

Quantification of collagen fibre maturity in decellularized collagen matrices

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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). Decellularized scaffolds are successfully utilized to develop a variety of tissues, such as the bladder, arteries, esophagus, trachea and skin (Yoo et al., 1998; Dahl et al., 2003; Nieponice et al., 2006; Schechner et al., 2003; Macchiarini et al, 2008).

1. 1 Decellularization to Produce “ Scaffolds”

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. Ott et al. (2008) observes that “ transplant organ rejection is prevented with the

use of immunosuppressing drugs that should be taken by the recipient for the rest of their life”.

Transplantation of the recellularized liver graft

(Ott et al., 2008; Uygun et al., 2010).

The existence of natural antibodies is a considerable obstruction to xenotransplantation. Xenotransplantation is where animal organs 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 caused 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 that “repress this immune response and are deficient of 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). “More than 200 000 human patients have been implanted with xenogeneic ECM scaffolds” (Badylak, 2004). Grafts preparations required collating porcine intestinal grafts with collagen scaffold of porcine skin to “rebuild a variety of human ligaments and tendons” (Badylak, 2004).

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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 Gratzner, 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 immunosuppressants 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). Very often, as in the case of the tissue valves, this means removing the different types of complex sugars that can be identified upon the tissue surface. Examples of scaffolds being applied to produce fresh tissue include “ the use of acellular matrices for growing new heart valves” (Knight et al., 2008).

A particular advantage of tissue engineered valves for younger patients is that they can adapt internally which can prevent repeat operations (Lichtenberg et al, 2006). Tissue engineering has been shown improved quality of life. Macchiarini et al (2008) observed that a “ successful implantation of new trachea that was grown from stem cells on a collagen-based scaffold into a patient who had end-stage bronchomalacia.”

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In this instance, a scaffold was generated as a result of the removal of cells and antigens from a donated trachea which was infused with epithelial and mesenchymal stem cells which are cultured from patients. As a result, a graft was generated in vitro and inserted to a recipient to provide the recipient with a functional airway. Subsequently, the graft was grown in vitro and was grafted into the patient provided the recipient with a functional airway and improved the life quality. The patient was able to stop immunosuppressive drugs (Baiguera et al., 2010). According to BBC (2010) discovered “ It is the first time a child has received stem cell organ treatment, and it’s the longest airway that has 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 engineered tissue was successfully contained by a collagen structure donor scaffold that was infused with the boy’s stem cells. The scaffold was then transplanted into the patient at the same time as the cells were still growing “ so the recipient’s body was used it as the bioreactor” (Baiguera et al., 2010).

1. 2 Scaffolds for Tissue Engineering

In tissue engineering synthetic scaffolds can be utilized in a similar fashion as donor organs. 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 media cells that can be integrated

alongside a host recipient. The tissue rebirth of extra cellular matrices and collagen organization are thus similar to the native valve, and Knight et al. (2008) observed that “ the graft was prepared in vitro before injecting and letting it to change and grow by the dynamic nature of tissue engineering when was revealed by study.”

Choi et al. (2011) observe that human adipose tissue, such as scaffolds and which are widely available, “ can be used to derive extra cellular matrices.” A “ 3 dimensional scaffold” could be produced by the successful decellularization of adipose tissue. Choi et al., (2011) also observed that “ having been treated with a combination of physical, chemical and enzymatic procedures, the adipose tissue generated ECM.” Consequently, this successfully removed all components of the cell, leaving only collagen, sulfated glycosaminoglycan and elastin. This preserved the structure and morphology of the matrix allowing for the successful implementation of the matrix. Similarly, Flynn (2010) observed that the “ decellularization of adipose tissue with maintenance of the basement membrane was obtained.” Laminin and type IV collagen of the conserved structure was shown to offer an environment that facilitated adipogenesis.

Kian et al. (2007) outlines how the “ production of engineering dermal tissues have used hybrid collagen-polymer meshes” and this supports the “ growth of mesenchymal stem cells, which are derived from bone marrow” (Tan et al., 2011).

