Determination of Cryoprotectant Behavior, Physiological and Anatomical Characterization of the Porcine Meniscus as a Model for Future Meniscal Vitrification Studies

by

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Abstract

The menisci hold significant, protective biomechanical function in the human knee and loss of meniscal functions have been linked to the development of osteoarthritis. Currently, irreparable or non-salvageable meniscal injuries undergo partial or total removal of injured menisci to alleviate patients' symptoms. Unfortunately, in the long term both procedures lead to knee degenerative changes. Investigators are seeking techniques to bank donor meniscal tissue for future allograft transplantation, by means of tissue vitrification while using cryoprotectant agents (CPAs). However, achieving this goal would require extensive investigation and intact human meniscal tissue is not readily available for this purpose. Thereby, animal models are indispensable tools for meniscus investigation. The reliability and applicability of these models to replicate findings in humans depends on determining the most suitable animal proxy. The porcine model is commonly used in our laboratory for Orthopaedic tissue experimentation. Before the porcine meniscus can be regarded as a suitable model for human meniscal investigations, it has to be examined from a physiologic, anatomical and functional perspective.

First, we examined the behavior of two commonly used CPAs (EG and glycerol) with the porcine meniscus by determining the post-incubation efflux rates. Second, we performed biochemical analysis to investigate the glycosaminoglycans (GAGs) and DNA content of the porcine meniscus. Finally, through a descriptive anatomical study, we compared the weight, volume and dimensions of the human meniscus with two commonly used animal models: sheep and pig.

Our findings provided preliminary data on the efflux amount of EG and glycerol from the porcine meniscus for different experimental conditions. Those findings would aid in choosing meniscus incubation duration and the choice of experimental temperature when designing future studies. Moreover, our structural study revealed a higher concentration of sulfated GAGs in the inner two-thirds region of the porcine meniscus. Additionally, based on anatomical features, the sheep meniscus showed more resemblance to the human meniscus than the porcine meniscus, suggesting that it might be a more suitable model than the current model that was used.

Preface

This thesis is an original work by Talal Takroni. All of the work presented henceforth was conducted in Dr. Jomha's laboratory on the 3rd floor of the Li Ka Shing Building at the University of Alberta. Approval of the Research Ethics Board of the University of Alberta, Edmonton, Canada was obtained and institutional safety and ethical guidelines were followed. All human specimens, in Chapter 4, came from deceased, unidentified organ and tissue donors. The experimental work involving animal tissue, covered in Chapters 2-4, received ethics approval from the Animal Care & Use Committee: Health Sciences at the University of Alberta under category A to acquire and handle animal tissues. This experimental work included no direct contact with livestock. Animal joints, used in experimental work, were acquired from a local abattoir where pigs and sheep were euthanized for commercial meat consumption.

I was responsible for tissue collection, performing experimental work, data gathering and analysis, and writing of manuscript including the literature review in Chapter 1. Dr. Nadr Jomha and Dr. Janet Elliott did experiment design and concept formation for Chapter 2; Dr. Nadr Jomha, Dr. Adetola Adesida, Dr. Leila Laouar and myself designed Chapter 3; Dr. Nadr Jomha, Dr. Adetola Adesida, Dr. Janet Elliott and myself designed Chapter 4. The biochemical analysis described in section 3.2.4 was carried out with the assistance of Dr. Leila Laouar. Hana Yu created tables 2-1, 2-2 and 2-3. Also Hana Yu modified figures 2-1 through 2-8 to have the final layout shown in Chapter 2. All other parts of the thesis are my original work.

Versions (as per selected journal article guidelines) of the work described in Chapters 2-4 will be submitted for publication. A version of Chapter 4 was submitted for publication but was not accepted. All reported authors have contributed to the manuscript edits.

Dedication

To my parents: Abdulkareem A. Takroni and Aisha A. Hausawi, for their endless love and for teaching me hard work, dedication, and passion for success.

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First of all I would like to thank to my supervisors: Dr. Nadr Jomha, Dr. Janet Elliott, Dr. Adetola Adesida and Dr. Leila Laouar for their guidance, inspiration, and dedication to my learning and development. Profound thanks to Dr. Nadr Jomha for his heartfelt and inspiring scientific and surgical mentorship; Dr. Janet Elliott and Dr. Adetola Adesida for their insightful discussions that provoked my scientific curiosity; Dr. Leila Laouar for her patience and for teaching me everything I know about working in a laboratory when I showed up with no prior experience. My special thanks extend to Dr. Thomas Churchill and Dr. Samer Adeeb for reviewing the thesis and their valuable input. My gratitude also extends to Ms. Aillete Mulet-Sierra for her technical support and teaching. Special thanks to all current and past colleagues from the lab with whom I shared friendship, motivation and scientific knowledge. To family and friends thank you so much for your support. I wish also to thank my sponsors: King Abdulaziz University, Jeddah, Saudi Arabia and the Saudi Cultural Bureau in Canada. Thank you all!

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List of Abbreviations

AC: articular cartilage
ACL: anterior cruciate ligament
CHO: Chinese hamster ovaries
COO^-: carboxyl group
CPA: cryoprotectant agent
CS: chondroitin sulphate
C_v: concentration needed to vitrify
D: diffusion coefficient
DMMB: dimethylmethylene blue dye
DMSO: dimethyl sulfoxide
DNA: deoxyribonucleic acid
DS: dermatan sulphate
ECM: extracellular matrix
EDTA: ethylenediaminetetraacetic acid
EG: ethylene glycol
GAG: glycosaminoglycan
HA: hyaluronic acid
H-NMR: proton nuclear magnetic resonance
IL-1β: interleukin-1beta
KS: keratan sulphate
LN_2: liquid nitrogen
L_p: hydraulic permeability
M: molarity
m: molality
MAT: meniscal allograft transplantation
Me_2SO: dimethyl sulfoxide
MFC: meniscus fibrochondrocytes
MRI: magnetic resonance imaging
OA: osteoarthritis
PBS: phosphate buffered saline
PCL: posterior cruciate ligament
PG: propylene glycol
PSG: penicillin, streptomycin and L-glutamine
PVP: polyvinylpyrrolidone
SD: standard deviation
SEM: standard error of the mean
SMA: smooth muscle actin
SO^-_3: sulphate group
Tg: glass transition temperature
VEGF: vascular endothelial growth factor

Chapter 1. General Introduction

1.1 Meniscus Basic Science

1.1.1 Gross Anatomy

The menisci are two crescent-shaped, fibrocartilagenous structures sitting inside the knee joint between the articular surfaces of the femoral condyles and the tibial plateaus. They are vital structures for normal knee function and their role in the knee joint includes: load bearing, shock absorption, joint stabilization, joint lubrication, and they play some role in joint proprioception and articular cartilage nutrition. Upon gross inspection they are glossy white structures with smooth glistening surfaces [1]. Individually, each meniscus has two surfaces and two borders. The proximal (concave) surface is in contact with the femoral condyles, while the distal (flat) surface sits on the tibial plateau. These surfaces allow the menisci to increase the congruence between the femoral condyles and tibial plateaus for better joint function as they act to deepen the tibial articular surface and accommodate the convexity of the femoral condyles [2, 3]. The peripheral (outer) border of each meniscus is thick, convex and attached to the inside of the joint capsule, while the inner border tapers down to a thin freely mobile margin centrally, thereby, giving the menisci a triangular shape on cross-section [4]. This triangular cross-sectional shape is important in the load bearing and stress distribution functions [5]. Each meniscus can be divided into: meniscal body, anterior horn and posterior horn. The anterior and posterior horns provide a secure bony anchorage via their attachments to the subchondral bone of the tibial plateau. The anterior meniscal horns are usually narrower than the posterior horns. At the front of the knee joint, the two menisci are connected to each other via the transverse ligament, which is a transverse fibrous band of tissue, found in 64 % of people [6] that connects the anterior horn of

the medial meniscus to the lateral meniscus. Their bony, capsular and various ligamentous attachments provide mechanical stabilization to the menisci at multiple points in the knee range of motion. There are two menisci in each knee joint, namely, medial and lateral. Although both menisci are functionally and structurally the same, they have different sizes and attachments to appropriately meet the load in their respective knee joint compartments.

