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QUANTIFICATION OF COLLAGEN FIBRE MATURITY IN DECELLULARIZED COLLAGEN MATRICES

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Abstract

Bioengineering of tissue for transplantation is a growing area. As technology improves it is becoming increasingly possible to produce new cells, tissues and organs to replace damaged or diseased ones. Growing tissues alleviates the reliance on compatible donors and producing immunologically compatible material is possible. Growing new tissues involves the seeding of a supporting structure, or scaffold, with cells taken from the recipient. The scaffold needs to be made of an inert material that doesn't initiate an immune response, it must have the structure and mechanical properties of the tissue that it is replacing and lastly it must provide an environment that is conducive to cell proliferation. Collagen has emerged as the scaffold material of choice for several reasons: few people possess humoral immunity against it, it is one of the most abundant proteins on earth and, as it is found in many tissues, it can provide a three dimensional structure of the correct spatial arrangement. Preparation of the collagen scaffold involves removal of the cellular content of the tissue leaving behind the extracellular matrix. This is achieved by decellularization using chemical, physical or biological reagents. The decellularization process must remove any material that could illicit a immune response, yet not damage the collagen content or structure. Certain dyes can be used to selectively stain collagen and one of these, picrosirius red, was used here to identify the collagen and study its structure. Samples of three potential scaffolds: tendon, aorta and carotid artery, were examined before and after decellularization to see how the process had affected the amount and structure of the collagen present. It was found that the decellularization process had little effect on the collagen of the tendon or carotid artery, but the aortic collagen was altered by the treatment.

Key words, Transplantation, Collagen, Decellularization.

Chapter 1
1.0 Introduction

Tissue engineering can be used to resolve the issues of replacing organs and tissue. Chemical polymers are used as "scaffolds" to support the growth of cells. Yet a "complex of three-dimensional structures and full organs" can be produced by taking different approach (Gilbert et al. 2006). Decellularization and recellularization techniques have been produced to help the removal of parenchymal cells from a specific organ, leaving an extracellular matrix (ECM) that comprises "structural and connective tissue" (Atala and Weston, 1999). The eliminating cells may be utilized as a guide to steer the development of cellular growth to develop a "copy structure" (Gilbert et al., 2006). Variety of tissue types such as bladder, arteries, trachea and skin are successfully developed by the use of decellularized scaffolds (Dahl et al., 2003; Macchiarini et al, 2008; ; Nieponice et al., 2006; Schechner et al., 2003; Yoo et al., 1998).

1.1 Benefits of Decellularization

Producing a new organ can be done by using decellurization of an organ or tissue that provided a three dimensional scaffold. To treat conditions such as end stage organ failure has been explored for years by generating replacement or alternative organs. At present, patient who have conditions such as end-stage failure of the the liver, lungs, or heart can only be treated by organ transplant. There is a disadvantage of this form of treatment is that a deficit in suitable organ donors as well as the transplant procedure because it exposes the patient to the risks of blood transfer diseases. The use of immunosuppressant drugs prevent the rejection of transplanted organs, however it's required that they are taken for life (Ott et al., 2008).

Transplantation of The Recellularized Liver Graft

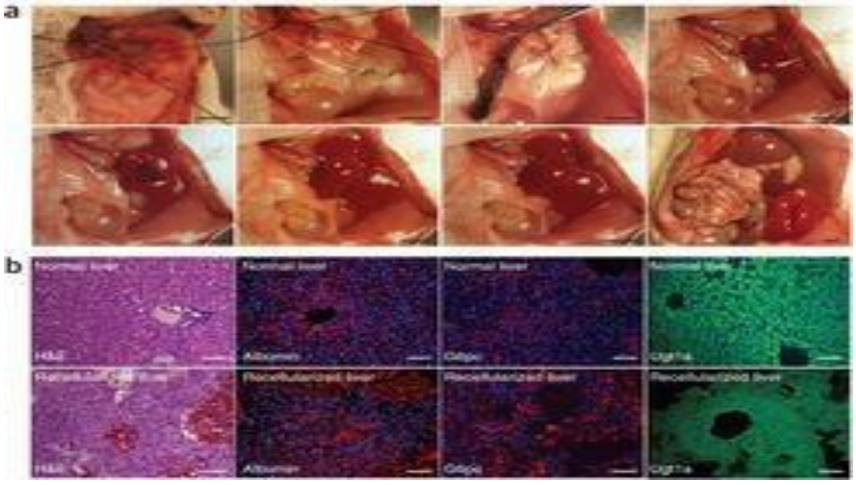


Figure 1. In this example of transplantation using a recellularized graft, a rat liver graft was (a) observed from placement to blood reperfusion. Immunohistochemical staining of native (bottom) and graft (top) was then conducted to show the differences between the two. Figure courtesy of Uygun B.E., Soto-Gutierrez A., Yagi H., Izamis M-L., Guzzardi M.A., Shulman C., Milwid J., Kobayashi N., Tilles A., Berthiaume F., Hertl M., Nahmias Y., Yarmush M.L., UygunK., (2010). Organ reengineering through development of a transplantable recellularized liver graft using decellularized liver matrix. *Nature Med.*, (16), pp 814–820

The existence of natural antibodies is a considerable obstruction to xenotransplantation. Xenotransplanta¬tion is where animal prgans are transplanted into human beings, and has come under a lot of scrutiny. Yet, difficulties were found due to the immune response. The size and physiology of the human have been investigated that is similar to the pig organs, which is an excellent candidate for xenotransplantation. Unfortunately, pig cells display a sugar antigen (Galactose 1-3 Galactose) upon its surface including kidneys and hearts that correspond to that of humans. Transferring pig cells

or organ to the human or non- human primates was causesd hyperacute rejection minutes to hours of the pig cells after transplantation because of presence of the combination of the Gal epitope and the preexisting antibodies. As a result, pig genes have been appropriated to be used with human genes, which are capable of suppressing the immune response and lack the antigen (Prather, 2007; Lai et al., 2002). Pig scaffolds have been used to produce fresh bladder, ureteral and vaginal tissues which have been grafted to human beings (Atala et al., 1999). 200 000 or more human patients underwent insertion of xenogeneic ECM based scaffold and graft preparations required assembly of small intestinal graft from pig and collagen scaffold obtained from porcine skin to reconstruct diverse ligaments and tendons of human nature (Badylak, 2004). The anterior cruciate ligament (ACL) of damage is a very frequent ligament knee injury. The use of porcine cruciate ligament bone grafts have been used as suitable treatment to help these ligaments because they maintain the mechanical characteristics which permits the "successful repopulation by human fibroblasts" (Woods and Gratzer, 2005).

The advent of stem cell technology has meant that the prospect of growing tailor made organs from the recipient's own cells is possible. This has the advantage that the patient's body will accept an organ made up of the original cells which means immunosupressants isn't required. Growing new tissue from stem cells requires the use of a scaffold for the cells to develop around. This scaffold may consist of biological or artificial material, but will become part of the final structure and so will also be implanted in the recipient's body. Therefore the scaffolding material needs to not elicit an immune response (Chen et al., 2002). This means removal of the various complex sugars that are found on the surface of the tissue, and can bind to antibodies and cell-surface receptors of immune cells. Examples of scaffolds being applied to produce fresh tissue include using acellular matrices to grow heart valves (Knight et al., 2008).

Particular advantages of tissue engineered valves for younger patients in that they can adapt internally which can prevent repeat operations (Licheten berg et al, 2006). Tissue engineering has been shown improved quality of life. Successful transplantation of newly formed trachea from stem cells, which was cultivated in collagen-based scaffold was inserted into a patient suffered from end-stage bronchomalacia was demonstrated by researchers (Macchiarini et al., 2008)

In this instance, a scaffold was generated as a result of the removal of cells and antigens from trachea donated by human and introduced into mesenchymal and epithelial stem cells, which were belonging to the recipient. Afterwards, the graft was allowed to grow in vitro and was then transplanted into the patient. As a result, the inserted grafts provided the recipient with functional airways and improve life quality. The patient was able to stop immunosuppressive drugs (Baiguera et al., 2010). According to BBC (2010) discovered the initial stem cell organ transplant into a child. They documented the longest airway to have ever been replaced. The successful transplant of trachea engineered tissue to a child was first accomplished in 2010 by a Great Ormond Street Hospital medical team. A very expensive process is when the new grown trachea been used by a bioreactor. The genetically engineered trachea tissue has been contained in a collagen structure obtained from a donor, which was impregnated with stem cells from the patient. Subsequently, the scaffold was injected into the patient even as the cells were undergoing active division and growth. Therefore, the body of the recipient was used as bioreactor (Baiguera et al., 2010).

1.2 Types of Scaffold Production

In tissue engineering uses synthetic scaffolds can be the same method as donor organs mentioned above. Employing "Biodegradable scaffold poly-4-hydroxybutyrate-coated polyglycolic acid "has been used in producing new heart valves was examined by research (Rabkin et al., 2002). Ovine endothelial and carotid artery were planted by biodegradable scaffold cells and transplanted into active host. It was observed that the tissue regenerated extracellular matrices and collagen arrangement similar to that of the native valve. Preparation of the graft was carried out in vitro prior implanting. This allowed the graft to grow and change by the active nature of engineered tissues according to study (Knight et al., 2008).

A three-dimensional scaffold of well-preserved extracellular matrix composed of collagen, sulfated glycosaminoglycan and elastin could be produced by successful decellularization of adipose tissue using a combination of physical, chemical and enzymatic procedures (Choi et al., 2011). Flynn (2010), whereby decellularization of adipose tissue along with preserving of basement membrane was carried out, adopted similar method. Laminin and type IV collagen of the conserved structure has been conformed to offer an environment, which is positive for cell differentiation.
Hybrid collagen-polymer mashes are essential to genetically engineer dermal tissues (Kian et al., 2007) as it is used to support the advancement of mesenchymal stem cells derived from bone marrow (Tan et al., 2011). Kian et al., (2007) placed mesh along with dermal fibroblast, which were from human and was subsequently attached to blood supply by arteriovenous. This process allows cell proliferation and differentiation as well as collagen formation to occur (Kian et al., 2007).
Tan et al have cultivated artificial scaffolds, which allow and sustain growth of mesenchymal stem cells, from bone marrow. A major extracellular matrix component in cartilaginous tissues is Collagen II, undergoes fibrillogenesis under different physiological conditions in body (Tan et al., 2011). This type of collagen has also been combined with biodegradable polyester to improve its scaffolding function (Chang et al., 2010).

Engineering scaffolds of other dermal tissue have been found to use and collagen-glycosaminoglycan (CG) and type I collagen matrices. The productions of low-density biodegradable supports have the ability to stimulate skin conjunctive nerve peripherеral nerves regeneration in vivo (Harley et al., 2004; Yannas et al., 1989; Yannas et al., 1990). Tendons and ligaments, which are connective tissue in nature, have been arranged from dermal extracellular matrices. This is because extracellular matrices are capable of recruiting progenitor cells and they can use in repairing selective tissues such as those of the heart and liver. Therefore, flexible scaffold for numerous tissues engineering application can be used by porcine skin (Chen et al., 2004).

The use of the decellularized scaffolds helped to produce cardiovascular tissue and can also retain the cell's biological composition and structure. While natural scaffold composition assists with the cell-in-cell adhesion, there exist several drawbacks due to the density of collagen fibers. By removing certain elements of the additional cellular matrix, this permits the generation of a permeable architecture which can maintain the structure of the parent tissues. Additional chemical cross-links across the different fibers helps to develop the strengths and stability. Samples of porcine aorta were decellularized, extracted and cross-linked. When the fibroblast penetrates the scaffold, cells mature in a controlled way (Lu et al., 2004).

