bundles of collagen fiber distributions are align and well adhere to each other. Native porcine adipose tissue revealed indigenous microstructure where all the matrix is surrounded by the lipids and cellular components and structure is completely logged.

3.4 HISTOLOGICAL ANALYSIS

The confirmation of remaining cells after decellularization was evaluated using H&E staining and compared with native porcine tissue. Decellularization efficiency showed positive correlation with the native tissue as cell were completely eliminated. Fig 12 shows and confirm that the cells were significantly removed from decellularized ECM while native porcine adipose tissue witness of cell nuclei clusters and can be seen as blue or purple stain. cytoplasm and extracellular matrix, however can be seen as dark red and pale pink respectively.

3.5 DNA QUANTIFICATION

DNA content was evaluated after decellularization treatment. Study confirm significant reduction in DNA content of decellularized ECM than native porcine tissue. 14-day protocol of ECM extraction proved elimination of almost 4^{th} times of the DNA content of native porcine adipose tissue represented in Fig 13. DNA content in native porcine tissue found to have 60±5 ng/mg while the decellularized ECM was detected 14±2 ng/mg dry weight tissue.

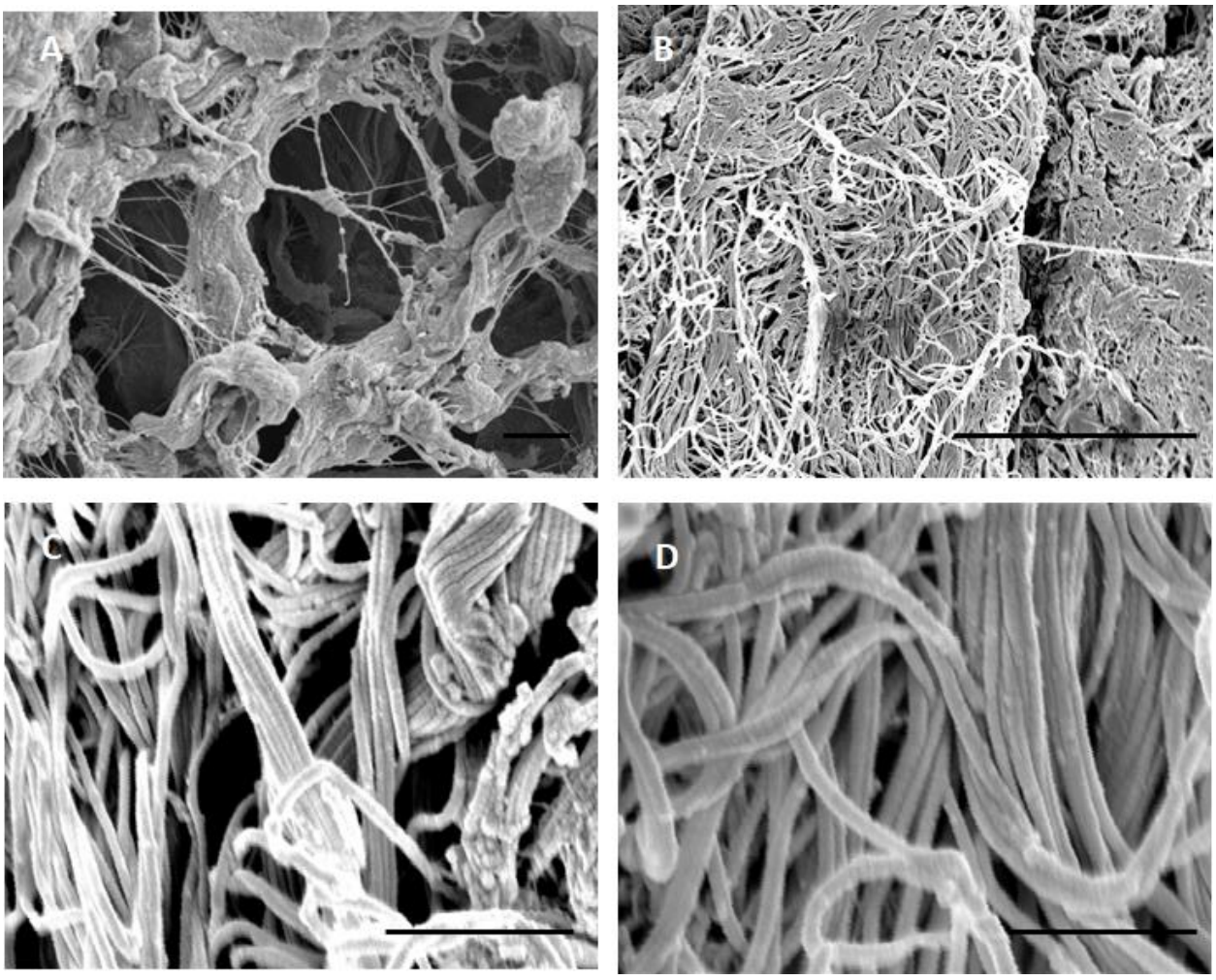

Figure 11 SEM images of native porcine tissue (A) and decellularized ECM (B, C & D). scale bar represents 10 µm (A x1400), 10 µm (B x 5.0k) and 1 µm (C 30k & D 40k)

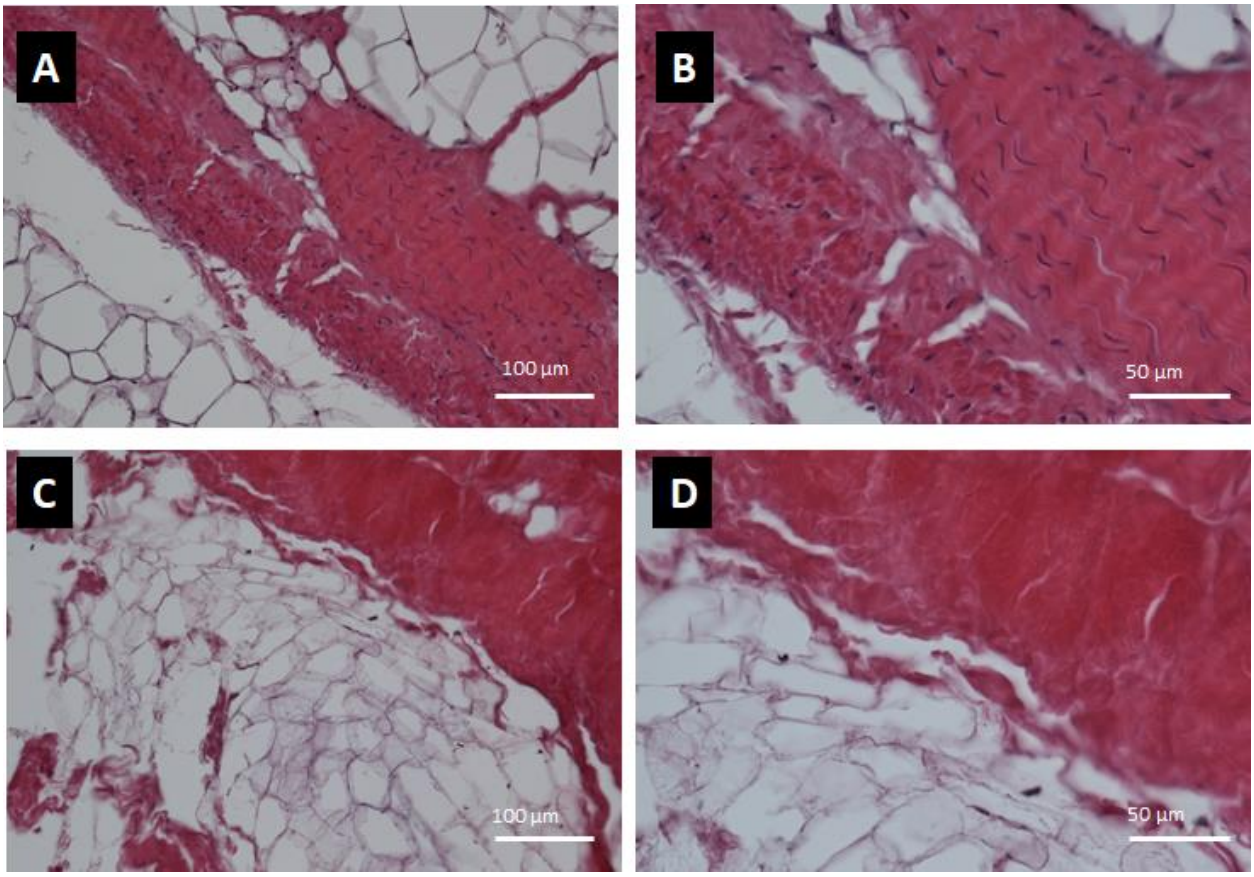

Figure 12 Histological images of native tissue (A and B) and decellularized ECM (C and D). scale bar represents 100 µm (A and c) and 50 µm (B and D)