The medial meniscus is semilunar in shape and it is narrower anteriorly than posteriorly. It covers approximately 60 % of the tibial surface. It is approximately 3.5 cm in length in the anterior-posterior direction with a width that increases towards the posterior horn attachment [7]. Ferrer-Roca and Vilata [8] measured the width of the medial meniscus horizontally along the tibial plateau and their results showed that the width at the posterior horn (widest point) averages 10.6 mm, at the midpoint it averages 9.6 mm and at the anterior horn it averages 7.7 mm [8, 9]. The anterior horn of the medial meniscus attaches to the anterior intercondylar fossa of the tibia in front of the base of the anterior cruciate ligament (ACL) [10]. While, the wider posterior horn attaches to the posterior intercondylar fossa of the tibia anteromedial to the posterior cruciate ligament (PCL) insertion site [10]. The anterior and posterior horn attachment sites of the medial meniscus are larger than those of the lateral meniscus [11]. The medial meniscus is firmly attached to the joint capsule throughout its peripheral border, and the tibial portion of the joint capsule is referred to as the coronary ligament. Additionally, the mid-portion of the medial meniscus is attached to the deep medial collateral ligament, which is a condensation in the joint capsule that attaches to both the femur and tibia [4]. The firm attachment of the meniscus to the joint was demonstrated to result in limited excursion (motion) of the medial meniscus compared to the lateral meniscus during knee flexion [12] leading to decreased tolerance to rotational

movement and, therefore the medial meniscus is at increased risk of injury especially in athletically active individuals who have a higher chance of sustaining rotational forces on a partially flexed knee. Clinically, the medial meniscus holds a special importance in the ACL-deficient knees by acting as a “chock” or a “buttress” that prevents anterior tibial translation in response to anterior tibial load [13, 14].

The lateral meniscus is almost circular and it covers a larger portion of the tibial surface (80 %) than the medial meniscus (60 %) [3, 15]. The lateral meniscus has a more uniform width from front to back which was found to be 10.6 mm at the posterior horn, 11.6 mm at the mid-point and 10.2 mm at the anterior horn [8]. The anterior horn of the lateral meniscus attaches to the lateral inter-condylar eminence of the tibia parallel to the ACL insertion site and has a very close proximity to ACL or, sometimes it might even blend with it [9]. The posterior horn attaches to the lateral inter-condylar eminence of the tibia at the posterior border of the ACL insertion site anterior to the attachment site of the posterior horn of the medial meniscus [9]. Compared to the medial meniscus, the entire circumferential peripheral border of the lateral meniscus is loosely attached to the joint capsule, and has no lateral collateral ligament fixation. Moreover, at the posterior convexity of the lateral meniscus, the popliteal tendon creates an indentation in the body of the meniscus and it receives an aponeurotic extension from the upper half of the popliteus muscle. The arcuate ligament is also attached to the upper end of the posterior convexity of the lateral meniscus [5]. The posterior horn of the lateral meniscus provides origin for two ligamentous divisions, called the meniscofemoral ligaments that pass, upward, anterior and posterior to the PCL to be finally inserted into the lateral aspect of the medial femoral condyle. The anterior meniscofemoral ligament is called the ligament of Humphery and the posterior

division is called Wrisberg's ligament. There is variability in the presence and sizes of the divisions of the meniscofemoral ligament [4]. Kusayama et al [16] in a cadaveric study, reported that, 46 % of people have both of these ligaments and 100 % of people have at least one. Another cadaveric study by Poyton et al [17] reported the presence of both ligaments in 27 out of 42 (64 %) and all 42 (18 males and 24 females) had one of the ligaments present in the dissected knees. The anterior meniscofemoral ligament was found in all 18 males and 17 females while, the posterior meniscofemoral ligament was present in 16 males and 22 females. The exact function of the meniscofemoral ligaments is an issue of debate but they are assumed to act as restraint to posterior translation of the tibia in PCL-deficient knees as well as increasing the congruity between the lateral meniscus and lateral femoral condyle at different ranges of motion [9].

1.1.2 Embryology and Early Development

The menisci start to develop around the 8^{th} to 10^{th} week of fetal gestation [3, 18, 19]. They arise from a condensation of the intermediate layer of the mesenchymal tissue and they take on their characteristic shape around the 4^{th} month of gestation [3, 9, 20]. The developing menisci start off as highly cellular and highly vascular structures with blood vessels extending throughout the whole width of the meniscus horizontally. Histologically, in the fetal developmental stage, the menisci have a high content of tightly packed fibroblasts with no matrix formation and an empty intracellular cavity leading to a large nucleus-to-cytoplasm ratio [3]. As the gestational age progresses and the fetus enlarges, cells start to mature and become more differentiated and more collagenous with an extracellular matrix formation leading to a decrease in the nucleus-to-

cytoplasm ratio. With further growth the menisci showed more prominent vascularity. There is also a uniform rate of growth between the menisci and their corresponding tibial plateaus [3].

In the post-natal period, the menisci continue to grow constantly as do their enclosing femoral condyles and tibial plateaus [3]. Histological changes witnessed after birth involve a gradual decrease in the number of cells, an increase in the collagen content with relatively smaller nuclei and retraction of vascularity from the inner region towards the periphery [2]. The presence of blood supply along the entire width of the meniscus helps to insure the delivery of nutrients to all parts of the meniscus before the infant can develop the ability for erect standing and walking [21]. As the child becomes ambulatory, between 1-3 years of age, there is a change in the orientation of the collagen bundles into a more circumferential fashion to fit the biomechanical functions. Additionally, the resultant load from the body weight and muscular forces compress the blood vessels causing them to retract until they finally become confined to the outer region of the meniscus around the age of 10-11 years old [1, 2, 21]. Interestingly, Pufe et al. [22] investigated the mechanism regulating the gradual loss of vascularity in the innermost part of the meniscus and they reported higher concentrations of endostatin/collagen XVIII in the avascular inner two-thirds of adult menisci compared to fetal menisci. Endostatin is a portion of the C-terminal region of collagen XVIII with antiangiogenic properties leading to the inhibition of vascular endothelial growth factor (VEGF) in the menisci. Early in life, this molecule is evenly distributed throughout the meniscus, but with aging its levels increase in the inner two-thirds and decrease in the outer one-third. Its expression is influenced by mechanical factors suggesting a strong relation between weight bearing and the increase in endostatin's concentration [22, 23]. The collagen content continues to increase in the post-natal period, influenced by the joint motion

until approximately the age of 30 years and remains stable until it begins to decrease at around the age of 80 years [9].

The structural and vascular patterns of the menisci before the vascularity retracts may have a role in the low prevalence of meniscal injuries in children, due to the high reparative potential, compared to adults [3]. Nonetheless, the increasing number of children participating in physically demanding sport activities may lead to an increase in incidence of meniscal tears in children [24].

1.1.3 Vascular Anatomy

At maturity, vascular supply is confined to the outer-third of the meniscal body [25, 26]. The body of the meniscus receives vasculature from a confluence of geniculate arteries around the knee [25, 26]. The superior and inferior branches of the medial and lateral geniculate arteries form a perimeniscal capillary plexus within the synovial and capsular tissue arranged in a circumferential manner to supply the peripheral border of the menisci around their attachment to the joint capsule [4]. These vessels penetrate 10-30 % of the width of the medial meniscus and 10-25 % of the width of the lateral meniscus [25]. Based on the pattern of vascularity difference across the body, the menisci can be divided into two regions. The outer vascular region (red-red zone) and the inner completely avascular region (white-white zone) and, in between these two zones is the red-white transition zone.

The horns of the menisci, additionally, receive vascular supply from the middle geniculate artery, along with a few terminal branches of the medial and the lateral geniculate arteries. Blood vessels penetrate the anterior and posterior horn attachments through their vascular synovial covering, making the horns more vascular than the body. Ligaments attached to the menisci receive vascularization through the endo-ligamentous vessels around the meniscal horns, but their bony insertions (entheses) are avascular.

Aside from the horns and the peripheral border, the remainder of the meniscus is avascular (65 % to 75 %). With aging there is a further decline in vascularity leading to unfavorable results even after repair [27, 28]. Another avascular portion of the meniscus is the posterolateral convexity of the lateral meniscus next to the popliteal tendon [21, 28].

Nutrition is supplied to the meniscus through penetrating blood vessels in the outer vascular portion [29]. The avascular portion derives its transudative nutrition from the synovial fluid by diffusion and mechanical pumping during loading conditions through a system of microcanals (~ 10-200 μm in diameter) between the collagen bundles [4, 21, 30].

1.1.4 Neuroanatomy

The menisci have different branching nerve fibers (myelinated and unmyelinated) arising from nerves that supply the knee joint. The knee joint gets innervation from the posterior articular branch of the posterior tibial nerve in the popliteal fossa (the main source of innervation), the

terminal potion of the obturator nerve and the terminal branch of the femoral nerve to the quadriceps muscle [9, 29]. However, the recurrent peroneal branch of the common peroneal nerve innervates the inside of the capsule. Those nerve fibers from the capsule follow the vascular supply (paravascular) to the peripheral portion of the menisci and branch to extend to the horns, anteriorly and posteriorly [4]. Meniscal horns, particularly the posterior horn, have the highest concentration of neural ending and the requirement to provide nutrients for the concentrated neural elements could be the reason for their increased vascularity compared to the body of the meniscus [21]. This paravascular pattern of nerve fibers suggests a vasomotor or a vasosensory function of those neural endings. Moreover, free nerve endings (not surrounded by blood vessels) have been found in the middle third of the meniscus (where there is no blood) and have been implicated in slow pain conduction [21]. Free nerve endings have also been found in the meniscofemoral ligament and the transverse ligament [7, 31].