1.3 The Common Scaffold Material: Collagen

Collagen has been identified as a key protein that comprises a quarter of the total proteins in a human body; and can be identified within strong molecular cables. Collagens facilitate the functional conditions of connective tissue, for example tendons, and also aid the organs and skin. Collagen helps in maintain structure, support and helps the softer parenchmal tissues, which link them to bones (Badylak, 2004). A fibrosis of tissues may arise within chronic inflammations, although this is because of excessive depositions of connective tissues. A scar may form from the structural remodeling within particular organs; for example, in the heart. Zhao et al. (2008) observed that stiffness of the ventricular wall and reduced blood pump by the heart is due to extra cellular matrix deposition (Zhao et al., 2008; van Heerebeek et al., 2008), which Hadi et al. (2011) explains "interferes electrochemical signaling causes arrhythmias."

Collagen comprises 3 main protein chains, with every one containing more than 1400 amino acids. A polyproline II helix is folded by individual polypeptides and then a right-handed triple helix is formed via a combination of 3 of these helices. Contacts are required as the triple helix is folded and transformed into trimerization domains. A single starting point for triple helix formation has been confirmed by these domains and was also responsible for the selected chain in heterotrimeric

collagens. Proline rich domains are found on all collagen types and contain tripeptide Gly-X-Y repeats. (Boudko et al., 2011). Helix and proline contain a small glycine residue, and X and Y positions are filled with hydroxyproline (Xu et al., 2011). A twist in the peptide chain was done in proline residues and repeats are completely made in order to allow the proteins of each collagen to produce triple helix (Bella et al., 1994). This structure is vital for the collagen as it gives its properties such as strength (Buehler, 2006). On top of its crucial role in maintaining structure, collagens also work with other cellular proteins called discoidin domain receptors. Cell surface receptor called tyrosine kinases are activated by triple helical collagen, which plays significant part in remodeling extracellular matrices, adhesion and migration of cells and cell proliferation (Vogel et al., 1997). Recently Xu et al., (2011) showed that non-fibrillar collagen type IV contain binding sites for certain members of the discoidin domain receptor family.
All collagens have a long triple helix. These triple helices are attached to different additional domains of different members of the collagen family and these domains give the different members their own unique properties. Space between the greater part of cells were found Type I collagen. Large amounts of type I of collagen found in dermal layer of the skin, tendons and artery walls (Di Lullo et al., 2002). Collagen type II is found in cartilaginous tissues as the major component of the ECM (Holmes and Kadler, 2006). Type III of collagen is found in reticular tissue whereas types I collagen in the skin and artery walls. These collagen family members are all fibrillar being created of continual stretches of triple helices. Type IV collagen is different in that; it is formed of a mesh like structure and found in the base layer of many membranes of skin. This type of collagen has a circular head at one end and tail at the other. Other collagen molecules were associated with the circular domains and the tails resulting in a crisscross structure. An extended network is formed through these interactions which laminin and proteoglycans are associated to form a dense sheet (NCBI, 2000). All together the collagen family are contained of different members found in various tissues (Holmes et al., 2001).

Particular structure and mutations was essential in the molecular structure of collagen that resulted in alternate amino acids in different genetic conditions with exchange of residues, for example, epidermolysis bullosa (Almaani et al., 2011). Hydroxyprolines can be altered by glycosylation with glucose or galactose (Motooka et al., 2011). Shoulders and Raines (2011) observe that “the conformational stability of the triple helix is affected by hydroxylation” and the protein stability bundle is increased by the interactions between the hydroxyl groups of different strands; the formation of the triple helix is unfavourable when the prolines conformation is forced (Motooka et al., 2011).

1.4 Decellularization of Organs

To produce the collagen scaffold present in cellular structures are taken and treated to leave only the scaffolding molecules behind. Lysis of the cellular membranes can be done by decellularization usually pursued by the division of the cell’s components via the ECM. The cytoplasmic and nuclear components were solubilised and all this cellular remnants was washed away to leave the central structure (Gilbert et al., 2006). This can be achieved by physical, chemical and enzymatic methods and consisted of a mixture of them. Some examples of the various processes are described below:

1.4.1 Physical methods

Physical such as agitation, freeze-thawing, application of direct pressure and sonication were applied to produce scaffolds. Combination of ultrasonication has been used with detergent and nucleases to generate the ECM of porcine nucleus pulposus tissue that can actually be used to treat intervertebral discs. After the process, all the cellular structures have been removed, and the end result consisted of a solid, homogenous mixture containing aggrecan, chondroitin-6-sulfate and type II, IX, and XI collagen. This, in turn, is able to support the growth of stem cells derived from human adipose tissue seeded in this scaffold (Mercuri et al., 2011).

Snap freezing is often used to decellularize tendons and ligaments (for example see Roberts et al., 1991). Breaking the cellular membranes and causing the cells to lyse which can be done by the ice

crystals formed from the rapid freezing process. However, care must be taken not to cause damage to the ECM too. Collagen-glycosaminoglycan scaffolds have been made by freeze drying and quenching techniques (O'Brien et al., 2004). These functions can be as similarities of the dermal ECM (Yannas et al., 1989). The scaffold provides a surface of natural ligands is porous and can be seeded with dermal fibroblasts. These rounded fibroblasts elongate over time and their contractile behaviour causes deformation of the flexible scaffold. These can be used as valuable models to mimic the wound healing process as well as to support skin regeneration in vivo (Harley et al., 2007).

To lyse cells of application of the direct pressure can be used where a tissue does not have a dense or highly organised ECM For matrices that do not have a dense or highly organized structure, application of direct pressure can be conducted to lyse cells. Such process has been used on small intestine, and bladder (Badylak, 2004). To lyse the cells, it can be done by using mechanical removal, but it is usually used to help remove the displaced cellular material (Lin et al., 2004).

1.4.2 Chemical methods

Chemical methods contain of different acid and alkaline treatments, non-ionic, ionic and zwitter ionic detergents and also osmotic shock systems. Cell membranes are broken down when they are subjected to high PH, also this will damage the glycosylamino glycans (GAGs) structures (Gilbert et al., 2006). The organic solvent tributyl phosphate (TnBP) is a chemical that has been used to remove cells from tough structural tissues such as cruciate ligaments (Woods and Gratzer, 2005). Triton X-100 is used to study from non-ionic detergents commonly used, as they are effective decellularization agents. However, they can cause damage to the structure of the tissue and results depending upon the tissue type involved. Sodium dodecyl sulfate (SDS) is ionic detergents that remove cellular material effectively but the native tissue structure can be disrupted by SDS. . Zwitter ionic detergents are commonly used in decellularization of nerve tissue e.g. sulfobetaine-10 has been used successfully to decellularize peripheral nerves (Gilbert et al., 2006).

Osmotic shock can be conducted by transferring tissue from low to high ionic strength buffer solutions, which lead to lyse within the cell or organs. Chelating agents are included in buffers that are not only do they inhibit the activities of metal containing proteases, but they also inhibit cell adhesion to the ECM. Another common technique is enzymatic methods and includes the use of trypsin and endonucleases. Disruption of the ECM and reduce the elastin and fibronectin content can be caused by the Treatment with trypsin, while these methods are efficient at decellularization (Gilbert et al., 2006). Fetal bovine is serum that is commonly used in cell culture, has also been used to remove DNA from detergent that treated scaffolds due to the nucleases it contains (Gui et al., 2010).

It is essential to avoid immune mediated failure of allografts thorough decellularization of biological scaffolds. According to Meyer et al., (2006) have revealed that three alternate techniques compared to decellularize aortic valves: Triton X-100 in Tris buffers, osmotic based (hypotonic and hypertonic Tris buffers) and enzyme based (trypsin). The detergent based method gave near complete decellularization was found by them with maintenance of the ECM, whilst the detergent absence caused remaining of some cell material. Damage to the ECM is caused when the enzymatic method did remove the cells.

Using detergent methods are done to decellularize structures such as ligaments and tendons. In a study by Vavken et al., (2009) have found that the detergents Triton-X and SDS evaluated to form using trypsin. Reducing the content of DNA was found by all of their decellularization protocols and the levels of collagen or total protein did not affect. They observed some decrease in the amounts of GAGs, but using of the Triton-X regime was helped to minimise.

Quint et al., (2011) have been shown that a two-step process comprising of detergents and hypertonic solutions to decellularize human vascular tissue. The collagen matrix stayed undamaged and was able

to support the growth of a vascular graft by the analysis have been done for following decellularization.
In another study on vascular tissue, Grandi et al., (2011) have been used a detergent-enzymatic method. Decellularization of bovine vessels were done by using detergent and partially trypsinised then were cross-linked with poly (ethylene glycol) diglycidyl ether. An efficient way to prepare vascular scaffolds was done by this method that maintained a large amount of collagen and elastic fibres. Removing the HLA antigens had an advantage that happened by the provided mechanical of the subsequent cross-linking. However, detergent based cell removal from porcine derived scaffolds has been found by detergent based cell removal to destabilise the primary scaffold in the case of pulmonary valves (Naso et al., 2010) indicating that different techniques may suit different tissue types.

Proteases and certain detergents could make breaking and damaging the essential structure of the ECM can be done by Proteases and certain detergents and alternate methods have been identified. Gillies et al., (2011) have been studied various decellularization techniques on skeletal muscle. Techniques include sequential incubation of latrunculin B found in muscles, salt solution that contains great ionic force, and a solution of the enzyme DNase I was able to completely remove DNA, myofibres and the majority of contractile proteins (myosin and actin). There was no loss of collagen, and only a slight loss of GAGs, and the mechanical function was preserved and developed successfully. Recently Zhao et al., (2011) have been shown that a combination of the enzymes pepsin, DNase and RNase used to enzymatically decellularize their tissue followed by treatment with varying amounts of glutaraldehyde to form cross links and strengthen the structure. All the cellular material removed by to leave the structure of collagen behind. The crosslinking process helped the scaffold to resist enzymatic degradation. Implants were able to facilitate the development of endothelium as well as “smooth” muscle cells.

An increasingly common way of applying decellularizing chemicals osmotic buffers and enzyme solutions to the tissue were made by perfusion. This technique was used to decellularize the hearts and liver successfully (Ott et al., 2008; Uygun et al., 2010). The increased surface area of tissue that in contact with the decellularization solution was occured due to enabling perfusion thorough and rapid decellularization. According to Ott et al., (2008) have obtained that the perfusion of rat hearts generate the cell free scaffold was used by detergents and antibiotics. The structural components of the heart (collagens I and III, laminin, and fibronectin) were retained allowing the formation of a new organ that exhibited pumping activity. Using decellularized liver matrix was found to produce transplantable liver grafts in vitro (Uygun et al., 2010). The structure and function of microvascular system were preserved by Perfusion based decellularization that allowed the recellularization of liver matrix with hepatocytes obtained from adult. The resulting graft was showed that a liver functioned by recording cytochrome P450 expression, albumin secretion and urea synthesis (Uygun et al., 2010).

Tissue types such as vascular tissue can make difficulties with the decellularization procedure. A large radial expansion can be caused by the removal of the smooth muscle cells (Roy et al., 2008). The multifaceted composition and 3 dimensional ultra structure of the ECM can be preserved ideally; on the other hand all methods of decellularization could have resulted in some dgree of disruption to the architecture. The surface structure and composition can be damaged potentially by the disruption to the architecture. The tissue density and organisation of scaffold source have affected by any decellularization process, and the properties and potential use of the end product (Crapo et al., 2011).

1.5 Verification of Cell Removal

Histological analyses of the decellularized tissue samples are performed to confirm that all cellular material has been removed. Histological methods are employed with standard stains such as Hematoxylin and Eosin These identify any nuclear structures and find cytoplasmic and extra cellular molecules by another histological stains such as Safrin O and Masson’s Trichome can be used.