Kian et al., (2007) “ planted their mesh with dermal fibroblasts that were of human nature and was then attached with an arteriovenous blood supply”

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and consequently, this cell proliferation, collagen formation and angiogenesis have been allowed to take place.

Tan et al., (2011) developed “ artificial scaffolds that can be used to support the growth of mesenchymal stem cells, which are derived from bone marrow.” Collagen II is a considerable extracellular matrix component within cartilaginous tissue that experiences fibrillogenesis from different “ physiological conditions” in the body (Tan et al., 2011).

Change et al. (2010) observed that the “ combinations of collagen type II and biodegradable polyester have been used to produce cartilage formation from scaffolds.”

Engineering scaffolds of other dermal tissue use type I collagen and collagen-glycosaminoglycan (CG) matrices. Yannas et al. (1989; 1990) observed that “ low density biodegradable supports have been formed and they have been used to stimulate renewal of the skin conjunctiva periphereral nerves in vivo.”

Tendon and ligament connective tissues are prepared via dermal matrices due to the additional cell matrices which possess the capability to employ circulating progenitor cells as well as ECM in the reparation of damaged tissue. As a result,

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. Lu et al. (2004) observed that "cells grew in an organized manner when the scaffold was penetrated with fibroblasts."

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. Badylak (2004) identifies that "collagen has a vital role in providing structure, support and assists the softer parenchymal tissues, which connect them to bones." 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 an "increased deposition of extra cellular matrix can cause stiffening of the ventricular wall and reduce the pumping of blood by the heart" which Hadi et al. (2011) explains "interferes electrochemical signaling causes arrhythmias."

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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 which allow the proteins of collagens to make triple helix (Bella et al., 1994); " This structure is important to the properties of collagen such as its strength" (Buehler, 2006).

Additional proteins work together with collagen called discoidin domain receptors. Vogel et al. (1997) states that " cell surface receptor called tyrosine kinases are activated by triple helical collagen that plays a role in the remodeling of extra cellular matrices, cell proliferation, adhesion and migration."

Recently Xu et al., (2011) showed that non-fibrillar collagen type IV contain binding sites for certain members of the discoidin domain receptorfamily. 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.

Type I collagen can be found in the space between the majority of cells.

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. The treatment of the scaffold was found to consist of aggrecan, chondroitin-6-sulfate and type II, IX, and XI collagen in a relation like to the starting material. Mercuri et al (2011) observe how stem cells “ are derived from human adipose tissue” that used to seed the “ scaffold and show its ability to support cell growth.”

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. Removing certain cellular layers from tissues is also used by the direct pressure such as the 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 Gratzner, 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 could be caused by the transfer of the 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 cellculture, 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

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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.

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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 muscles in latrunculin B, a high ionic strength salt solution, 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

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al., 2008; Uygun et al., 2010). The increased surface area of tissue that in contact with the decellularization solution was occurred 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 architecture and purpose of the microvascular network were preserved by Perfusion based decellularization that allowed the recellularization of the liver matrix with adult hepatocytes. The resulting graft was showed that a liver functioned with albumin secretion, urea synthesis and cytochrome P450 expression being recorded (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 degree 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 samples are performed by the decellularization process to confirm that all the 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 known 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, the capability to become any type of cell. 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 (Korbling 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 (Korbling 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 germinativum. 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 supports lymph and blood vessels, nerves, muscle cells, sweat and sebaceous glands and hair follicles. It is in dermal layer that fibroblasts involved in the synthesis of collagen and elastin are found; “ Collagen and elastin give the skin its elasticity, tone and texture and 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). As the synthesis of collagen declines with age, and the collagen content of the skin decreases and becomes more disorganised in nature (Uitto et al., 1989; Oikarenen, 1994). Changes are also found within all relative proportions types of collagen with an 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 picosirius 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

Disruption of the ECM can be caused by decellurisation treatment depending on the techniques and types of tissue used. For example, the use of trypsin altered the collagen structure and reduced the elastin and fibronectin content (Schenke-Layland et al., 2003; Gilbert et al., 2006), as well as the detergent-based cell removal from porcine scaffolds destabilise the scaffold (Naso et al., 2010).

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 detrimental 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., 2004). 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 picosirius 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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