Furthermore, three morphologically different mechanoreceptors are found in the horns and during the extremes of knee flexion and extension they become stimulated to send proprioceptive inputs to the central nervous system [32]. Type I mechanoreceptors (Ruffini corpuscle) have static and dynamic qualities, and provide inputs to the central nervous system on joint position, change in position and intra-articular pressure changes. Type II mechanoreceptors (Pacinian corpuscle) provide inputs on acceleration and deceleration. Type III mechanoreceptors (Golgi tendon organ) provide protective postural and muscular reflexes [4, 32].

1.1.5 Microanatomy: Biochemical Composition and Structure

The meniscus is a very hydrous tissue with water constituting 70-73 % of the wet weight, and the remaining 27-30 % consisting of organic matter (cells and extracellular matrix). The meniscus is biphasic in nature with fluid and solid components interacting with each other to give the tissue its characteristic functions. The densely packed dry extracellular matrix is made up of collagen (~75 %), glycosaminoglycans (15 %), DNA (~2 %), adhesion glycoprotein (< 1 %) and elastin (< 1 %) [2]. Collagen and elastin make the fibrous components of the ECM, hence, the name fibrocartilage due to the dominance of collagen fibers. The fractions of the extracellular matrix components and water within the meniscus vary with age, injury or pathology affecting the meniscus. For instance, the water content is higher in the posterior areas than in the central or anterior areas. Interestingly, all mammalian menisci have similar shapes with minor biochemical differences and several experimental animal (e.g., canine, rabbits and sheep) models have been used to study many aspects of this tissue [33-41].

1.1.5.1 Collagen

Collagen is the main component of the ECM (75 % of the dry weight) and forms the macromolecular framework of the menisci [31]. As mentioned earlier, the collagen content increases after birth as a result of weight bearing and joint motion until 30 years of age, and reaches a plateau state until it starts to decrease at around 80 years of age [29, 31]. Type I collagen makes up approximately 90 % of the total collagen content in the meniscus. For this reason, the predominance of type I collagen is what differentiates the fibrocartilage of the menisci

from hyaline articular cartilage that shows predominance of type II collagen [42]. Other types of collagen also present in the meniscus in small amounts including: types II, III, V, and VI [42]. The different types of collagen are not evenly distributed throughout the meniscus with some regional variation in concentrations. In a study to determine the distribution and percentage of collagen in the outer and the inner parts of the body of bovine menisci, tissues were purified (removing proteoglycans) with the use of 4 M guanidine hydrochloride followed by pepsin digestion to solubilize collagen for the electrophoresis. Cheung [43] reported that the outer meniscus showed predominance of type I collagen (95 %) with trace amounts (< 1 %) of collagen types III or V; while in the inner (avascular) meniscus, collagen type II made up 60 % and type I collagen made up 40 % of the total collagen content. Collagen type VI is another type of collagen that was identified within the matrix of the meniscus but it is classified as a matrix glycoprotein that helps to stabilize collagens type I and type II, and maintain cell attachment to the matrix [44].

Collagen fibers are oriented in a fashion that enables them to perform their function. In the superficial layer of the menisci, collagen fibers are parallel to the surface and oriented randomly in a mesh-like fashion, while in the deeper layer collagen fibers are oriented circumferentially extending to the horn attachments [45, 46]. Also there are some radially oriented fibers extending to the deeper layer known as “radial tie fibers”. These radial tie fibers are located in between the circumferential fibers helping to limit the motion of these circumferential fibers leading to reinforced stability [45, 46]. As a result of this pattern of collagen fiber orientation, the vertical (axial) load from the femoral condyles is converted to a circumferential (hoop) stress that dissipates throughout the meniscus [42]. Collagen fibers

provide the meniscus with its tensile strength and their interaction with elastin fibers gives resiliency that allows the collagen fibers to return to their original shape [47].

1.1.5.2 Proteoglycans

Proteoglycans are polypeptides located between the collagen fibers with a core protein that is covalently bound to one or more glycosaminoglycans (GAGs), and they are commonly classified based on the GAGs present [2]. They make up 1-2 % of the dry weight of the meniscus [1]. Based on the ability of the core protein portion to aggregate noncovalently with hyaluronic acid, proteoglycans can be classified into large molecules (high density) and smaller molecules (low density) [42]. Aggrecan is the major large proteoglycan found in the human menisci, while biglycan and decorin represent the small proteoglycans. Different types of GAGs are found in normal human menisci including: chondroitin-6-sulfate (40 %), dermatan sulfate (20-30 %), chondroitin-4-sulfate (10-20 %), and keratin sulfate (15 %) [10]. The presence of sulfate groups makes glycosaminoglycans negatively charged molecules with great hydrophilic property. As a result they are able to absorb water into the structure of the menisci, which helps in maintaining tissue hydration, firmness and elasticity [48]. Moreover, the negative charge allows them to exchange mobile ions (e.g., Na+, Ca+) between the tissue and the surrounding fluids. Regional distribution of glycosaminoglycans depends on the type of force sustained by the different parts of the meniscus. For instance, dermatan sulfate is commonly condensed in areas that are subjected to tensile forces, while chondroitin sulfate is more condensed in areas that are subjected to compressive forces [42]. Therefore, the meniscal horns, which are subjected to tensile forces, contain mostly dermatan sulfate and some chondroitin sulfate, while the body of the meniscus

contains mostly chondroitin sulfate and keratin sulfate, and the fibrous peripheries contain mostly keratin sulfate and hyaluronate with almost no dermatan sulfate. Overall, the inner two-thirds and horns contain higher concentration of proteoglycans than the outer third. The inner avascular/aneural portion of the meniscus resembles articular cartilage in the abundance of GAGs and the presence of collagen type II. By comparison, the amount of proteoglycans within the meniscus is only 8-10 % that of the articular cartilage, meaning the articular cartilage is more hydrous than the meniscus.

1.1.5.3 Cells

Cells are embedded within the extracellular matrix of the menisci. These cells are responsible for synthesizing and maintaining the matrix components. The main cells in the meniscus are termed meniscal fibrochondrocytes (MFCs) as they exhibit both fibroblastic and chondrocytic characteristics [48]. However, based on morphology, location and presence of pericellular matrix there appear to be four distinct types of cells [49]. First, cells of the superficial zone, at the interface with the synovial fluid, have a fusiform shape with spindle like morphology and no cytoplasmic projections [49]. They may play a role in tissue healing/remodeling due to their expression of alpha smooth muscle actin (SMA), which has been found to be elevated in areas of injury within the meniscus. Second are fibrochondrocytes; these cells have a round or oval shape, resembling the articular cartilage chondrocytes, and they make the main cells in the body of the inner and middle meniscus [2]. They are responsible for synthesizing both collagen type I and type II. Moreover, they can be distinguished from other cells in the meniscus by the presence of the pericellular matrix. Third, fibroblast-like cells have

no pericellular matrix and are located in the outer portion of the meniscus [49]. They also have projections that connect them to other cells and they mainly make collagen type I. Fourth, endothelial cells were identified in the lumen of the microvasculature of the outer-third and they help to maintain the vascularity [50]. Despite the differences between these cells in their synthetic activity and gene expression in response to mechanical stimuli, all cells contribute to the mechanical properties of the meniscus. However, the number of available cells and their synthetic ability deteriorates with aging and degenerative changes in the knee [51]

1.1.5.4 Other Components

Matrix glycoproteins (adhesion glycoproteins) make up < 1 % of the dry weight and help adhere matrix substances to one another and to the cells [42, 48]. The major adhesion glycoproteins include: link protein, fibronectin, thrombospondin and collagen type VI. Link protein is necessary to stabilize the aggregation of proteoglycans with hyaluronic acid [9]. Fibronectin is involved in tissue repair processes as it helps keep cells attached to the ECM; it also helps in cell migration/adhesion and with blood clotting. Elastin constitutes less than 1 % of the meniscus dry weight and together with collagen forms the fibrillar elements of the meniscus. Elastin provides some resilience to the meniscus in the form of elastic recoil allowing the meniscus to undergo deformation without being damaged [2].

1.1.6 Kinematics and Biomechanics

Knee kinematics is a mix of flexion, extension, rolling and gliding. During normal loading conditions such as walking, the knee is exposed to loads that can be 2.7 - 4.9 times the body weight [52]. Of the total load created in the knee joint, the menisci bear 45 - 50 % depending on the knee range of motion during loading. During load bearing, the femur press down on the meniscus, creating an axial load with radial displacement force that is counteracted by the stabilizing action of the horns, circumferential stiffness linked to the collagen fiber orientation and proteoglycan content. The degrees of the meniscal motion have been quantified, where the medial meniscus had lower excursion, especially in the posterolateral corner, than the lateral meniscus [5, 12]. In a study to assess the degree of meniscal motion through different knee ranges of motion (flexion 0˚- 120˚) the average excursion in the medial meniscus was 5.1 ± 0.96 mm, while for the lateral meniscus the average excursion was 11.2 ± 3.3 mm. This differential in the medial meniscus has been attributed to the tight capsular and deep medial collateral ligament attachments. The reduced motion in the medial meniscus plays a role in the increased rate of meniscal injuries at the posterolateral corner especially when strained during flexion [5, 12]. During periods of loading, the articular cartilage is subjected to compression. These compressive forces over the articulating surfaces result in contact stress (pressure) that is inversely proportional to the contact area. Thereby, and due to the incongruous shape of the bone ends in the knee joint, the contact stress cannot be minimized without the menisci [53]. Indeed, removal of the medial meniscus leads to 50 -70 % reduction in femoral condyle contact area resulting in 100 % increase in contact pressure, whereas lateral meniscus removal decreases the contact area by 40-50 % with a greater increase in the contact pressure of up to 200-300 % [54].