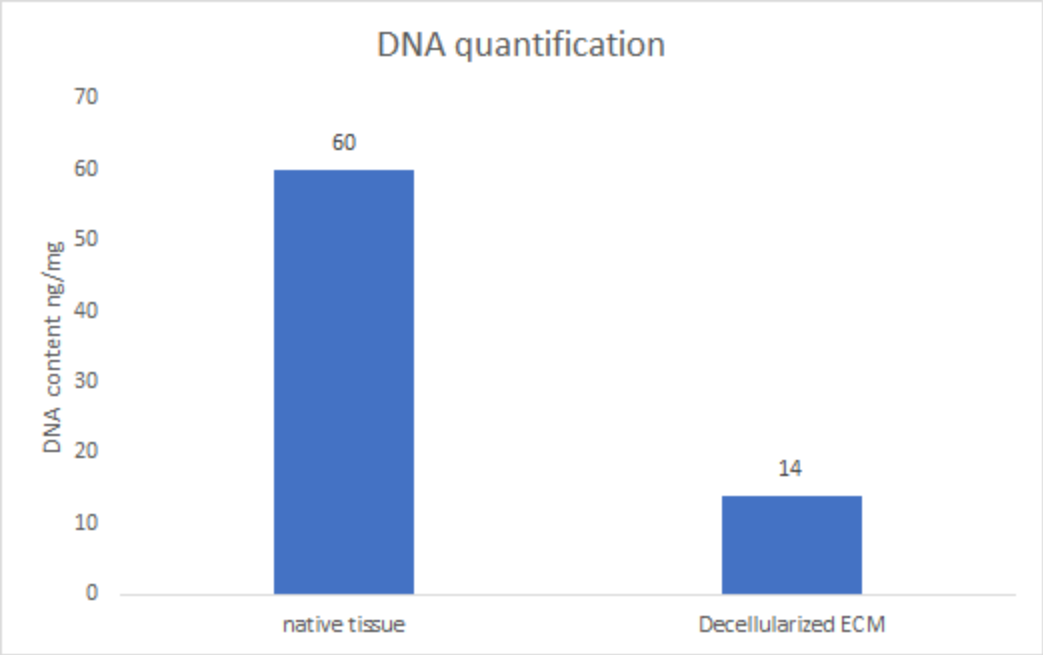

Figure 13 DNA quantification. native tissue shows 60 ng/mg while decellularized ECM shows 14 ng/mg DNA content

Decellularized ECM Component analysis

It has been found that most abundant protein collagen have remained intact in ECM matrix. The decellularized ECM was rich in acid-pepsin soluble collagen with amount of 127 ± 10 µg/mg ECM dry weight. A significant amount of Another main component of decellularized ECM which is elastin protein found to have remain 81 ± 5 µg/mg ECM dry weight. However, a very small amount of glycosaminoglycan was also detected in the matrix of ECM weighing 42 ± 4.4 µg/mg ECM dry weight. Fig 14 show the comparison of each component.