Staining sections of the tissue with the fluorescent stain DAPI had easily identified by DNA (Gilbert et al., 2006). For example, Fitzpatrick et al., (2010) took proximal and distal samples of their acellular arterial scaffold and compared this to untreated material. Sucrose saturation and embedding, areas are taken and blemished with Mayer's Hematoxylin and Eosin stains. Examine the sections were used by light microscopy to find any residual cellular components.

1.6 Tissue Regeneration

Generated ECM acts as the basic structure to grow a new organ. Stem cells are taken from patients to produce new organ that are allogenic. As result, in an allogenic organ that is produced will not trigger an immune response when implanted. Generation of allogenic tissue is made through the recipient's own cells that being used to impregnate the decellularized support. The cells that act as a support for the growth of new tissues can be supplied directly from the recipient when adult stem cells are appropriate or therapeutic cloning is used. In this approach taking a patient's cells or its nucleus and transferring it into a denoted egg cell, with an individual nucleus, carry out therapeutic cloning and this procedure is referred to as "Somatic Cell Nuclear Transfer (SCNT)". A blastocyst formed from the cultured egg, from which the internal layers of cells are extracted. Generating new tissue can be used from providing stem cells (Yang and Smith, 2007). This method produces pluripotent stem cells, which have the ability to develop into any type of cell upon proper induction. Repairing damaged tissues and grow new organs can be done by using embryonic stem cells. For example, Damaged tissues have been repaired with the use embryonic stem cells (Hall et al., 2010) and to produce new liver cells from a patient's skin cells (Rashid et al., 2010).

Adult stem cells can be used in some situations and these can be indentified within human bone marrow, brain tissue and heart of mature adults to maintain and repair the tissue (Körbling and Estrov, 2003). However, some types of stem cells wield the capability to distinguish into cells types different than those predicted from their source. This makes these stems cells potentially useful (Körbling and Estrov, 2003).

Scaffold that develops cell can be either static or through perfusion (Solchaga et al., 2006). Most commonly, it is used static seeding and the process involves the suspension of the scaffold into a solution that contains cells. Incubation of the scaffold in the cell solution in order to allow the cells to adhere is carried out and subsequently the scaffold is washed carefully. Conversely, perfusion techniques have been used to grow human dermal fibroblasts on a scaffold that is made of a collagen-chitosan and uses perfusion system (Chun-Mei et al., 2008). Scaffold using perfusion provides increased efficiency and increased distribution. Increased distribution of cells throughout the scaffold is thought to increased cell proliferation, which results in better homogeneous morphology of the produced tissue (Chun-Mei et al., 2008).

Allogenic tissues of human blood vessels were grown from smooth muscle cells that were supported on a biodegradable scaffold of ECM proteins (Quint et al., 2011). Decellularized structure formed was later inserted into a rat model as an aortic graft. The engineered vascular structure had similar mechanical properties to that of a human vein and functioned successfully as a graft. Further analysis of the implanted graft demonstrated that the remnant collagen fibres stayed intact and the elastin formed. Cardiovascular vessels have been grown using human umbilical arteries as the scaffold (Gui et al., 2009). Complex anterior cruciate ligaments (ACLs) have been grown from human ACL fibroblasts and seeded onto porcine scaffolds. (Vavken et al., 2009).

1.7 Skin Composition

The skin is a large organ composed of protein, lipids, minerals and water. Skin comprises of three layers: the epidermis, the dermis and the subcutis. The epidermis consists of four layers. The top layer has hardened, flattened dead cells that make the skin surface. These lie on top of larger living cells that contain the next three layers known as the stratum germinativium. Just below the hard outer layer

are found the squamous cells and the bottom basal layer produces the new cells. As new cells grow and push upwards towards the surface they transform from soft columnar cells to become flatter in shape with reduced water content due to dehydration and pressure. The epidermis contains three different types of cells: the keratinocytes that produce keratin, the melanocytes that produce melanin, and the Langerhans cells that play a role in immunity (Amirlak, 2011).

The dermis is a bulky and flexible network of connective tissues. It consists of the papillary dermis that lies under the epidermis and contains a subepidermal band of connective tissue below the basal lamina. The reticular dermis has superficial and deep zones that can be distinguished through the fibrous connective tissue is organised. Collagen and elastin form a matrix that sustains lymph and blood vessels, muscle cells, hair follicles amongst others. It is in dermal layer that fibroblasts involved in the synthesis of collagen and elastin are found. The skin gains its elasticity properties and tone and texture from elastin and collagen. The GAGs facilitate the retention of water (Amirlak, 2011).

1.8 Changes in Dermal Collagen due to Ageing

The aging of skin is resulted in changing of dermal collagen, elastin and glycosylaminoglycans. Collagen and elastin start to get worse as we age. Collagen becomes cross-linked in an enzyme-mediated process, and increasingly glycated both of which results in it becoming solid (Bailey, 2002). A reduction in the stretch of the skin/tissue caused by deterioration of elastin , as its elasticity is lost. Organisation of the collagen fibres and the overall dermal structure can be affected by the reduction of elastin (Holbrook et al., 1982).

Structures of the collagen and elastin fibres alter with age there is a reduction in the skin's space causing the fibrous components to become compacted. This results in decreasing in the width of the collagen bundles (Lavker et al., 1989; Lavker et al., 1987). With decline in collagen synthesis as age progresses and subsequent reduction in collagen content of the skin and becomes more disorganised in nature (Uitto et al., 1989; Oikarenen, 1994), changes are also found in the relative proportions of collagen types with the amount of type III collagen in the skin reducing relative to the amount of type I collagen (Mays et al., 1988). This is different to internal tissues such as heart and lung, and may be a result of the skin's environment.

1.9 Picosirius Red

When using combination with polarised light picrosirius red (PSR) can be used to selectively stain collagen. The collagen fibres will appear bright yellow or orange and the thinner (younger) fibres as green. Thus this compound enables the differentiation to be made between newly-made, maturing and mature collagen as the different states reflect plane polarised light in a different manner. This bi-refringence is highly specific for collagen (Junqueira et al., 1979).

1.10 Effect of Decellularisation on Collagen

Depending upon the technique and tissue type adopted, decellurisation treatment can cause disturbance in the ECM. Examples include employing trypsin changes collagen structure and as a result reduced the content of fibronectin and elastin (Schenke-Layland et al., 2003; Gilbert et al., 2006). In addition, cell removal from pig scaffold using detergent based technique causes the destabilization of the scaffold (Naso et al., 2010).

(Chapter 2)

1.0 Aims

Collagen, one of the most abundant proteins in the body, is the predominant fibrous protein in the extracellular matrix.

- This study aims to identify the presence of collagen in various tissues that can undergo in decellularization process.
- Quantify the amount of new, immature and mature collagen present to assess the effect of

decellularization on the different forms of collagen.

2.0 Materials and Methods

2.1 Equipment and chemicals

Slides of aorta, tendon and carotid artery both before and after the decellularization process were given from Dr Ian Kill.

2.2 Image Acquisition

- Images were using by a Zeiss Axioskop II light microscope fitted with an AxioCam. Images were processed using Zeiss Axiovision software. A condenser lens and a polarized light filter were used to capture images at 40x magnification. After a manual focusing the images were recorded digitally for subsequent analysis.

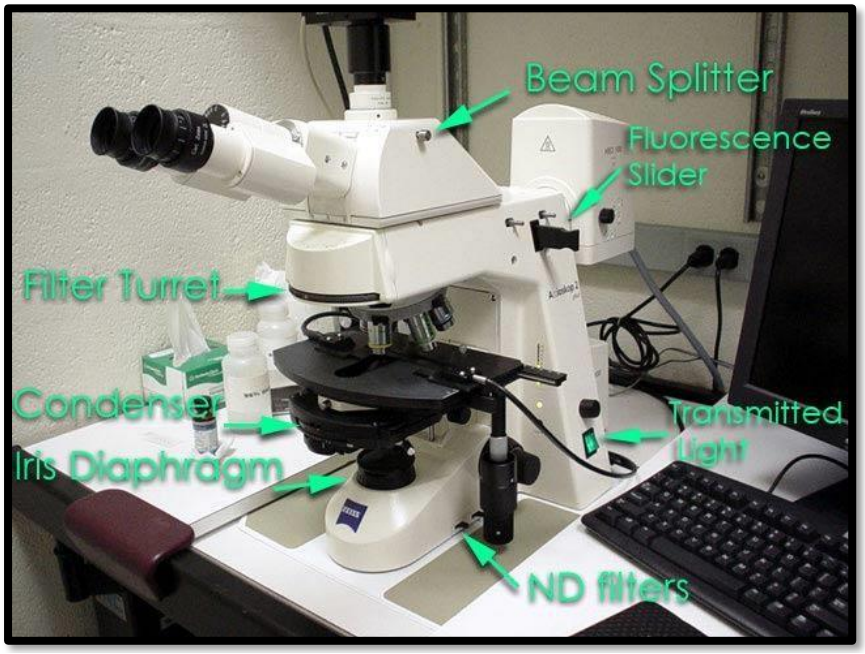


Figure 1: The image above shows Zeissa Axioskop II light microscope, which was adapted during the practical investigation. The image is found in http://www.einstein.yu.edu/aif/instructions/zeiss/axioskop2/index.htm

2.3 Image Processing

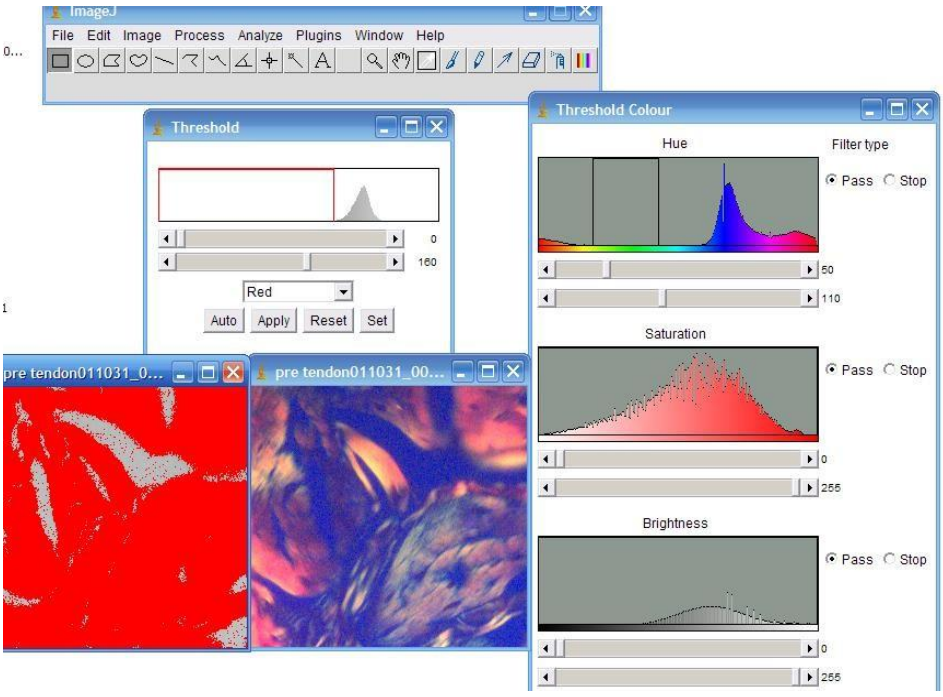
J software was used to process the obtained images and in turn to identify, quantify and measure the maturity of the collagen. The different forms of the collagen were identified with the use of Picrosirius red (PSR). Newly formed collagen appears as a green, however maturing collagen tissues appears as yellow and mature ones as red. The procedure was as follows

Using Image J software to calculate the total amount of collagen carried the analysis of the bright field image and all forms of collagen appeared as red in the bright field view in PSR. Each individual types of collagen that were present were measured through the analysis of the obtained images with the polarized light filter. Every individual collagen is colored in a different way in the polarized light and subsequently each form of the collagen was measured through segmentation of the image using colour-based thresholding.