The shock absorbing function is attributed to the viscoelastic nature of the meniscus [42]. This is evident during loading where the frictional drag force that is developed as the fluid tries to escape the tissue provides the ability for shock absorption [42]. Additionally, the biphasic nature of the meniscus allows it to transport nutrition and removes waste via diffusion, and provides lubrication to the joint surfaces.

1.2 Meniscal Injury, Overview of the Problem and the Need for Meniscal Allograft Transplantation

Meniscal defects and removal of knee menisci (partial or total meniscectomy) lead to a decrease in the protective role of the meniscus in the knee joint leaving the articular cartilage exposed to increased static loading strain from increased pressure inside the joint [1, 55, 56] and to dynamic deformation [55] with resultant deterioration in the joint leading to early onset osteoarthritis. The name osteoarthritis may imply that the pathological process is limited to the bone and joint cartilage. In fact, studies have proven osteoarthritis to be a whole-joint disorder and it would be expected to affect the meniscus, subchondral bone, synovial membrane and ligament integrity as well [57]. Normal menisci are not seen in knees with osteoarthritis. In fact, menisci in knees with osteoarthritis are often torn, macerated or otherwise destroyed suggesting a strong association between the meniscus and the disorder [57-59]. Loss of meniscal function is associated with an increased risk for knee pain and osteoarthritis [58-60].

In the United States, meniscal lesions are considered the most common knee injury that requires surgical intervention by orthopedic surgeons [1, 61, 62]. The mean annual incidence of meniscal lesions was found to be 66 per 100,000 inhabitants with 61 of which resulting in meniscectomy [63, 64] with a male-to-female ratio of 3:1. The highest incidences were found in the 3rd, 4th and 5th decades of life. Medial meniscus injuries were found to be four times more common than lateral meniscus injuries with the posterior horn of the medial meniscus as the most frequent location [65]. The right knee was found to be at a greater risk of meniscal injuries especially in athletically active individuals [63, 66]. Meniscal lesions are among the clearly

identified causes for the development of arthritis. In 2007-2008, 4.2 million Canadians (16 % of the population) aged 15 years or older reported having arthritis [67, 68]. This number is expected to increase to approximately 7 million (20 %) by 2031 [67, 69]. Furthermore, the total cost related to arthritis was estimated to be $6.4 billion, which can be attributed to expenditure on hospital care, drug cost and physician care [67]. This disorder affects the sufferers' quality of life. Work leaves and absenteeism due to arthritis disability lead to a decrease in the workforce productivity that can also affect people's ability to make a living and the labor system as well.

Mechanisms of meniscal pathology (as have been described in the standard textbooks) [70], include traumatic injuries and degenerative lesions. Traumatic lesions are commonly seen in young, physically active individuals as a result of the meniscus being compressed between the femoral condyle and the tibial plateau while the knee is bent. These injuries are classified based on their relation to the orientation of the collagen fibers as either vertical, longitudinal, oblique, radial or horizontal [66]. On the other hand, degenerative lesions dominate in elderly patients and are described as horizontal cleavages, oblique flaps, complex tears or meniscal maceration. Other less common forms of meniscal pathology include: a very rare developmental abnormality known as discoid meniscus, or metabolic diseases such as calcium pyrophosphate crystal deposition and hemochromatosis [70, 71]. Nonetheless, meniscal tears in the vascular periphery have a potential for healing aided by fibrin clot formation and vascular proliferation through the fibrin clot that lead to filling of the gap with a fibrovascular scar tissue that can promote further vascularization [4]. Whereas, meniscal tears in the avascular portion tend not to heal due to the absence of hematoma and its contained chemotactic and mitogenic factors (e.g. platelet-derived growth factor and fibronectin), known to stimulate the reparative process [4, 72]. As meniscal tears in the

vascular zone have the potential to heal, sometimes even on their own without surgical intervention, this led to new therapeutic approaches being suggested to evaluate the ability to enhance a healing response in the avascular zone by simulating the vascular environment using experimental animal models [73]. The most common of these approaches, include creation of vascular access channel, exogenous fibrin clot, synovial flap, and trephination of the edge of the meniscus with suturing the tear [74-78]. Results have shown that the previous innovations succeeded to fill the surgically inflicted defects (in experimental animal models) but the new tissue contained calcified regions, unattached collagen fragments and pools of proteoglycans, making them non-optimal to undertake the biomechanical functions. Furthermore, these approaches did not receive an appreciable acceptance due to the deficiency in long-term follow up and replication of results [1, 2, 74-77].

Historically, meniscal resection or removal used to be an acceptable option for meniscal injury as the meniscus was considered to a functionless remnant of the leg muscle and the preconceived notion that it will regenerate following its removal provided the rationale towards total resection of an injured meniscus [79, 80]. In total meniscectomy, the knee is opened up surgically and the meniscus is removed fully. This procedure leaves the articular surfaces exposed to rub against each other during loading with resultant wear and tear that accelerates degenerative changes [81]. As a matter of fact, in 1948, Fairbank [60] described the knee joint changes observed after total meniscectomy by comparing knees that had total meniscectomy to the contralateral normal knees of the same patients. Those changes include: formation of a ridge projecting from the margin of the femoral condyle to the site of the removed meniscus, narrowing of the joint space and flattening of the femoral condyles [60]. With these changes in mind, and in

an effort to retain as much of the healthy tissue as possible, open partial meniscectomy became superior to total removal of the meniscus [82, 83]. Furthermore, a biomechanical study has proven that contact stresses in the knee increased in proportion to the amount of the meniscus removed [56]. Later on, as arthroscopic techniques were introduced, arthroscopic partial meniscectomy gained special attention as it provided good intra-articular visualization, decreased the length of hospital stay and caused minimal scaring following surgery [84]. Soon arthroscopy became prevalent as a diagnostic and treatment modality [85]. Burks et al. [83], reported results that were 88 % good (especially in the ACL-intact knees) and 12 % poor results in patients who had undergone arthroscopic partial meniscectomy after a fifteen-year follow-up with no significant difference between lateral and medial meniscectomies. On the contrary, other studies have shown good early results followed by deterioration over time with longer follow-ups [84, 86-88]. Correspondingly, partial meniscectomy has been shown to alter knee mechanics by changing intra-articular fluid pressurization and lead to > 350 % increase in contact forces on the articular cartilage as well [52, 88]. Therefore, partial meniscectomy leads to excellent short-term results with knee deterioration seen at long-term follow-up. Furthermore, the overall frequency of Osteoarthritis has not changed dramatically, meaning that partial meniscectomy is not the absolute solution for meniscal injuries [57, 86, 89].

Even though, arthroscopic repair and arthroscopic partial resection both led to reduction in the total meniscectomy cases being performed, there are conditions where meniscectomy becomes an inevitable procedure. Due to the lack of satisfactory results following meniscectomy, orthopedic surgeons started to look for alternative treatment options, and some of the suggested options included meniscal repair and tissue transplantation. Meniscal repair involves suturing the

meniscus at the tear site using different surgical techniques, which include open, outside-in, inside-out, and all-inside arthroscopic methods [29, 90]. Certain factors have to be considered before attempting a meniscal repair including patient factors, overall stability of the knee and, morphology and location of the tear as tears in the peripheral portion will initiate a healing response [29]. In general, best results were seen in acute tears that were vertical and longitudinal in the peripheral third of the meniscus in young patients with stable knees [91]. In an animal study, 20 dogs and 12 monkeys, Cabaud et al. [34] reported that sutured meniscus was strong enough to protect the underlying cartilage in 94 % of the cases. While Horibe et al. [92] reported that some of the patients with initial good or excellent arthroscopic results subsequently tore their meniscus at the site of repair and some patients suffered a new tear in a different region following a completely healed repair. In addition, the latter group stated that sutured menisci were found to be weaker than normal healthy menisci [92]. All things considered, results from meniscal repair studies showed variability that could be attributed to the variety of surgical techniques and different suture materials used by different surgeons [21]. Then the idea of meniscal tissue replacement started to gain more acceptance as an alternative treatment option. Different approaches were suggested in regard to the tissue source for replacing a damaged meniscus, including: synthetic matrix grafts, tissue engineering modalities and donor meniscal graft transplantation [29]. The first two options are currently evolving and studies are being published on what tissue source will be optimal; however, the question that has not been answered is whether the new tissue will be just a replica of the meniscus or can the engineered tissue actually undertake functions of the native meniscus [1, 29]. For this reason, properly selected allograft transplantation will be considered as the most reliable option with similar functional and structural characteristics. The first published study on replacing the meniscus tissue was done by Lexer [93], who used an autogenous fat tissue interposition inside the knee in an attempt to

restore normal joint function [94]. Numerous other animal model studies followed using different autogenous tissues for meniscal replacement [95-99]. Unfortunately, degenerative changes with poor clinical results were evident following those innovations, indicating that autologous tissues had only temporary protective effects and they were not suitable options as meniscal substitutes [96-98].