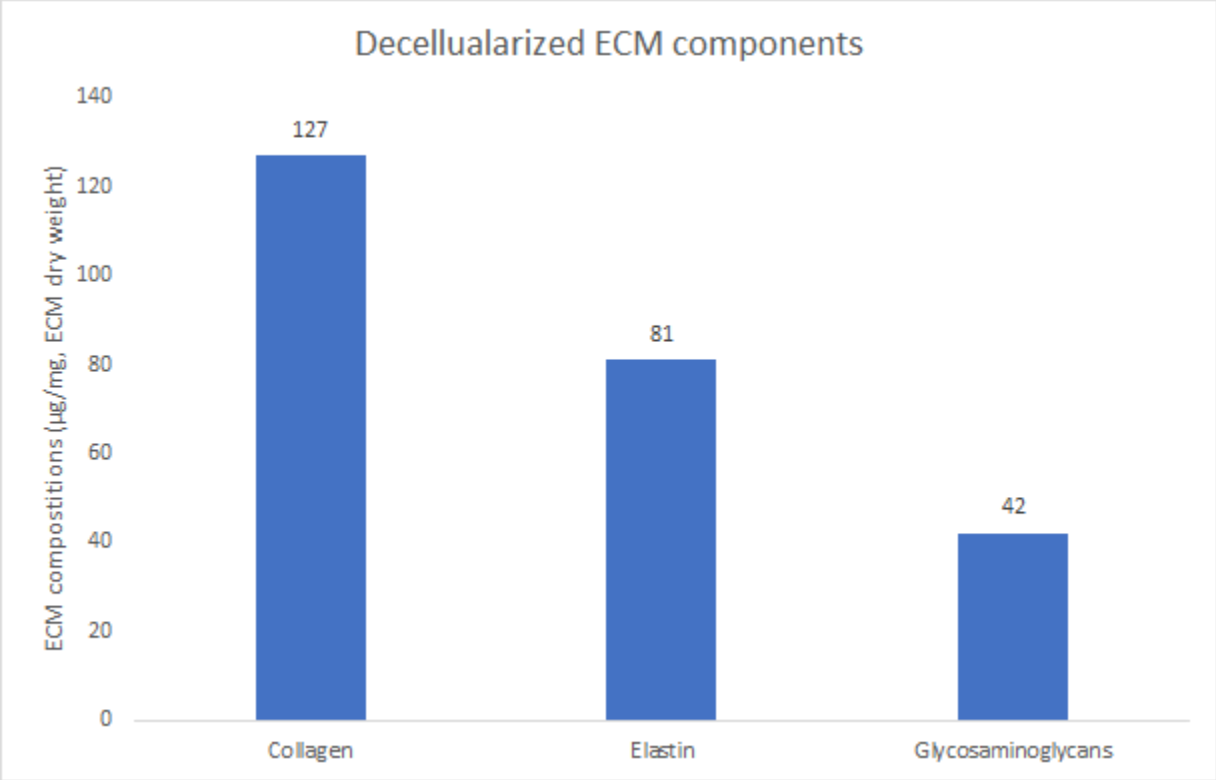

Figure 14 Decellularize ECM component analysis such as collagen, elastin and GAG

Chapter 5 : Discussion

Porcine adipose tissue was treated using our protocol aiming to removal of the xenogeneic epitopes like cells and DNA with minimal disruption of Extracellular matrix to maintain structural integrity and biochemical integrity. ECM is a regulator of cellular behaviors including cell survival, proliferation, morphogenesis, and cell differentiation. [29] It has been proved that decellularized ECM can mimic the compositions, microstructure and biomechanical properties of the native ECM. [30,31] Animal derived ECM can have concern over negative immune response, as well xenogeneic disease transmission. Considering all above factors, new and more efficient techniques to isolate the ECM should be develop to eliminate xenogeneic epitopes. There have been several protocols establish by various researchers to optimize the decellularization effect with minimal adverse effect on ECM compositions. [22] Methods also found to decellularized human adipose tissue. [32] They have used isopropanol as solvent removal, as well chemical, physical and enzymatic treatment was used for effectiveness of protocol. Here in current study non-ionic detergent 1% Triton X-100 was used optimize the protocol. Trypsin which is best known for cell removal, however trypsin is disruptive and exposure time should be well controlled. In another study, ECM was extracted from porcine adipose tissue by simple pulverization method under high temperature and effectively decellularized after a relatively short exposure to sodium dodecyl sulfate and enzymes, Unlike human adipose tissue, porcine adipose tissue cannot be easily decellularized because it is thick and dens, in addition at low temperature and at room temperature, lipid may have trapped within the adipose tissue may congeal, which may affect decellularization and freeze drying.[33]

Primary focus of the current research was to isolate ECM from porcine adipose tissue which is complex network of cells and matrix attached and adhere to each other that makes isolation even

difficult, therefore chemical and enzymatic treatment can be used to remove cellular remnants and xenogeneic epitopes. [34] Although, these chemicals are harsh for the human body and may raise concern of toxicity in decellularized ECM. However, this combination of chemical, physical and enzymatic treatment may not be efficiently remove cellular components from tissue. Even commercially available biological scaffold and ECM material contains a trace amount of remnant DNA, despite their use in clinical operation. [35]