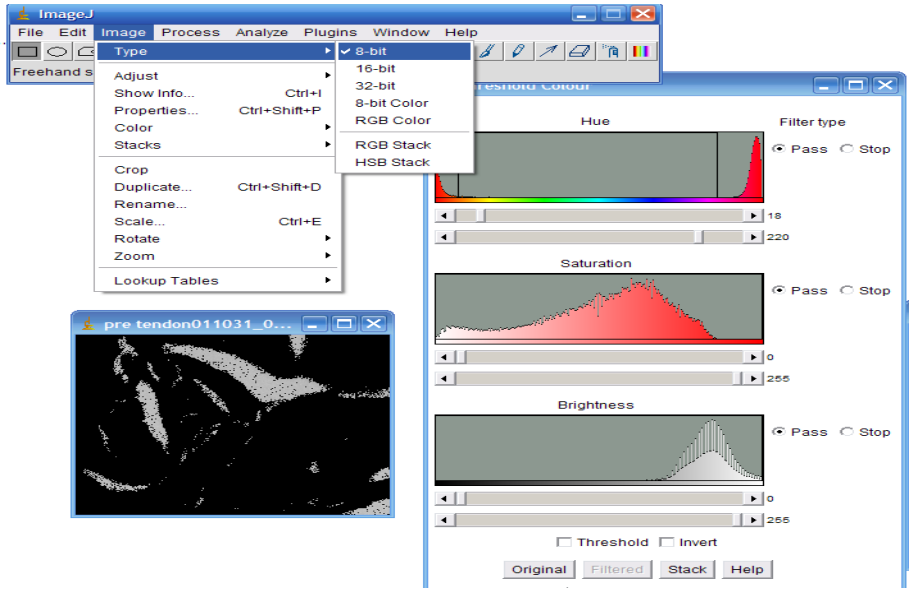
The observed colours of collagen fibres are different upon staining with the PSR in a polarized light and the detected colour is heavily dependent on the thickness of the collagen fibre (Hiss et al., 1988; Junqueira et al., 1982). With increasing thickness of the fibre, it can be observed changes of the colour from green to yellow to red consecutively. Resolving the segmented images into its hue concluded the amount, saturation and value components of the different coloured of collagen fibres. The hue of

each pixel within the subtracted image is obtained (Junqueira et al., 1982). A graph that shows the "hue frequency was obtained from the 8-bit hue images". The numbers, which were used, are the following: red 0 – 25 and 225 – 255, yellow 26-50 and green 50-110. Applying colour threshold was particularly important as it allowed the isolation of fibres from different hues. "The number of pixels within each hue range was determined and expressed as a percentage of the total number of collagen pixels" (Junqueira et al., 1982).

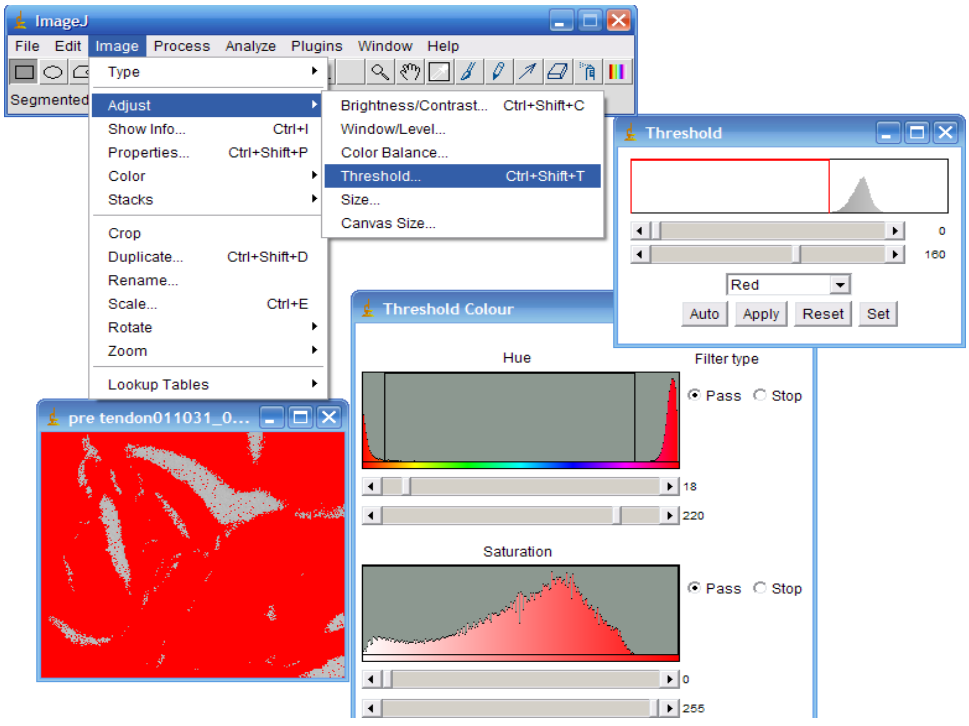
The digital images were segmented into many sets of pixels for analysis. Segmenting the image give a label to every pixel meaning, each pixel with the same label has the same visual characteristics. Here segments were defined as being comprised of similar pixels with respect to the colour. This allows the image analysis software to calculate the amount of pixels in a segment and therefore the amount of a collagen form (Rugesan and Palaniswami, 2010).
Once the wanted image (using pre tendon for demonstration) was opened, the first step was to select plugins on the top tool bar. This was followed by selecting segmentation, the first and most important action involved in image analysis and pattern recognition. Image segmentation entails the dividing of the image into distinct region, which are supposed to strongly correlate with features of interest in the real image (Gonzales and Woods, 1993). Each region is homogenous in that they are their pixel attributes e.g. intensity, colour and texture are similar uniform. The pixels can then be clustered together in accordance to these attributes, such as grey levels in a process termed thresholding. This is the simplest method of segmentation; hence the next step being the selection colour based thresholding. The threshold is colour based as the images are in colour. This opened a window that gave sections called Hue**,** Saturation**,** and Brightness.



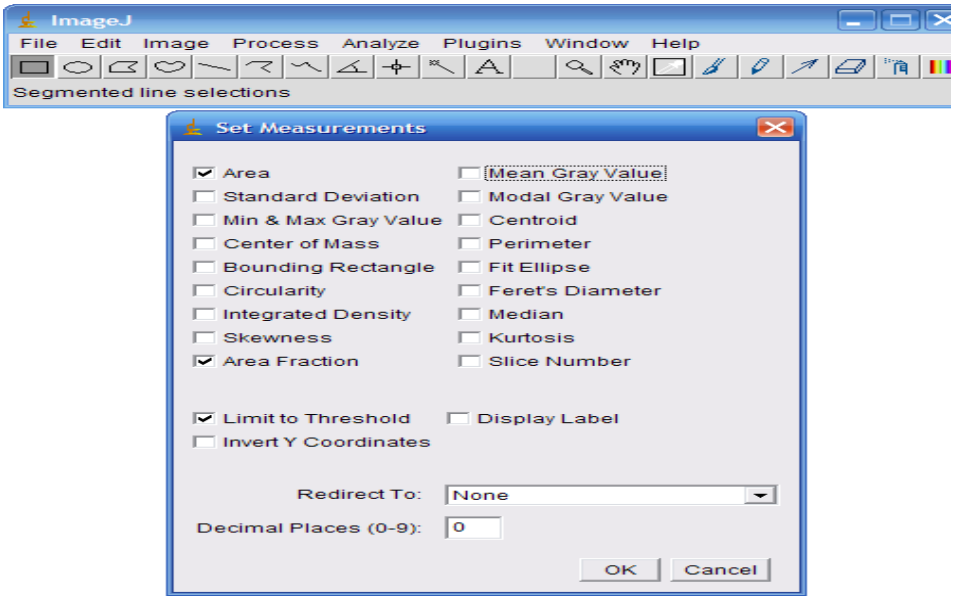
After selecting image from the top tool bar, the type submenu was selected and after that 8-bit. As the previous final thresholded image type was HSB, selecting 8- bit allows conversion of this image into a format that enables analysis. At this stage the image was no longer in colour.



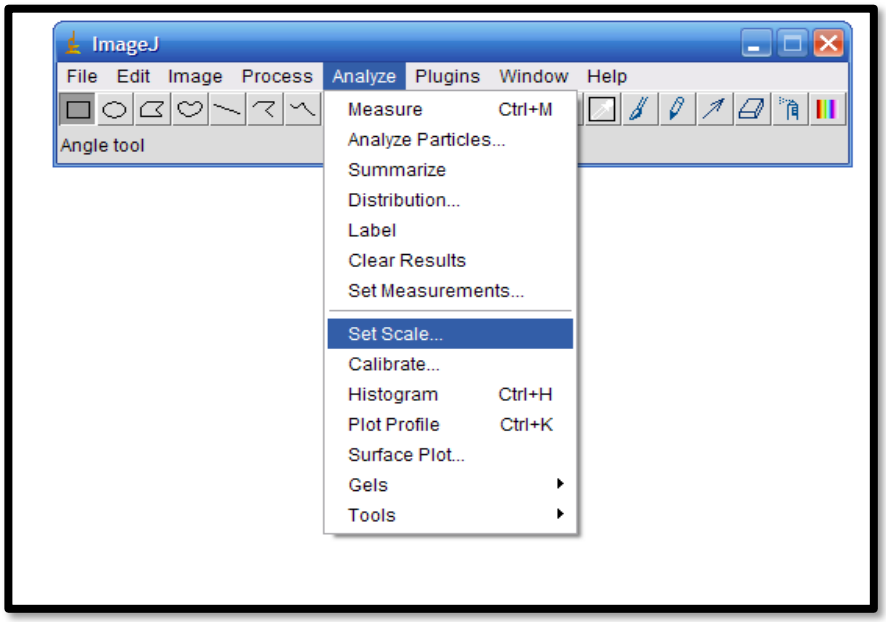
Subsequently, the top tool bar was used to choose the images. Adjust was selected with the subsequent selection of threshold. The threshold tool allowed adjustment to be made and it was employed to set lower and upper threshold values. This allows section of the image to be placed into features of relevance and setting. As seen below, the threshold features "pixels with brightness values greater than or equal to the lesser threshold and less than or equal to the upper threshold" exist in red, however the background is revealed in gray.