It was clear that autograft tissues were functionally inadequate at replacing the meniscus; therefore, meniscal transplantation was suggested for patients who had undergone total meniscectomy to prevent the joint degeneration that follows meniscectomy [100]. The concept of donor meniscus transplantation was first brought into attention in 1984 by Milachowski [101] who performed the procedure on sheep and human knees. Since then, several clinical studies have been carried out examining the feasibility and efficacy of human meniscus transplantation. Patients involved in most of the published studies reported pain relief, functional improvement and overall improvement in the functional assessment score used in that study [102-107]. A potential chondroprotective effect has also been suggested [104, 108] although some debate exists [109] due to the lack of a control group in most of those studies, indicating that there is no explicit evidence that meniscus allografting stops the progression of an already started articular degeneration.

Clinical guidelines for meniscal transplantation have been determined [110, 111]. Indications include: 1) history of total meniscectomy with pain localized to the meniscus-deficient compartment in young patients (< 50 years of age) in a stable and well aligned knee

with no advanced cartilage degeneration; 2) history of medial meniscectomy in an ACL-deficient knee which provides additional stabilization if combined with ACL reconstruction; and 3) concomitant osteotomy in patients with axial malalignment as it helps to enhance the result of high tibial osteotomy [111]. Prophylactic transplantation is not routinely recommended in patients with no symptoms because patients usually have no symptoms in the early years after meniscectomy [110] and there is no long-term evidence that the transplantation will prevent articular degeneration. Results of meniscal allograft transplantation (MAT) are dependent on technique used for surgical fixation, the clinical scoring systems and patients' compliance with follow-up times [111]. Interestingly, with recent advances in surgical techniques for fixation, utilizing new devices that facilitate repair, good outcomes have been reported [112]. Moreover, in a meta-analysis study of 44 published English-language trials reporting outcomes of meniscal allograft transplantation in humans, the authors concluded that "*Meniscal allograft transplantation is a safe, reliable procedure and should no longer be considered experimental" [113]*.

Success of MAT depends on proper size and shape matching of the transplanted meniscus to the native meniscus as well as the presence of viable cells to maintain the biosynthetic activity and biomechanical properties of the tissue [15, 114]. Based on this, fresh-harvested grafts will provide the highest number of live cells with no structural deterioration. However, fresh grafts can maintain cell viability for only a short period of time and require proper size matching to the recipient's knee. Currently, meniscal allograft transplantation is not so widely applied due to the limited number of available grafts, need for allograft sizing, screening for blood borne disease, loss of cells during storage and difficulty to treat partial meniscectomy (as only whole meniscus

transplantation has been carried out) [115]. Those limitations can be mitigated by appropriate meniscal preservation techniques. The aim of proper preservation methods is to create a meniscus tissue bank where donor menisci can be stored after screening that will allow surgeons to choose size-matched allografts from a pool of available shapes and sizes [116].

1.3 Cryobiology and its Potential Role to Enhance Meniscal Tissue Banking

1.3.1 Cryopreservation

The shortfall in meniscal tissue availability in relation to the total number of transplants that are needed requires the presence of a meniscus tissue bank. In the past, different meniscal preservation methods were investigated including: freeze-drying, lyophilization and simple cryopreservation. All of those methods resulted in alteration in the tissue structure with less than optimal functional characteristics. Consequently, there is still a need for a proper preservation method that can prevent those issues during and after storage. Vitrification, as an alternative to conventional cryopreservation, holds a promising option that maintains cells viability and preserves the complex matrix architectural characteristics for indefinite storage by using cryoprotectant agents (CPAs) [117]. Glycerol and dimethyl sulofoxide (DMSO) are the two most commonly used CPAs to preserve the human menisci and have been shown to cause very minimal alteration in the meniscal collagen array [118]. This section will provide an overview to the science of cryobiology and the potential to improve meniscal tissue banking.

In all biological systems, water acts as a solvent and medium for biochemical reactions. Since cellular metabolic reactions are temperature dependent, a decrease in the water temperature can either slow down or stop those reactions. These low temperatures can either destroy cellular material, due to ice formation, or preserve it for a longtime with no detectable biochemical activity [119, 120]. Cryopreservation involves exposing biologic cells and tissues to subzero temperatures (to -80 ºC or even to -196 ºC) with the purpose of minimizing damage to cells and

the matrix [121]. One form of cryopreservation involves the cooling of the biologic cells and tissues under controlled conditions in the presence of chemical compounds termed "cryoprotectant agents (CPAs)". These agents have the ability to lower the freezing point of the solution resulting in relatively unfrozen liquids at subzero temperatures. These agents are also known as anti-freeze when used for non-biological purposes. The term cryoprotectant is functionally based and is used to describe any chemical compound that when introduced to cells before cooling can protect them from freezing injury and improve their post-thaw survival [121]. Moreover, they have the ability to dissolve in water (by forming hydrogen bonds with water molecules) and penetrate through the cell membrane to prevent freezing in the intracellular environment [120]. The process of successful cryopreservation involves many steps that are based on biophysical and chemical aspects related to the cells and CPAs being used [122, 123]. Having a basic idea about the process of cryopreservation will simplify our understanding of some of the common mechanisms of cell damage during this process. Generally, cells are incubated for a pre-determined period of time in solutions into which the cryoprotectant agents have been dissolved. Once a sufficient concentration of the CPAs is reached within the cells, they are cooled to subzero temperatures, either slowly or rapidly, then kept mostly in liquid nitrogen (LN_2) for storage [119]. Finally, prior to use, the specimen must be warmed to normal temperature and washed free of CPAs. Nevertheless, the process is not straightforward and involves many potentially damaging effects that occur to the cooled cells [122]. The damage is not purely due to the storage at the subzero temperature but will most likely happen during the progression to low temperatures and when trying to warm up the cells while removing the CPAs [124]. This damaging effect is termed "cryoinjury", which has been studied in many cell lines [125-127]. A significant amount of studies have been produced to describe the mechanisms by which cooling can induce cell damage [126, 127]. Most of these studies investigated individual

cell lines in suspension. In the surrounding solution containing CPAs water will crystallize, thus creating two phases: unfrozen (liquid) and frozen (solid) [119]. This will lead to high osmotic concentration in the the unfrozen portion resulting in an osmotic gradient to force water out of the cells by exosmosis [128, 129]. At this stage, ice probably has not formed inside the cells and the cytoplasm will be in a supercooled (unfrozen) state. If, at this point, cooling happens slow enough to allow exosmosis to neutralize the concentration gradient, ice crystals will not form inside the cell [119, 130]. Whereas if the cells are coooled very rapidly there will not be enough time for the water to leave the cells in order to equilibrate the concentration outside the cells and ice crystals will form inside the cells [130]. In practical application it has been found that the use of two-stage cooling (slow cooling to relatively low subzero temperture followed by rapid cooling) is better with some limitations in different cell lines. Ice formation does not allow the inclusion of impurities, thus all the solutes, including the unbound CPA, in the solution will be excluded from the growing ice crystals leading to a hypertonic environment in parts of the solution (on the sides, between cells) with a pocketing effect [119]. Ice formation, especially intracellularly, usually leads to cell death [129, 131]. Moreover, the high electrolyte concentrations are also damaging to cells [132, 133]. As solute concentration plays an important role in the ability to lower the freezing point until lower temperatures are reached, higher concentrations of one or more CPAs are needed to achieve good penetration into the cells. Unfortunately, these high concentrations expose the cell to toxic effects. Given these points, injury to cells occurs by the mechanical effect of crystallized water, high solute concentration and CPA toxicity [134]. Moreover, it has been reported those mechanical forces can induce an apoptotic pathway resulting in cell death [38, 135]. Meanwhile, when trying to apply this knowledge to cryopreserve complex multicellular tissues, such as the meniscus, the process appears to be more complicated and difficult compared to individual cells [136]. In complex

tissues, difficulty stems from the presence of different cell types, which may respond differently, the presence of extracellular matrix and sometimes the presence of vascular channels [137, 138]. This means that the cryoprotectant agent must penetrate (non-uniformly) all cells and, reach equilibrium with the surrounding components and all the cells that are important for the function of the tissue [137]. Also, when ice forms outside the cell it is still inside the system and can directly damage the tissue [136, 139]. Thereby, damage must be avoided in the extracellular matrix to maintain structural integrity and maintain connections between cells [125]. To complicate the matter, in vascularity-dependent tissues, when water freezes its volume increases by 4-10 %, which can lead to permanent mechanical damage that will prevent the blood flow to the tissue following thawing or after transplantation [125, 139, 140]. In this case, even if the cells survive the process, the tissue will still be considered damaged due to the lack of vascular supply. Furthermore, the high solute concentration in blood vessels, due to ice formation, will draw water out of the rest of the tissue resulting in harmful shrinkage and dehydration [139]. Other complicating factors that need to be taken into consideration when designing studies aimed at cryopreservation of complex tissues include: tissue water content, volume of the tissue and the fact that ultra-rapid cooling is technically problematic for tissues or organs due to heat transfer [122].