The current research however, was highly effective in removing DNA, cellular components, lipids. Approximately, 14 ± 3 ng/mg (dry weight) DNA was quantified compared to native porcine adipose tissue 60 ± 4 ng/mg (dry weight) was measured. However, another study for the extraction of ECM from porcine adipose tissue evaluated the DNA content of native tissue 1173 ±1.75 ng/mg (dry weight) while the decellularized ECM DNA content 43.2 ± 3.23 ng/mg (dry weight).[33] Interestingly, matrix structure of ECM remains intact which was observed in native porcine tissue. Our result suggest that the protocol is effective eliminating xenogeneic epitopes and preserve the fibrous structure of the native tissue. Current research shows a descent amount of decellularized fibrous component such as collagen 127 ± 10 µg/mg (dry weight), soluble elastin 81 ± 5 µg/mg dry weight and small amount of GAG 42 ± 4.4 µg/mg dry weight. while in another study 332.9 ± 12.1 µg/mg (dry weight), 152.6 ± 4.5 µg/mg (dry weight) and 85 ± 0.7 µg/mg (dry weight) of collagen, elastin and GAG was quantified respectively.[33] Collagen was quantified in Various research on human and porcine adipose tissue reported containing many ECM component, including collagen I, III and IV, elastin, laminin, fibronectin, and various bioactive molecules. [21,32,36]

The aim of this project is to decellularization of porcine adipose tissue retaining the ECM composition. Histological analysis such as H&E staining showed that cellular component and

DNA was successfully removed. DNA quantification was witness for reduction of DNA content than the native porcine adipose tissue. Important decellularized ECM component such as collagen, GAG and elastin was found well preserved.

Chapter 6 **:** Conclusion

Discarded waste of porcine adipose tissue can be used as biomaterial. The results of the study showed that the decellularization protocol able to successfully removed Immunogenic component such as cellular content, DNA and lipids, were significantly removed while preserving decellularized ECM matrix. Decellularized ECM may provide a suitable biochemical environment for the growth, proliferation, adhesion and differentiation of human cells in the in vitro and in vivo study for tissue engineering applications. However, the presence of nucleic acid remnants, including excess lipids and DNA, may affect the ability of an adipose ECM material to function as a template for constructive remodeling in vivo. The ECM derived from porcine adipose tissue can serve as a xenogeneic biomaterial and can have great potential for biomaterials application.

References

1. RegenerativeMedicine.net - What is Tissue Enginnering? Available at: http://www.regenerativemedicine.net/Tissue.html. (Accessed: 15th December 2017)
2. Tissue Engineering and Regenerative Medicine | National Institute of Biomedical Imaging and Bioengineering. Available at: https://www.nibib.nih.gov/science-education/science-topics/tissue-engineering-and-regenerative-medicine. (Accessed: 15th December 2017)
3. Langer, R. & Vacanti, J. P. Tissue engineering. *Science* **260,** 920–6 (1993).
4. The Extracellular Matrix and cell wall. *Khanacademy* Available at: https://www.khanacademy.org/science/biology/structure-of-a-cell/cytoskeleton-junctions-and-extracellular-structures/a/the-extracellular-matrix-and-cell-wall.
5. Fausto, K. A. Robbins and Cotran: Pathologic Basis of Disease(7th ed.). *Philadelphia: Elsevier.*
6. Di Lullo GA, Sweeney SM, Korkko J, Ala-Kokko L, S. A. J. Mapping the ligand-binding sites and disease-associated mutations on the most abundant protein in the human, type I collagen. *J. Biol. Chem.* **277 (6): 4,** (2002).
7. collagen structure triple helix - Google Search. Available at: https://www.google.com/search?q=collagen+structure+triple+helix&rlz=1C1CHZL_enMX699MX699&source=lnms&tbm=isch&sa=X&ved=0ahUKEwi5w_brivnXAhXM3YMKHZIeAvoQ_AUICigB&biw=1366&bih=662#imgrc=cSpf77hD-IRvAM: (Accessed: 7th December 2017)
8. Fischer GM, L. J. Collagen and elastin content in canine arteries selected from functionally