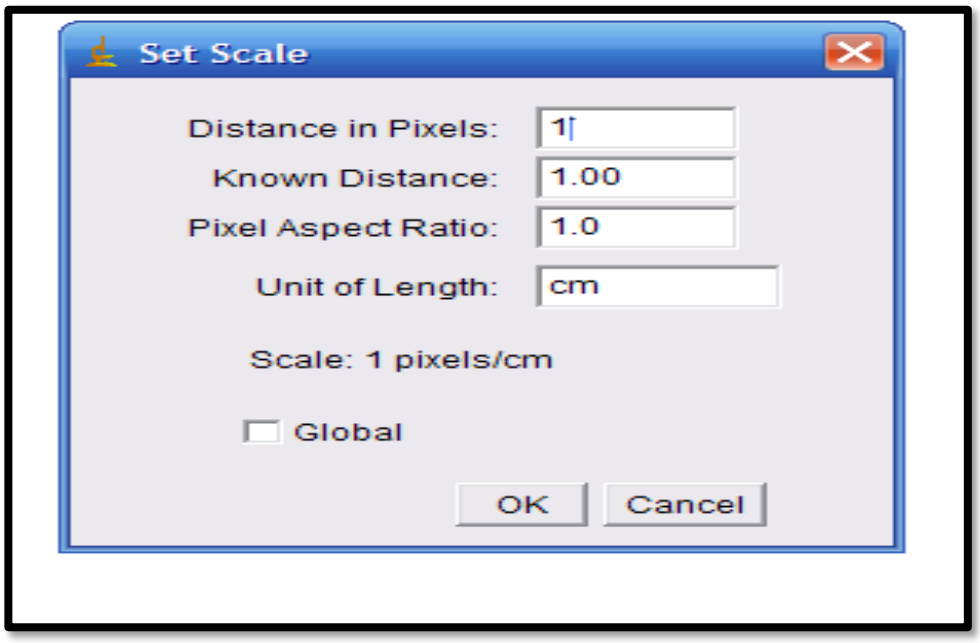


The 'Set Measurements' menu allows the area of selection to be set as square pixels or cm2. In order to specify the recorded measurements, Analyze followed by set measurement was used. After that, the Area box, representing the area of selection in square pixels was checked. The Area Fraction box, representing the percentage of pixels that have previously been highlighted in red was also selected. Within the threshold boundary was set to allow only threshold pixels to be included in the measurement calculations.

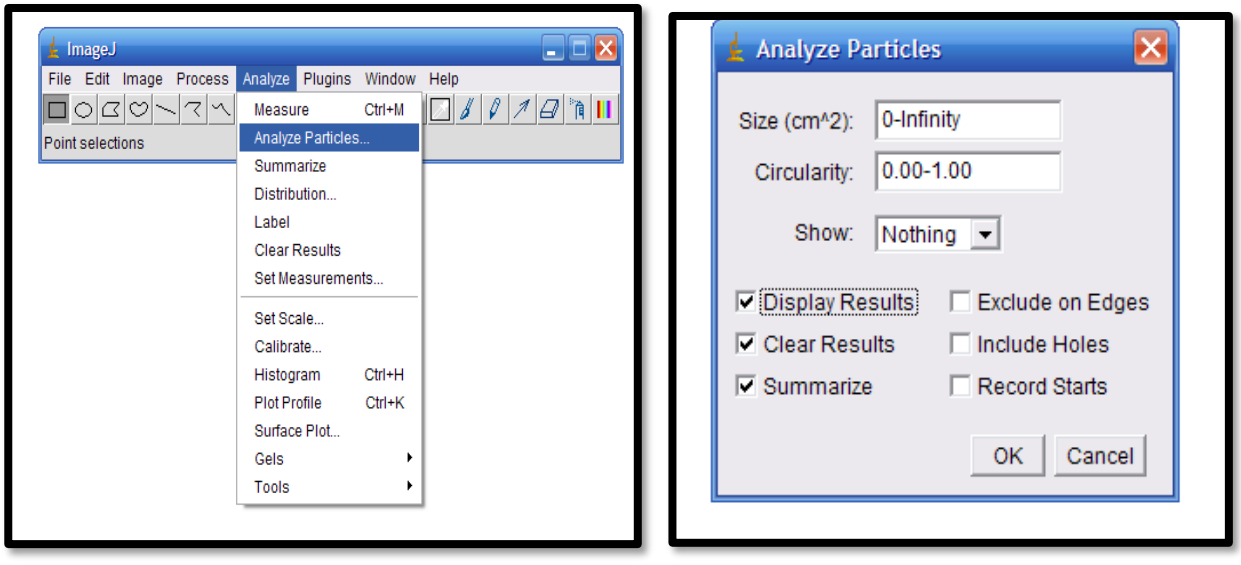


Selecting analyze on the top tool bar, then set scale allowed was done so the measurements would be given in calibrated units. The spatial scale of the image was recorded in calibrated units (centimetres) via the 'Set Scale' command. The length of the scale bar was measured and entered to spatially calibrate the image. This allowed the number of pixels per known distance to be measured (pixel aspect ratio) so defining the pixels per cm^2.

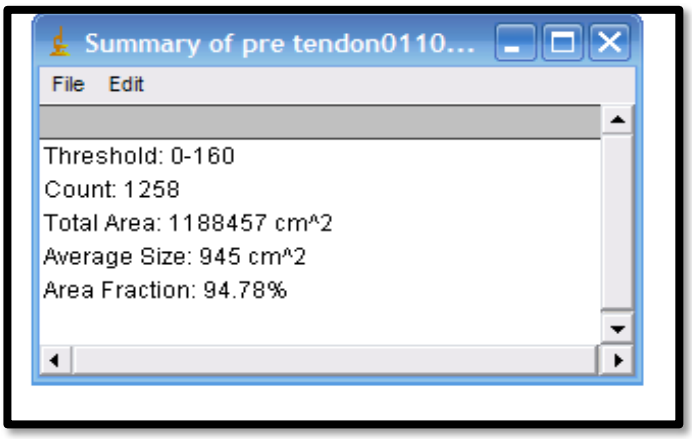




The quantification of the segmented collagen was performed through the 'Analyze' function. Analyzing particles enabled the measuring and counting of features individually. This happens by the scanning of the image and outlining the edge of the defined object (ImageJ, 2011). The analyze option was selected at the top tool bar and then analyze particles.



The final steps involved simply selecting display results in order to obtain the measurements for each particle, clear results to wipe and prior measurements and summarize. The fragment count, total area and average fragment size as well as area fraction was concluded in a segregated window. These sections are examined by pressing the 'Analyze' menu to obtain the area fraction, which is displayed in percentage, of the pixel in the picture that are beyond the threshold value.



2.4 Processing of the Data

The analyzed image data was inserted into Excel (Microsoft) to calculate the average of the amount of the collagen in each tissue (pre- and post-treatment). The amount of the collagen present was calculated as a percentage of the area of each image, which was expressed in pixels. The method of analysis is in accordance to the methods described by Rich and Whittaker (2005) (e.g. (new collagen / total collagen) x 100).

Standard deviations of the samples were calculated to illustrate the variance in the amount of collagen per sample. The effects that fall outside the range, which is defined by the standard deviation, are considered statically significant (Campbell, 1989). Statistically, this allows random errors or variance in the sample to be overlooked.

Independent two-sample t-test statistical analysis was carried out to assess if there is significance discrepancy in the amount of collagen that was present before and after treatment. The more significant the difference between the two groups, the larger the t-ratio (i.e. the closer to 1) (Campbell, 1989). The P value is found by statistically calculating the t-values for each collagen type per tissue and referring to statistical table (Statcalc3, 2011). The P value was established by using a two-tailed approach since the extent of change was not known. P values demonstrate the chance of receiving the same mean for each group under random sampling methods. Consequently, the smaller the P value obtained, the more significantly different the two groups are (Campbell, 1989).

(Chapter 3)

3.0 Result:

3.1 Presence of Total Collagen

The structural protein collagen is abundant in the human body found in structural tissues such as the skin, tendons and vascular tissue. Procollagen is secreted by fibroblasts and folded allowing interchain disulphide bonds to form that trigger the formation of the collagen triple helix (Canty and Kadler, 2002). This specialized helix undergoes a series of post-translational modifications to give rise to the mature collagen fibres. By staining with picrosirius red (PSR) new (green), immature (yellow) and mature (red) collagen can be differentiated based upon the colour when viewed using polarised light. Here examined decellularised collagen matrices from various tissues stained with PSR.

PSR has been used in the brightfield to detect collagen (e.g. Ophof et al., 2004). In the brightfield thinner fibres appear bright pink and can be difficult to see leading to underestimation of collagen content (Rich and Whittaker, 2005). Here the amount of collagen in the PSR stained samples differed between tissue types as detailed below:

Examples of captured images: (1)

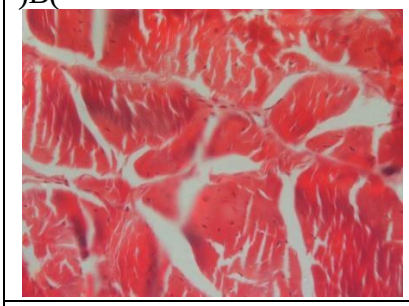
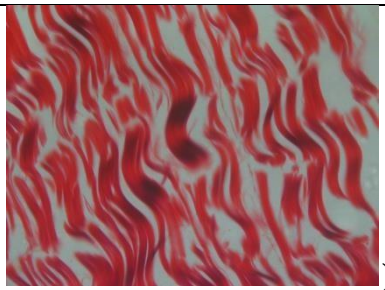
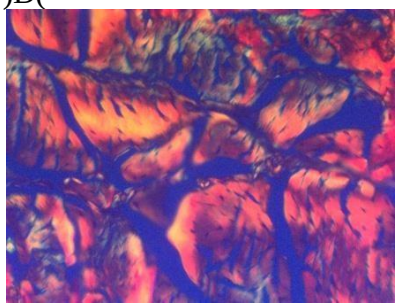
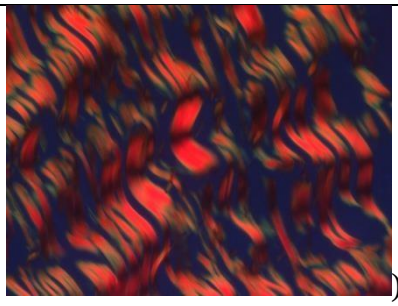
Pre tendon	**Post tendon**	)1(
)B(	)A(	Bright image
)D(	)C(	Polorized light image

Figure 1) A sample of tendon is staining with Picosirius red. Top row are the bright field images: (A) Cross-section showing the total collagen content as stained red. (B) The long collagen fibres can be seen as parallel strands. Bottom row are the birefingent collagen as shown with circularly polarized light: (C) Mature collagen is red, immature as yellow and new as green with other cellular material / gaps in blue. (D) Showing the longditudinal view the new and immature forms of collagen can be seen located at the ends of the collagen strands.

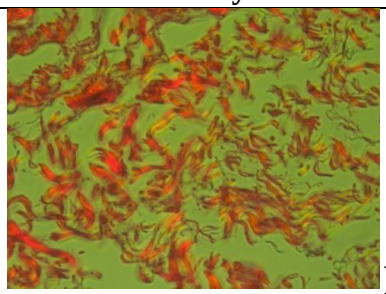
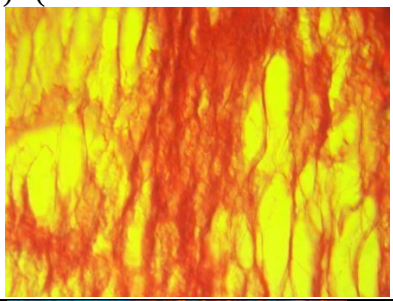
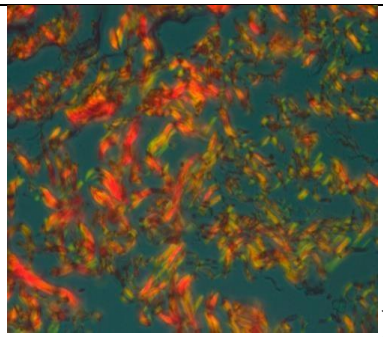
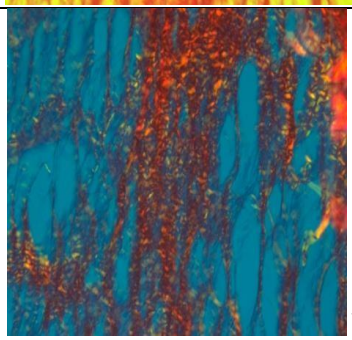
Pre carotid artery	**Post carotid artery**	)2(
)B(	)A(	Bright image
)D(	)C(	Polorized light image

Figure 2) A sample of arterial stained with a picosirius red stained. Top row shows the brightfield images: (A) Original brightfield image showing all forms of collagen stained as red. (B) Section of

the artery showing collagen as red and smooth muscle cells as yellow. Bottom Row shows the circularly polarized light images showing birefringent collagen: (C) Image after colour separation. Mature collagen appears as red, immature as yellow and new as green. (D) The section of the carotid artery showing the collagen between the muscle layers (blue).