Despite high throughput studies, attempts at cryopreserving complex tissues by extrapolating techniques from single-cell systems have proven unsuccessful. A few studies have attempted to cryopreserve the meniscus. Gelber et al. [141] studied frozen menisci (-80ºC for 7 days) in the absence of cryoprotectant agents using twenty-six human menisci harvested during total knee replacement procedures. The authors reported a disruption of the collagen architecture

with thinning of the fibril diameter compared to the control group. Interestingly, two years following the previous study [141], the same group, Gelber et al. [118] investigated the effect of cryopreservation in the presence of CPA. This time the authors investigated cell viability in addition to collagen architecture disarray and collagen fibril diameter. Ten human menisci were immersed in 10 % DMSO at 4 ºC for 30 minutes and then slowly cooled to -80 ºC. After 14 days, the samples were thawed by immersion in a 37 ºC sterile saline solution for 2 minutes. Their results showed a disarray of the fibril diameter with a mean cell survival of 23 % (ranging from 4 to 54 %). The range of cell survival varied greatly and that was attributed to the fact that cell viability was significantly different from one location to another within the meniscal samples [118]. Villalba et al. [142] investigated meniscal chondrocyte apoptosis as a possible factor contributing to cryopreservation failure. Menisci were kept in 10 % DMSO and slowly cooled in two steps (-1 ºC/min until -80 ºC and then -5 ºC/min until -120 ºC) followed by storage in liquid nitrogen. Following the removal of DMSO, results showed cell death evidenced by cytoplasmic changes with abnormal nuclei [142]. No clear pathway of cell loss was identified and the authors speculated that the physical stress exerted on cell membranes during the freeze-thaw process might be the one possible route. Apoptotic activation (molecular-based cell death) in response to low temperature exposure has also been reported in a variety of systems including but not limited to renal cells, fibroblasts, vascular tissue, hepatocytes, ovarian tissue, etc. and it often takes hours to days to manifest [121]. Similar results were reported in implanted aortic allograft valves, which showed endothelial cell loss and the contributing factors were suggested as immunological and chemical injury, hypoxic damage, and reperfusion injury at the time of implantation [143]. Interestingly, it has been reported that cell viability can be improved by inhibiting parts of the apoptotic pathway [38] via inhibition of the intracellular enzymes that lead to cleavage of intracellular proteins and DNA and, the formation of apoptotic bodies [144]. Laouar et al. [131]

reported structural alteration and biochemical changes, due to ice formation, in porcine cartilage cryopreserved using 1M DMSO. All things considered, clearly, there is a need for an alternative method of cryopreservation that can prevent ice formation to solve the issue of human meniscal tissue cryopreservation and banking.

1.3.2 Vitrification

Vitrification is an alternative form of cryopreservation that can be achieved by increasing the solution's viscosity, using very high concentrations of CPAs, sufficiently enough that the solution solidifies with rapid cooling without permitting ice formation [145, 146]. The cryoprotectant agents in the solution block the water molecules from bonding with each other until the temperature is low enough to prevent further molecular motion [117, 147]. This will allow the solution to cool down below the glass transition temperature (Tg), which was found to be approximately -130 ºC for DMSO, resulting in the creation of an amorphous glassy state [147]. At this state, all biochemical reactions stop and the biologic tissues are essentially in suspended animation with no aging or damage occurring until the temperature is increased; thus preserving cell viability and matrix integrity [147]. It is also believed that any material can be vitrified as long as it is cooled fast enough to prevent crystallization [146]. One main obstacle to vitrification of biologic tissues is that a rapid cooling rate is difficult to achieve. Therefore, to compensate for this, very high concentrations of cryoprotectant agents are required to eliminate intracellular ice formation [148]. Consequently, for vitrification purposes, solutions with low CPA concentrations are almost impossible to use, as crystallization becomes unavoidable using typical cooling rates [148]. Although, in comparison to conventional cryopreservation,

vitrification avoids the problem of ice formation, other forms of cyoinjury can still take place. For instance, biologic systems cannot tolerate the very high and toxic levels of CPAs needed to vitrify making those levels less practical [149]. This indicates the need to reduce the amount of CPAs needed to vitrify to result in less toxic levels. Several methods have been suggested to counteract this toxic effect of the CPAs [124, 150].

A discussion about those methods will require a brief introduction to some of the basic principles of vitrification. Studies on vitrification were based on the understanding of the solid-liquid phase equilibrium of solutions under certain thermodynamic conditions that can be represented in a phase diagram. Parts of the phase diagram include two temperatures that will be of interest here [117]. First, the homogenous nucleation temperature, which is below the freezing point, is the lowest temperature at which super-cooled liquids start to nucleate as they form ice crystals that can grow to result in ice formation [145]. Second, is the glass transition temperature (Tg) at which supercooled liquids start to vitrify, at or below -130 ºC as in the case of DMSO. On the diagram, the point in concentration that results in an intersection between these two points is termed "concentration needed to vitrify (C_v)" [117]. The first suggested method to reduce (C_v), includes a temporary increase in the solution's hydrostatic pressure during cooling. This increase in pressure will lower the nucleation temperature and increase the glass transition, thereby shifting the point of interaction between the two temperatures to a lower concentration of CPA [139, 145]. Once the experimental temperature has been lowered below Tg, then the pressure can be released with no risk of crystallization. However, this must be done carefully as the release of the pressure below Tg can cause a degree of tension that may result in fracturing of the formed glass-like structure. Thereby, this method is practically challenging and also not readily available

in most laboratories [148]. The second method is to use a combination (cocktail) of penetrating CPAs, each of which is below the toxic level [151]. The third method is to use CPA toxicity neutralizers (e.g., acetamide with DMSO). The fourth method is to add extracellular solutes in the form of non-penetrating agents (at lower concentrations) to reduce excessive exposure to the penetrating agents [150]. In this mixture, the permeating agents will facilitate intracellular vitrification, while the non-permeating agents will facilitate extracellular vitrification both leading to vitrification of the entire tissue [124]. However, enough time must be permitted for those agents to equilibrate with the tissue structures [152]. The fifth method is to reduce the time and temperature at which tissues are exposed to CPAs [124]. The sixth and final method advocates appropriate selection of carrier solutions [124]. It is also important to mention that these, and other modalities, must be used judiciously because low concentrations of the CPAs will increase the chance of devitrification during warming [148].

Currently, vitrification appears to be the most likely approach to successful cryopreservation of tissues. Much progress has been made towards achieving this goal with different tissues [149, 153-155]. A common factor that can be seen in most of those studies that successfully achieved vitrification is: having enough understanding of the cryoprotectants behavior in these tissues. Fahy et al. achieved successful vitrification of rabbit kidneys. Although the kidneys showed some minimal damage and small areas of devitrification, those kidneys recovered fully and were able to support life after re-implantation [156]. Through experimental work on the CPAs scientist were able to further classify CPAs based on their glass-forming ability.

1.3.3 Cryoprotectant Agents

Cryoprotetcant agents are water soluble chemical solutions that can block water molecules from binding to each other by forming hydrogen bonds with water, until the temperature is low enough to prevent further molecular motion [147]. In 1949, Polge et al. [157] discovered the cryoprotective action of glycerol during their efforts to preserve ovine spermatozoa, making it the first CPA to be discovered. Following the discovery of the glycerol action, many other solutes were found to have a cryoprotective activity [158]. Examples of these solute include: alcohols, sugars, amides and even amino acids. All of these solutes differ in their water binding affinity, effciciency in preveting ice formation, glass forming ability and toxicity. It has been reported that these agents have multifactorial modes of action but still there is limited understanding of all aspects of protection offered by these agents [159]. Interestingly, due to their ability to protect biological tissues from freezing injuries, some of the proposed modes of action have been linked to the theories of freezing injury [158]. Suggested modes of action include: ability to form hydrogen bonds with water molecules, freezing point depression, the tendency to interact with cellular proteins (to aid with intracellular vitrification) [159, 160], ability to stabilise the cellular phospholipid membrane [159, 161], mild anti-bacterial action (especialy alcohol-based solutes) [159, 162] and hydroxyl free-radical scavengers [163].