different vascular beds. *Circ. Res.* **19 (2): 39,**

9. G, P. *The extracellular matrix and cell adhesion*. (2007).
10. Esko, J. D. & Selleck, S. B. Order Out of Chaos: Assembly of Ligand Binding Sites in Heparan Sulfate. *Annu. Rev. Biochem.* **71,** 435–471 (2002).
11. Alberts, B. *Molecular biology of the cell*. (Garland Science., 2002).
12. Plotnikov, S. V, Pasapera, A. M., Sabass, B. & Waterman, C. M. Force fluctuations within focal adhesions mediate ECM-rigidity sensing to guide directed cell migration. *Cell* **151,** 1513–27 (2012).
13. Hadjipanayi, E., Mudera, V. & Brown, R. A. Close dependence of fibroblast proliferation on collagen scaffold matrix stiffness. *J. Tissue Eng. Regen. Med.* **3,** 77–84 (2009).
14. Engler, A. J., Sen, S., Sweeney, H. L. & Discher, D. E. Matrix Elasticity Directs Stem Cell Lineage Specification. *Cell* **126,** 677–689 (2006).
15. Lo, C. M., Wang, H. B., Dembo, M. & Wang, Y. L. Cell movement is guided by the rigidity of the substrate. *Biophys. J.* **79,** 144–52 (2000).
16. Tachi, M. & Yamada, A. Choice of flaps for breast reconstruction. *Int. J. Clin. Oncol.* **10,** 289–297 (2005).
17. Wu, L. C., Bajaj, A., Chang, D. W. & Chevray, P. M. Comparison of Donor-Site Morbidity of SIEA, DIEP, and Muscle-Sparing TRAM Flaps for Breast Reconstruction. *Plast. Reconstr. Surg.* **122,** 702–709 (2008).
18. Chan, B. P. & Leong, K. W. Scaffolding in tissue engineering: General approaches and tissue-specific considerations. *Eur. Spine J.* **17,** (2008).
19. Longo, U. G., Lamberti, A., Petrillo, S., Maffulli, N. & Denaro, V. Scaffolds in tendon tissue engineering. *Stem Cells Int.* **2012,** (2012).

20. Sellaro, T. L. *et al.* Maintenance of human hepatocyte function in vitro by liver-derived extracellular matrix gels. *Tissue Eng. Part A* **16,** 1075–82 (2010).

21. Flynn, L. E. The use of decellularized adipose tissue to provide an inductive microenvironment for the adipogenic differentiation of human adipose-derived stem cells. *Biomaterials* **31,** 4715–4724 (2010).

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

23. Information, A. Xenogeneic extracellular matrix grafts elicit a TH2-restricted immune response . Join Ovid Insights ! **71,** 3–5 (2017).

24. Zheng, M. H. *et al.* Porcine small intestine submucosa (SIS) is not an acellular collagenous matrix and contains porcine DNA: Possible implications in human implantation. *J. Biomed. Mater. Res. - Part B Appl. Biomater.* **73,** 61–67 (2005).

25. 'Pixie dust' helps man grow new finger - Telegraph. Available at: http://www.telegraph.co.uk/news/1915228/Pixie-dust-helps-man-grow-new-finger.html. (Accessed: 7th December 2017)

26. Hirano, Y. & Mooney, D. J. Peptide and Protein Presenting Materials for Tissue Engineering. *Adv. Mater.* **16,** 17–25 (2004).

27. Drury, J. L. & Mooney, D. J. Hydrogels for tissue engineering: scaffold design variables and applications. *Biomaterials* **24,** 4337–4351 (2003).

28. Seal, B. ., Otero, T. . & Panitch, A. Polymeric biomaterials for tissue and organ regeneration. *Mater. Sci. Eng. R Reports* **34,** 147–230 (2001).

29. Song, J. J. & Ott, H. C. Organ engineering based on decellularized matrix scaffolds. *Trends Mol. Med.* **17,** 424–432 (2011).

30. Keane, T. J., Swinehart, I. T. & Badylak, S. F. Methods of tissue decellularization used for preparation of biologic scaffolds and in vivo relevance. *Methods* **84,** 25–34 (2015).

31. Mosser, G., Anglo, A., Helary, C., Bouligand, Y. & Giraud-Guille, M. M. Dense tissue-like collagen matrices formed in cell-free conditions. *Matrix Biol.* **25,** 3–13 (2006).

32. Brown, B. N. *et al.* Comparison of Three Methods for the Derivation of a Biologic Scaffold Composed of Adipose Tissue Extracellular Matrix. *Tissue Eng. Part C Methods* **17,** 411–421 (2011).

33. Choi, Y. C. *et al.* Decellularized Extracellular Matrix Derived from Porcine Adipose Tissue as a Xenogeneic Biomaterial for Tissue Engineering. **18,** 866–876 (2012).

34. Information, A. Porcine cartilage transplants in the cynomolgus monkey . III . Transplantation of alpha-galactosidase-treated porcine cartilage . Join Ovid Insights ! **65,** 1–2 (2017).

35. Gilbert, T. W., Freund, J. M. & Badylak, S. F. Quantification of DNA in Biologic Scaffold Materials. *J. Surg. Res.* **152,** 135–139 (2009).

36. Choi, J. S. *et al.* Decellularized extracellular matrix derived from human adipose tissue as a potential scaffold for allograft tissue engineering. *J. Biomed. Mater. Res. - Part A* **97 A,** 292–299 (2011).

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