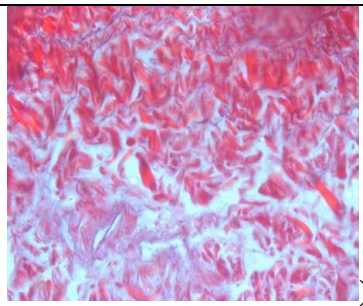
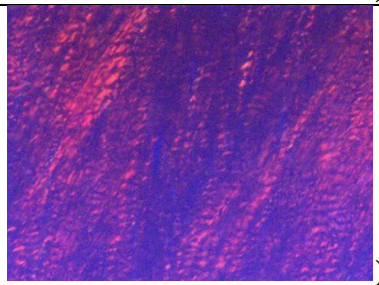
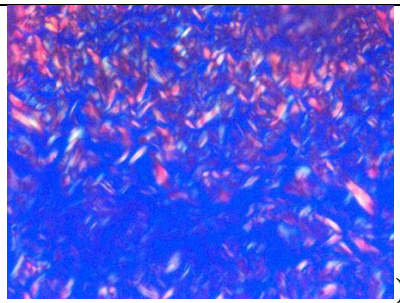
Pre aortic	**Post aortic**	)3(
)B(	)A(	Bright image
)D(	)C(	Polorized light image

Figure 3) Sample of Aorta stained with Picosirius red. Top row are the brightfield images: (A) shows a part of the aortic wall abundant with collagen stained red. (B) The cross section of the aorta shows collagen as the extracellular matrix between cells. Bottom row shows the birefringent collagen: (C) Removal of the background colouring shows the distribution of the collagen more clearly. (D) The aorta shows amounts of new and immature collagen to be present in the extracellular matrix.

3.2 Tendon Results

Ten images from two section of tendon both before and after decellularization were examined in accordance with the methods described above. Images were captured with the polarized light filter. By segmentation of the resulting images the presence of the three types of collagen (newly made, immature and mature) were quantified:

Table of pre tendon results

Image	Collagen	Red	Yellow	Green
1	94.8%	23.79%	1.03%	0.27%
2	88.12%	43.46%	0.36%	0.16%
3	64.31%	14.35%	0.54%	0.03%
4	96.71%	39.63%	0.72%	0.49%
5	93.13%	8.65%	1.67%	0.7%
6	94.16%	29.08%	1.18%	0.11%
7	94.99%	42.79%	0.19%	0.3%
8	96.4%	25.32%	0.97%	0.35%
9	83.24%	8.69%	1.01%	0.2%
10	93.73%	40.3%	0.03%	0%

Table of post tendon results

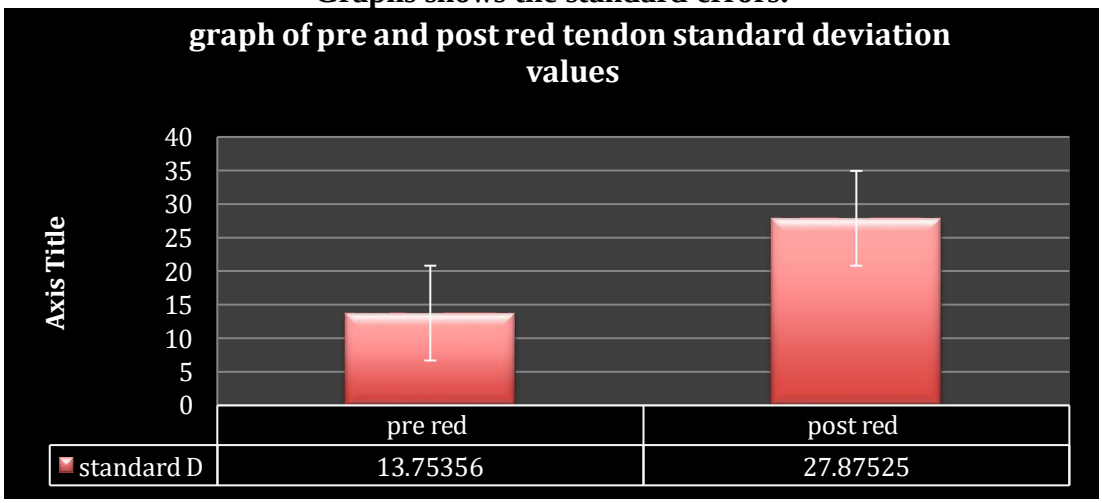
Image	Collagen	Red	Yellow	Green
1	81.33%	20.1%	1.7%	0.1%
2	93.27%	71.28%	0.1%	0%
3	71.02%	29.89%	0.5%	0%
4	90.12%	68.48%	0.5%	0.4%
5	61.93%	19.44%	3.3%	0.4%
6	80.94%	82.07%	3.9%	0%
7	70.73%	71.28%	1.8%	0.4%
8	78.69%	83.12%	0.2%	0%
9	91.87%	98.74%	0.1%	0%
10	60.59%	62.28%	1.5%	0.5%

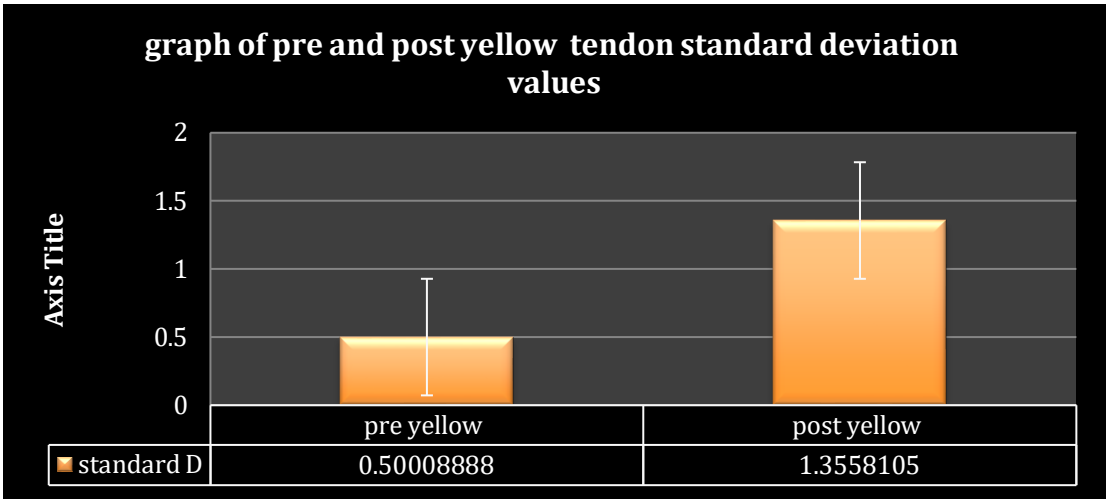
Tendon Image	Pre red	Post red	Pre Yellow	Post yellow	pre green	Post green
1	23.79	20.1	1.03	1.7	0.27	0.1
2	43.46	71.28	0.36	0.1	0.16	0
3	14.35	29.89	0.54	0.5	0.03	0
4	39.63	68.48	0.72	0.5	0.49	0.4
5	8.65	19.44	1.67	3.3	0.7	0.4
6	29.08	82.07	1.18	3.9	0.11	0
7	42.79	71.28	0.19	1.8	0.3	0.4
8	25.32	83.12	0.97	0.2	0.35	0
9	8.69	98.74	1.01	0.1	0.2	0
10	40.3	62.28	0.03	1.5	0	0.5
Average	27.606	60.668	0.77	1.36	0.261	0.18
Standard Deviation	13.75356	27.87525	0.50008888	1.3558105	0.214188	0.214994
Student t-test	0.00502		0.22223176		0.40973	

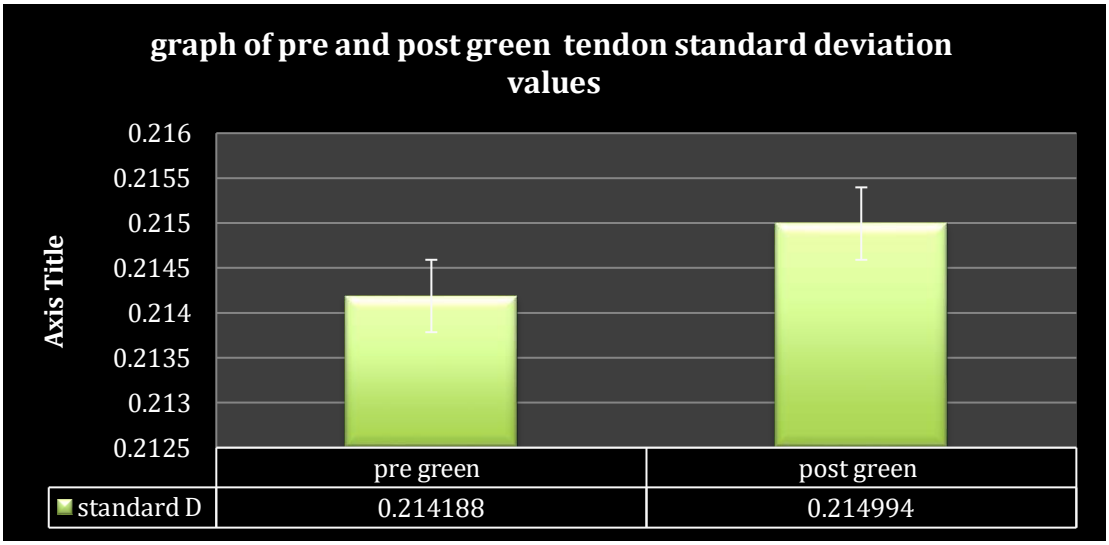
From the above table, it is evident that the p value for pre and post red is the only value that is statiscally significant where a statistically significant value would be $p \leq 0.05$. As a result, it can be accepted that the result were not due to chance and the null hypothesis that there is no difference between the two variables is to be rejected. . As for pre and post yellow and green, the null hypothesis is to be accepted.

The standard deviation (i.e. amount of variance in the samples) both pre and post processing are displayed as graphs below.