These agents can be divided into two main groups: permeating and non-permeating cryoprotectant agents [133, 164, 165]. Permeating (intracellular) cryoprotectant agents have low molecular weight which allows them to cross the cell membrane (e.g., glycerol and DMSO) and they are most effective in slowly cooled systems. Additionally, permeating agents require high

molar concentration to protect target tissues. These permeating agents usually are added gradually to prevent the osmotic stress and removed slowly to allow the gradual removal of CPAs from the tissue. On the other hand, non-permeating (extracellular) cryoprotective agents have relatively higher molecular weight and thereby, cannot penetrate cells (e.g., polyvinylpyrrolidone "PVP" and sucrose) and they are more effective at protecting biological systems cooled at a rapid rate [165, 166]. Non-permeating agents cannot be used alone for tissue preservation and most vitrification solutions contain a combination of permeating agents and low concentrations of non-permeating agents. Moreover, due to the low concentrations and toxicity margins of non-permeating agents, there are no elaborate studies focusing on investigating those agents. In 1959, Lovelck and Bishop introduced the use of DMSO as a CPA with better permeability compared to glycerol [158]. The most commonly used cryoprotectants are DMSO, propylene glycol and glycerol. In 1953, Lovelock et al. [167] stated that it was necessary for glycerol to permeate human erythrocytes if cell damage was to be reduced after slow freezing to -80 ºC, and that adding non-permeating agents may offer protection at -196 ºC [168]. In a study by Taylor et al. [169] comparing the protective effects of permeating agents (glycerol and DMSO) versus non-permeating agents (PVP and sucrose) by assessing the viability of Chinese hamster ovary (CHO) cells after cooling and storge. Their results showed superior protection in the permeating agent group compared to the non-permeating agent group. Additionally, they reported a more rapid uptake of radioactive carbon-14 glycerol (^{14}C glycerol) at 37 ºC and it decreased with the fall in temperature [169]. This pattern of low uptake at lower temperature shows similarity to published results by Bickis [170] who studied the permeation kinetics of glycerol and DMSO on Novikoff hepatoma ascites cells.

Successful vitrification is contingent upon the use of extremely high concentrations of permeating agents that have been dissolved in a carrier solution [148, 171]. These concentrations can be expressed in different forms as: percent by mass or volume, molarity or molality. Molarity (M) is the most commonly used method for expressing concentrations and it refers to the number of moles of solutes in 1 liter of solution, whereas molality (m) is the number of moles of solutes in 1 kilogram of solvent. Due to the temperature effect on the volume of the solution, molality can provide a measure of concentration that is independent of temperature changes.

Since the aim of tissue exposure to CPAs is to achieve sufficient concentration inside the tissue before cooling, it is very important for the tissue to reach equilibrium with the surrounding solution [152]. The final concentration inside the tissue, at equilibrium, can either be close to or lower than the concentration of the surrounding solution depending on certain factors [152]. Factors that have been reported to influence the permeation include (but are not limited to) duration of exposure, temperature at exposure, tissue water content, volume and geometry of the tissue. At higher temperatures, equilibrium happens more rapidly allowing shorter incubation times. However, cryorotectant related cytotoxicity has also been found to have a temperature dependancy [172]; a finding that had led to the introduction of CPAs into tissues at lower temperatures [172]. The extent of cryoprotectant permeation is also described in relation to the tissue water content as it determines the space available for the CPAs to perform their action and a potential dilution effect. For instance, if the tissue has 80 % water and 1 ml of the tissue is placed in 1 ml of 1M DMSO, the final concentration in the tissue will be close to 0.5 M due to the dilution effect by tissue water [139]. Additionally, the volume and geometry of the tissue have important roles in the ability of the CPA to equilibrate fully with parts of the tissue. In

studies performed on rabbit and dog kidneys, a discrepancy was reported in the equilibrium permeation rate of DMSO between the innermost (medulla) and outer portion (cortex) of the kidney [173, 174]. Mulderw et al. [175] reported that, due to the thickness of the cartilage, DMSO had insuffienct protection to the deeper layer while providing protection to the suprficial layers. This indicates that the CPA has to travel through all layers to achieve adequate permeation. Moreover, the edge of the cartilage was not an effective port of permeation [175].

The movement of CPAs into tissue happens as a diffusion process that is based on the concentration gradient. However, it has been reported for certain tissue such as the articular cartilage that factors other than the concentration may have a role in the process [152, 176]. Quantative analysis of CPA diffusion in cryobiology commonly uses Fick's laws of diffusion [177, 178]. Fick described two laws that govern the diffusion of particles across a semi-permeable membrane [179]. These laws mainly describe the amount of particles that will move through a unit area per time. The flux goes from areas of higher concentration to those of lower concentration and the driving force is proportional to the concentration gradient. This means that the rate of flux will decrease with increased time, due to lowering of the concentration gradient as more particles move to the other area [179]. Factors that can lead to more particles flowing to the lower concentration area: 1) decreasing the thickness, which will decrease the distance travelled by the particles, 2) use molecules with a smaller molecular weight as they can move faster, 3) increase the concentration by adding more molecules, and 4) having a larger surface area of exposure. Several methods can be used to measure the CPA concentration inside the tissue (permeation) whether directly or indirectly [170, 176, 180-188]. Some of the methods are technically demanding with variable accuracy [124]. Proton nuclear magnetic resonance is a non-

invasive and accurate method that measures the spatial and temporal concentrations of CPAs in the tissue being investigated.

1.3.4 Cryoprotectant Agent Permeation into Joint Tissues

Currently in the literature there are no reported data on the CPA diffusion and permeation kinetics into the human meniscus. Fortunately, the meniscus and the articular cartilage (AC) share some structural similarities. By using permeation data reported for articular cartilage [176, 182, 183, 189], a tissue that has been proven to be extremely difficult to cryopreserve [132, 147, 190-196], we will have a basis for comparison and extrapolating points of relevance. To add to the dificulty, in hydrated tissues such as articular cartilage, the permeation process is additionally influenced by the fixed charge density and the pressure difference [152, 176]. The presence of GAGs in both the articular cartilage and the menisci gives them a fixed negative charge. Shaozhi and Pegg investigated the effect of this negative charge and their findings showed the higher negative charge densities to result in difficult permeation of glycerol and DMSO into ovine articular cartilage [176]. The average CPA concentrations were 9.8 %, 9.1 % and 8.2 % for negative charge densities of 1000, 2000 and 3000, respectively [176]. From a structural point of veiw, compared to the menisicus, the articular cartilage contains more proteoglycans (eight to ten times more) [42]. This makes the cartilage more hydrous which reflects the volume inside the tissue available for the CPAs to diffuse to [192]; however, the meniscus has a larger volume compared to cartilage samples. Moreover, the harvesting process of articular cartilage samples, in the form of cored cylindrical osteochondral dowels, requires the inclusion of a bone base at the bottom of the cartilage. When cartilage is exposed to CPAs, the bone at the base acts as barrier

for permeation [183, 197]. Unfortunately, cryopreserved cartilage dowels still showed low post-thaw survival of the chondrocytes, especially in the middle layer of the cartilage [175]. Jomha et al. [190], in an effort to overcome the barrier issue and to improve the chondrocytes recovery in this layer, provided access to the CPA by drilling a 2 mm hole in the base of the bone. Their findings showed improved central and overall recovery of chondrocytes in the dowel but it was still less than satisfactory [190]. Nevertheless, in a permeation study aimed at measuring CPA concentrations inside porcine cartilage, the authors reported that removing the bone base had no significant impact on the diffusion coefficients [182]. Although, removing the cartilage from its bony attachment had no significant effect on the diffusion coefficient, there was a more rapid permeation without the bony attachment [182].

Several studies have been performed investigating the permeation of cryoprotectant agents into articular cartilage. Our focus will be on studies that used a methodology adopted by our laboratory, which have led to successful vitrification of articular cartilage [155]. Sharma et al. [182] described a novel method to examine the time-dependent permeation of DMSO and PG into six cartilage dowels of mature pigs. Cartilage samples were exposed to vitrifiable concentrations, 6.8 M (51 % wt/wt), of DMSO and PG. To determine the time dependency of the permeation six time-points were used (0, 15, 30, 60 min, 3, 6, and 24 hr); while temperature dependency was determined by using three above-zero experimental temperatures (4 ºC, 22 ºC, and 37 ºC). After determining the water content in those dowels, the final concentrations of CPA were expressed in moles of CPA per volume of space available for aqueous solution inside the cartilage. The effect of water within the AC matrix on lowering the concentration of the surrounding solution was negligible [182]. The authors used a backtracking method to determine