Graphs shows the standard errors:







3.3 Aortic Tissue

A sample of aortic tissue was then analysed in a similar manner to that described above for the tendon and the results are shown below:

Table of pre aortic results

Image	Collagen	Red	Yellow	Green
1	100%	7.91%	0.01%	0.000239%
2	100%	0.51%	0.000398%	0
3	100%	10.63%	0	0.000239%
4	100%	30.63	0.01%	0.000478%
5	99.99%	6.18%	0.000319%	0
6	99.95%	11.24%	0	0
7	96.55%	42.79%	0	0

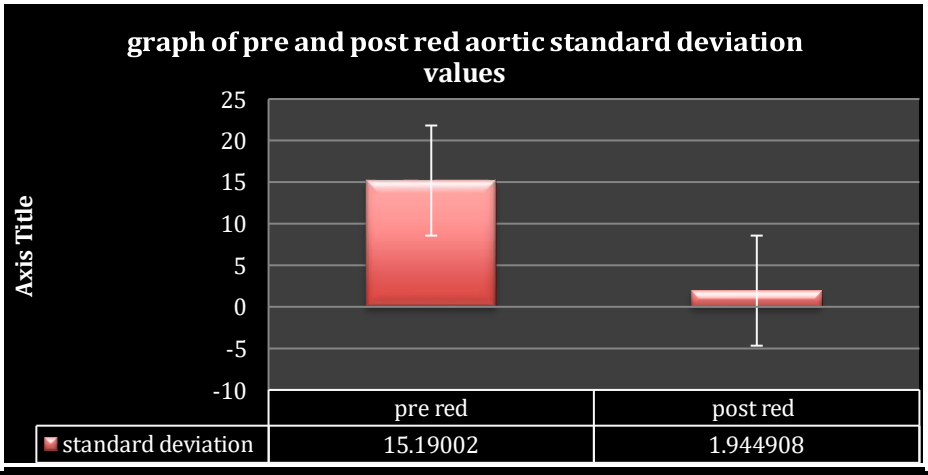
Table of post aortic results

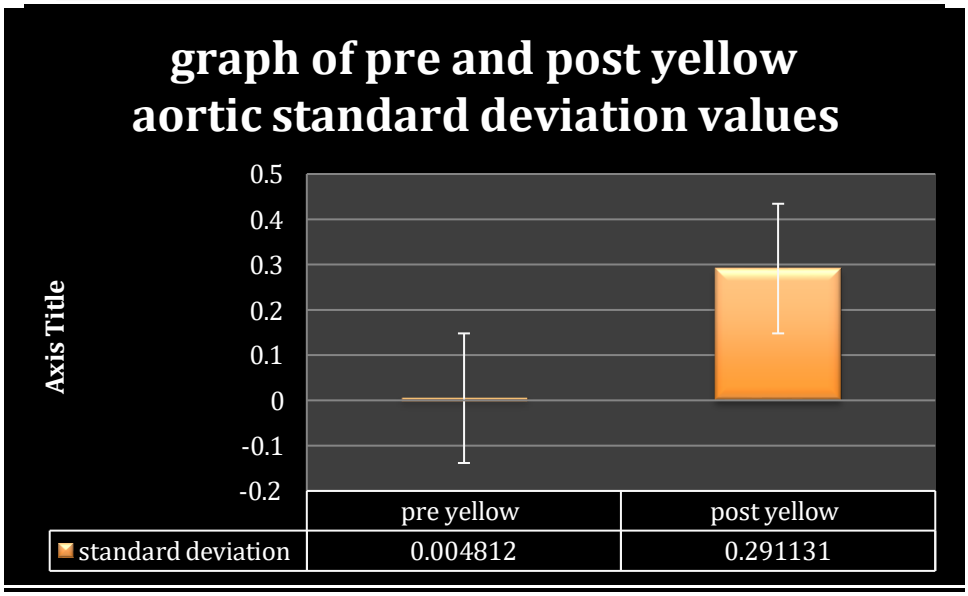
Image	Collagen	Red	Yellow	green
1	16.83%	2.31%	0.02%	0.01%
2	31.03%	0.54%	0.07%	0.04%
3	2.07%	1.93%	0	0.01%
4	77.39%	1.05%	0	0
5	14.13%	4.1%	0.8%	0.03%
6	13.6%	6.25%	0.05%	0.01%

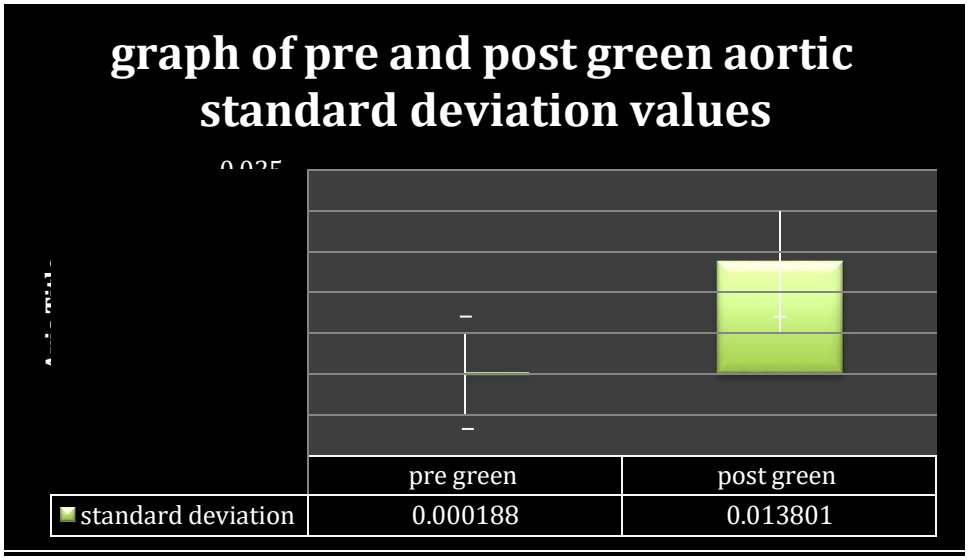
7	48.36%	2.72%	0.06%	0.02%

Aortic Image	Pre red	Post red	Pre Yellow	Post yellow	Pre green	Post green
1	7.91	2.31	0.01	0.02	0.000239	0.01
2	0.51	0.54	0.000398	0.07	0	0.04
3	10.63	1.93	0	0	0.000239	0.01
4	30.63	1.05	0.01	0	0.000478	0
5	6.18	4.1	0.000319	0.8	0	0.03
6	11.24	6.25	100	0.05	0	0.01
7	42.79	2.72	0	0.06	0	0.02
Average	15.6985714	2.7	14.2886739	0.14285714	0.00013657	0.01714286
Standard Deviation	15.19002	1.944908	37.79514	0.291131	53.45208	0.013801
Student t-test	0.064432		0.360303		0.017243	

From the above table, it is evident that the p value for pre and post green is the only value that is statistically significant where a statistically significant value would be $p \leq 0.05$. Therefore, it can be accepted that the result were not due to chance and the null hypothesis that there is no difference between the two variables is to be rejected. As for pre and post yellow and red, the null hypothesis is to be accepted







3.4 Carotid Artery Results

In addition to the aortic samples, further vascular tissue was examined. Slides of carotid artery were stained and analysed:

Table of pre carotid artery results

Image	Collagen	Red	Yellow	Green
1	69.55%	31.28%	0.4%	0.56%
2	65.55%	114.79%	1.38%	2.5%
3	16.61%	241.11%	0.49%	0.48%
4	28.65%	123.73%	0.86%	2.28%
5	100%	24.09%	0.61%	0.49%
6	51.06%	11.98%	0.35%	0.33%
7	14.46%	53.04%	0.35%	0.4%
8	55.36%	27.13%	0.4%	0.42%
9	75.57%	29.42%	0.39%	0.6%
10	18.49%	171.98%	17%	17.08%

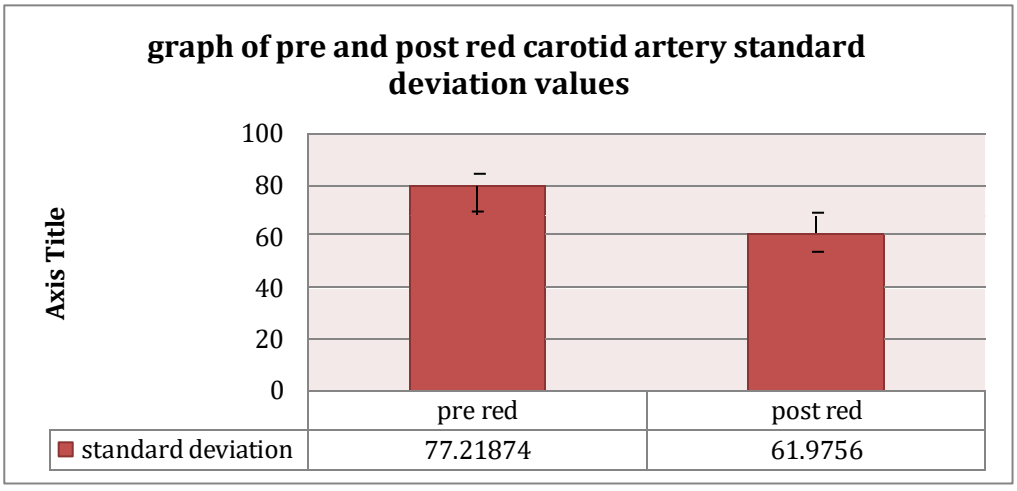
Table of post carotid artery results

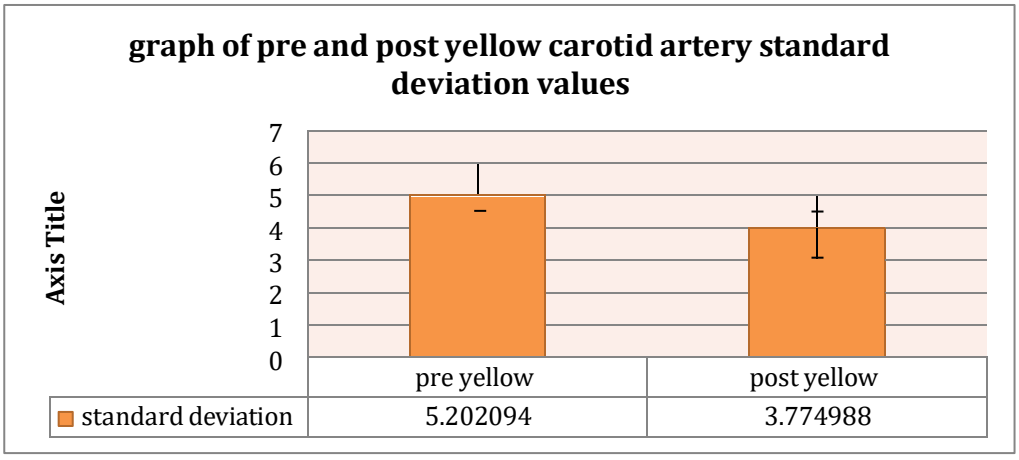
Image	Collagen	Red	Yellow	Green
1	67.86%	122.25%	2.05%	0.92%
2	52.35%	102.34%	3.36%	4.97%
3	36.09%	74.7%	1.65%	1.8%
4	28.34%	72.97%	13.23%	8.9%
5	2.67%	9.73%	0.03%	0.13%
6	12.06%	73.13%	2.02%	2.32%
7	8.76%	26.59%	0.56%	0.82%
8	5.88%	63.43%	1.28%	2.1%
9	99.89%	1.76%	1.55%	2.15%
10	5.61%	214.616%	3.24%	13.91%

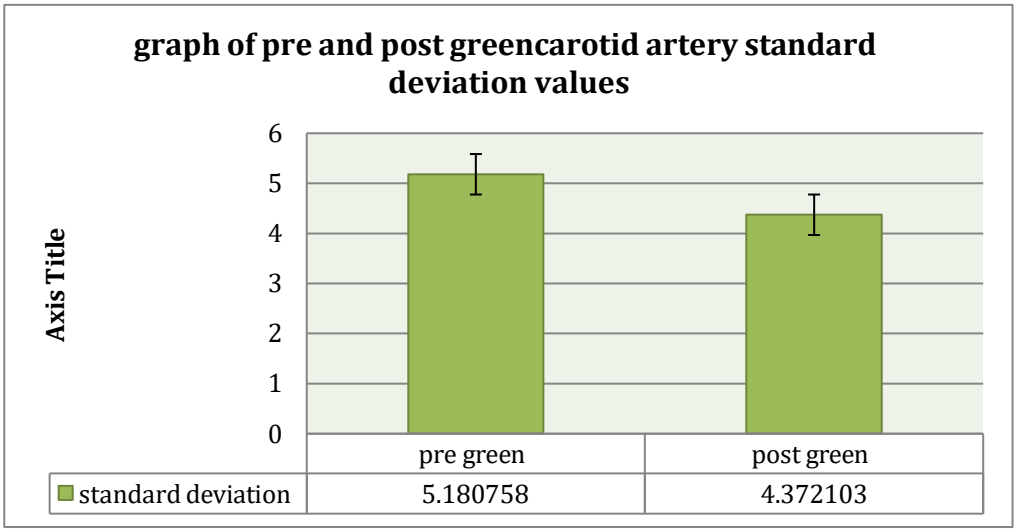
Carotid artery image	Pre red	Post red	Pre Yellow	Post yellow	pre green	Post green
1	31.28	122.25	0.4	2.05	0.56	0.92

2	114.79	102.34	1.38	3.36	2.5	4.97
3	241.11	74.7	0.49	1.65	0.48	1.8
4	123.73	72.97	0.86	13.23	2.28	8.9
5	24.09	9.73	0.61	0.03	0.49	0.13
6	11.98	73.13	0.35	2.02	0.33	2.32
7	53.04	26.59	0.35	0.56	0.4	0.82
8	27.13	63.43	0.4	1.28	0.42	2.1
9	29.42	1.76	0.39	1.55	0.6	2.15
10	171.98	214.616	17	3.24	17.08	13.91
Average	82.855		2.223		2.514	
Standard Deviation	77.21874	61.9756	5.202094	3.774988	5.180758	4.372103
Student t-test	0.832994		0.744383		0.555658	

From the above table, it is clear that the p values obtained from the student t test was insignificant for pre and post red, yellow and green, as a statistically significant result would be $p \leq 0.05$. Thus, it can be accepted that the result were due to chance and the null hypothesis that there is no difference between the two variables for all of them is to be accepted.







(Chapter 4)

4.0 Discussion

Collagen based scaffolds are becoming increasingly used within medical applications. Decellularization is the process of removing the cellular components from the ECM of a tissue whilst preserving its structural and mechanical properties (Parenteau-Bareil et al., 2010). As has been demonstrated in Chapter 1, there are a variety of approaches to decellularization which these can involve "chemical, physical and biological methods" (Gilbert et al., 2006). Through the removal of the cellular material, the antigenicity of the tissue can be reduced and the remaining structure therefore offers a three dimensional scaffold within which the recipient's own cells can proliferate (Parenteau-Bareil et al., 2010).

Different decellularization methods remove the cellular content with varying degrees of success. Whilst reducing the antigenicity of the graft, the techniques must "preserve the integrity of the ECM" (Fitzpatrick et al., 2010). SDS and other commonly used agents are good at removing cellular content but can also result in disintegration of the ECM (Kasimir et al., 2003). For the purpose of this study, 3 tissue samples have been analysed: tendon, aorta and carotid artery - before and after decellularization. The principle component of the ECM in these tissues is collagen, which is a protein that provides structure and strength; which means, as a result, that decellularization must not damage it if the mechanical properties are to be preserved. The amount and composition of the collagen was therefore assessed pre- and post-decellularization.

4.1 Tendon

The first tissue that is to be examined is the tendon. The tendons are strong, flexible and display a high level of elasticity. These properties are due to its composition: "structural proteins and little cellular content" (Di Lullo et al., 2002). Decellularization should not affect the collagen, therefore it was anticipated that post treatment the tendon would have a large amount of PSR stained material. This was observed to be the case with large amounts of collagen being seen in both before and after treatment (see fig. 1, Chapter 3).

The analysis of the tendon showed that the amount of mature collagen (red) had increased significantly by the decellularization process. This may be due to the nature of the material as tendon is composed mostly of parallel arrays of collagen fibres. Typically, the dry mass of a tendon is about 86 % collagen while the tendon collagen content of rats has been measured as 0.51 mg collagen per mg of tendon (wet weight) (Whitt et al., 2010). The removal of the additional cellular material allowed for more of the collagen to be accessed by the dye. There was no significant change in the amount of immature (yellow) or new (green) collagen observed in pre to post treatment. Whilst the mature form of collagen predominates in tendon, tenocytes are observed to synthesise collagen III and fibroblasts

collagen I following injury (Lin et al., 2004). Consequently, in areas of the tendon recovering from damage some younger collagen may be found.

The decellularization process did not cause a harmful effect on the collagen content of the tendon. In addition to the increase in the quantity detected the images showed that the strands of collagen within each cluster retained their orientation (parallel array) before and after the treatment (fig.1, chapter 3). The mature form of collagen in thick bundles may enhance its ability to resist degradation by the chemicals involved in decellularization. Its structure predisposes it to be a useful biological scaffold: able to withstand treatment without damaging the quality of the collagen.

4.2 Aorta

Aortic tissue contains less collagen in relation to tendons, because these are composed of smooth muscle, nerves, intimal, endothelial and fibroblast-like cells as well as the extracellular matrix. The collagen and elastin that composes the extracellular wall of the aorta provide mechanical support. The collagen content of rat aorta has been found to be around 28% of the dry weight, which corresponds to 0.03 mg/cm of aorta (Behmoaras et al., 2005). The decellularization of aortic tissue is important, as graft materials are required in arterial bypass operations. Currently, “about 40% of patients do not have an autologous vessel that can be used” (Salacinski et al., 2001) and so a synthetic graft is used. These have different mechanical properties and cause re-modelling to occur in the patient’s native artery. Excessive re-modelling can result in occlusion of the artery (Liu et al., 2000) and synthetic grafts can cause thrombotic complications (Budd et al., 1990). Thus, the use of decellularized arterial scaffolds that can be used to grow allogenic grafts can be considered as appealing.

The retention of the ECM’s structure and properties is important as differing mechanical properties between graft and native tissue can cause local flow disturbances. This can result in hyperplasia and end in occlusion of the vessel (Ballyk et al., 1998). The collagen and elastin fibres of the vessel’s ECM are arranged in a longitudinal direction imparting hyperelasticity (Holzapfel, 2006). Detergents can disrupt the interactions between structural proteins affecting the vessel’s tensile properties that are important due to the pressures they will experience (Fitzpatrick et al., 2010).

The measurements of the collagen content of the aortic artery before-and-after decellularization illustrated that the treatment did not demonstrate any significant effects over the level of collagen present. Whilst a large difference in the amount of mature (red) collagen was observed there was wide variance in the levels of collagen measured in the different samples both pre and post treatment. Therefore, the difference caused by the decellularization process was not found to be significant. Only the young, green collagen was significantly affected by the treatment. It has increased with the average value increasing approximately 100-fold. This was unexpected and could be as a result of the decellularization process removing the other cellular tissue and enabling the dye to access the thinner, younger and more inaccessible collagen. However, the total amount of green collagen visible is still very small.

Examination of the stained aorta showed, that similarly to the tendon, there was wide variation in the amount of collagen present from sample to sample. The variation in the quantities of collagen may be due to the aorta samples being from different sources and that the distribution of collagen around the aorta not being regular. Chronic hypertension is known to increase the extracellular matrix of the aorta, especially the amount of collagen (Benetos et al., 1997). As the maturation of collagen fibres involves several posttranslational modifications, such as hydroxylysines being glycosylated or processed by the enzyme lysyl oxidase to form interchain cross-links, (Van der Rest and Garrone, 1991) this can also differ from source to source.

Whilst the decellularization process had successfully removed the cellular tissue with retention of the collagen, the process affected the quality of the collagen. Sections of stained aorta show that the

collagen fibres were arranged in groups separating the smooth muscle and other cellular content (figure 3, chapter 3). Decellularization reveals larger groupings of collagen. It is unclear whether this is due to the decellularization process allowing the collagen to be seen more clearly or whether it is due to the ECM re-organising. From the increase in the amount of young green collagen found it is possible that the removal of cellular material is enabling this otherwise hidden form to be detected. However, it could be that the collagen has re-organised and that some smaller fragments are now being seen.

4.3 Carotid Artery

Carotid arteries can also provide useful grafts in medical procedures. A smaller vessel than the aorta it poses further challenges and decellularization processes can affect the mechanical properties of arterial tissue (Dahl et al., 2003). Comparison of native and decellularized samples of carotid artery by Williams et al., (2009) showed that decellularization disrupted the collagen fibre orientation causing stiffening. However, manipulation of reagents, such as replacing SDS with deoxycholic acid, has enabled the mechanics of carotid artery to be retained (Murase et al., 2006).

The amount of collagen was not affected by decellularization, as there was no statistical significance between the pre- and post-treatment samples ($p > 0.05$). By studying the images of the sections of carotid artery it can be seen that unlike the aortic tissue, the structural integrity of the collagen in the ECM also appears unchanged. When either the bright field or polarised light images are compared (see figure 2, chapter 3) the collagen appears to be of similar structure and configuration. This indicates that the decellularization method does not appear to have a detrimental effect on the quality of the collagen in the arterial ECM. Therefore the methods used here are applicable to preparing arterial scaffolds from excised carotid arteries. ?

4.4 Limitations of the Methodology

The methodology relies upon quantification of the collagen in the images by staining the tissues with picrosirius red (PSR). This dye identifies the different forms of collagen as the colour differs primarily according to strand thickness (Hiss et al., 1988). The younger, thinner strands appear green whilst the mature, thicker strands are red. However, as the thickness is the main determinant of the colouring, any degradation of mature collagen that reduces the strand's size could mean it is perceived as a younger form. A rough decellularization process could result in an increase in the amount of green collagen measured. The increase in the aorta's green collagen suggests that the decellularization is damaging the collagen causing an increase in smaller, thinner fibres. Other birefringent materials found in the ECM may affect the pixel count, e.g. keratin and fibrin appear green when stained with PSR (Rich and Whittaker, 2005). Whilst these will be a minor component of the ECM they still contribute to the results especially where the total collagen content is low.

The manual setting of the colour thresholds may also compromise the method (see chapter 2). The different collagen forms may have different amounts of pixels detected per weight of collagen due to the difference in range size. Certain assumptions have to be made that the pixel aspect ratio proportionately reflects the quantity of a form of collagen. If isolated and pure forms of the different types of collagen were available then the method could be calibrated by relating the pixels per cm2 to the amount of collagen. (?)

5.0 Conclusion

This study sought to identify collagen in tissue that can undergo decellularization and see how the process affected the quality of the collagen. The three tissues analysed all contained large amounts of collagen in their ECM (Di Lullo et al., 2002; Fitzpatrick et al., 2010). Tendon connects muscles to bone, being strong and flexible withstanding large forces. Aortic tissue experiences high pressures when the left ventricle contracts. Equally the carotid artery is mechanically strong as, being directly connected to the aorta, blood travels in highly pressurised surges. The high amounts of collagen in

their ECM make them useful biological scaffolds (Parenteau-Bareil et al., 2010). By staining with PSR the collagen was readily seen. Examination under polarized light enabled the different forms of collagen to be identified. Image J software allowed the quantification of collagen and assessment of the effect of decellularization. The tendon contained predominantly mature collagen and neither the amount nor quality was affected by decellularization. Similarly the carotid artery was not adversely affected by the removal of cellular material. The aortic tissue however was altered by decellularization with an increase in the amount of smaller collagen fibres seen and a change in the configuration of the collagen in the ECM. Therefore future work should examine alternate decellularization methods for aortic tissue.

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Conflict Of Interest
The authors declared that present study was performed in absence of any conflict of interest.

Author Contributions
SAk and RTQ designed, supervised the project and reviewed the manuscript. NKN, AHA, GRJ,OSM and AAS collected the samples and performed experiments and data analysis. All authors read and approved the final version.

8. Ethical statement
This study was conducted by permission granted from the research and studies in Genetics of human disease. The research includes human experiments. Experiments and study carried out in a special molecular laboratory under the supervision of the Dr and with the following laboratory and institutional guidelines for care and use of laboratory human

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