the total concentration of the CPA that went into the tissue. Knowledge of the initial concentration of the CPA allowed the determination of the total difference between the initial concentration and the sum of all the readings for a particular sample [182]. Maximal permeation after 24 hr of exposure was 99 % (37 ºC) and 88 % (22 ºC and 4 ºC). The CPA concentration within the matrix of the cartilage was calculated after calculating the diffusion coefficient *(D)* by applying Fick's second law of diffusion for a perfect cylinder. Diffusion coefficients were modeled on an Arrhenius plot and the temperature-dependent activation energies were then calculated. DMSO permeation was 75-90 % depending on the temperature and the bony attachment, while PG permeability was 62-84 % of the starting concentration [182]. Findings from this study were close but not exactly the same as other reported values in the literature. In a similar study, Jomha et al. [189], improved and optimized the previous study [182] by adding more parameters to the study. The number of CPAs was increased to four (DMSO, propylene glycol, ethylene glycol, and glycerol) at 6.5 M, more time points and determination of cartilage weights prior and after incubation for more accurate results. To be finally able to calculate the concentration of CPA that had permeated the AC disc certain mathematical equations had to be applied. Similarly, in correspondence with the results from the previous study [182], CPAs demonstrated an initial rapid increase in the concentration within the cartilage matrix, then gradually slowed down. Also, at 37 ºC in the early time points, the permeation rate was the fastest for all agents tested. Furthermore, calculated activation energies provided the ability to predict the permeation rates at any temperature leading to enhanced mathematical modeling [189]. Carsi et al. [198] performed a study to determine the permeation of DMSO and glycerol into intact human articular cartilage using proton nuclear magnetic resonance (H-NMR). They reported that human articular cartilage was more permeable to glycerol than DMSO; however, they both showed similar diffusion coefficients. This knowledge of the permeation kinetics of

each CPA will provide useful information regarding the best duration of tissue exposure that can be used to minimize toxicity due to prolonged exposure to achieve a specific concentration within the tissue. Based on this knowledge, different studies were designed to focus on CPA toxicity [199] and the ability to achieve vitrification of articular cartilage using less toxic concentrations of CPAs by utilizing combinations of these CPAs. Weiss et al. [200] used a statistical prediction method to assess the contribution of individual CPAs to the vitrifiability and glass stability of a solution containing 3-5 CPAs. They reported a large difference between different agents with propylene glycol displaying the most contribution to vitrifiability [200]. Moroever, of the 164 multi-CPA combinations created, certain combinations were more effective than others and this was partially linked to the contribution of the individual CPA in the combination to vitrification and the interaction between different CPAs [200]. Fortunately, in 2004 Song et al. [201] reported their success at vitrifying rabbit full-thickness cartilage plugs using a solution of three cryoprotetcive agents. However, it was not until 2012 when Jomha et al. [155] published their breakthrough study that achieved successful vitrification of intact, thick human articular cartilage. Looking back at the studies performed to achieve vitrification of the articular cartilage, clearly there has been a tremendous amount of work and investigations. Generally, those preliminary studies focused on studying the structure of the articular cartilage, tissue dimensions, diffusion of individual CPAs into the cartilage, determining the permeation kinetics, CPA toxicity (individually and in combinations), and then the ability to form glass structure.

1.4 Study Rationale and Hypotheses

The theoretical literature is currently lacking any report on the cryoprotectant agent behavior with the meniscus, a complex tissue that is expected to exhibit difficulty with vitrification. Moreover, the three-dimensional geometric shape of the meniscus adds to the existing complexity when compared to the articular cartilage. Thereby, based on our prior knoweldge of the cryoprotectant behavior with the articular cartilage, we hypothesized that if we preload intact porcine menisci with 6M ethylene glycol and glycerol, then we will show the difference in the efflux rate between EG and glycerol at three different temperatures to determine their permeation kinetics in the future. This hypothesis has led to the development of three major objectives, each will be presented in a separate chapter. The first objective was to understand the behaviour of the porcine meniscus to two cryoprotectant agents commonly used in vitrification solutions, namely ethylene glycol and glycerol. The secondobjective was to determine the overall and regional water content of the porcine meniscus. The third objective was to measure the meniscal tissue size and dimensions of the human meniscus and compare them to the porcine and ovine meniscus.

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2 Chapter 2. Ethylene Glycol and Glycerol Loading and Unloading in Porcine Meniscal Tissue

2.1 Introduction

The prevalence of meniscal injuries has led to the development of various approaches to alleviate patients' symptoms [1]. Arthroscopic meniscal repair and removal of all or damaged portions of the meniscus (total or partial meniscectomy) are two of the most commonly employed surgical approaches in meniscal injuries [2, 3]. Unfortunately, both of these approaches suffer from anatomical and biomechanical limitations, rendering them temporary solutions in relieving injury-related symptoms. For instance, arthroscopic repair is limited to the vascularized 10-30 % portion of the meniscus [4, 5] and arthroscopic meniscectomy creates an area inside the knee joint that is devoid from protective meniscal tissue [6, 7], resulting in poor long term outcomes with degenerative joint lesions [7-11] that will advance to osteoarthritis. However, as the presence of an intact meniscus is essential for normal function of the knee joint [12], meniscal allograft transplantation (MAT) is an effective alternative that can restore normal knee anatomy and biomechanics in patients with a non-salvageable meniscal injury [13-17]. Success of MAT depends on proper size and shape matching of the transplanted meniscus to the native meniscus [18, 19], as well as the presence of viable cells to maintain the biosynthetic activity and biomechanical properties of the tissue [20]. In addition, the procurement of infection-free allograft [21, 22] is another essential factor to ensure the recipient's safety. Freshly harvested meniscal allografts provide the highest number of viable cells [23] and, for best results, tissue recovery should be done within 4 to 6 hours after donor death [24]. However, fresh allografts can maintain live cells for up to 5 days at above 4 ºC [25, 26] thereby demanding fast transplantation.

This short time frame, along with the need for size matching and infectious disease testing may limit the clinical application of fresh meniscal grafts.

The availability of a long-term storage method for meniscal tissue would allow donor menisci to be stored in a tissue bank, leading to an increased number of available grafts, and would provide the necessary time for size matching, infectious disease screening and surgical team coordination [27, 28]. Commonly used meniscal preservation techniques such as deep freezing and conventional cryopreservation have been found to result in less than optimal survival and biochemical changes in human and animal menisci [23, 25, 29-33]. Gelber et al. [31], using transmission electron microscopy, reported thinning and disruption of collagen fibrils in 13 human menisci after deep-freezing to -80 ºC for 7 days without using cryoprotectant agents (CPAs). Likewise, Reckers et al. [25] reported progressive loss of cellularity in 180 rabbit menisci at different freezing temperatures (-7.2 ºC, -21.4 ºC and -73 ºC), in the absence of CPAs, for a total of 30 days. The authors reported that 80 % of the cells maintained their viability at all three temperatures for up to 14 days followed by gradual cell loss and structural damage at longer durations and lower freezing temperatures [25]. In fact, another study has indicated complete loss of cellularity and tissue destruction after 2 weeks of deep freezing [30]. Cryopreservation, due to the use of cryoprotectant agents (CPAs) and a slow freezing rate to inhibit ice crystal formation in the tissue, is expected to be less damaging than deep freezing. Several studies have investigated the effect of cryopreservation on the meniscal tissue; all were carried out using protocols adopted from articular cartilage (AC) studies, and have shown reduced cell viability with structural deterioration [23, 30, 33]. Fabbriciani et al. [30] studied the post-transplantation effect of cryopreservation using 15 Tibetan goat menisci that were cryopreserved, using an AC based method. In their study, the harvested goat menisci were incubated at 4 ºC in 10 % Me_2SO

(dimethyl sulfoxide) solution for 30 min then taken through a cooling profile: 0 ºC to -40 ºC at -1 ºC/min; -40 ºC to -80 ºC at -5 ºC/min followed by storage in liquid nitrogen (LN_2) at -196 ºC for 2 weeks [30]. At the time of transplantation, menisci were thawed in a water bath at 37 ºC for 2 min then washed with phosphate buffered saline (PBS) to remove excess Me_2SO from the tissue [30]. After euthanizing the animals, the authors reported normal appearance of menisci at different examination periods with best results observed at 2 weeks [30]; however, after the second week cell number started to decrease drastically with fluctuation in the water content and remarkable reduction in the glycosaminoglycan (GAG) content [30]. Jackson et al. [23] cryopreserved 10 goat medial menisci that were incubated at 4 ºC for 60 min in a solution containing 3 % glycerol and 3 % Me_2SO, followed by controlled rate freezing (4 ºC to -5 ºC at 0.5 ºC/min; -5 ºC to -35 ºC at 1.0 ºC/min; -35 ºC to -120 ºC at -10.0 ºC/min) and then held in the vapor phase of liquid nitrogen (-156 ºC) for 30 days. The authors reported a post-thawing cell viability of 30 % with increased water content and reduced GAGs content when compared to control menisci [23]. Arnoczky et al. [33] determined this effect in 5 dog menisci that were incubated in a 4 % Me_2SO solution for 120 min at room temperature followed by controlled rate freezing to -100 ºC (0 to -4.5 ºC at -0.5 ºC/min; -4.5 ºC to -35 ºC at -1 ºC/min; -35 ºC to -100º C at -10º C/min) and then stored in liquid nitrogen (-196 ºC) for 7 days. The authors reported that cryopreservation did not affect the morphological appearance of menisci; however, it caused alteration in the mechanical properties with only 10 % of the cells remaining metabolically active [33]. Additionally, cells that remained alive were those near the surface, closest to the CPA whereas cells in deeper layers, away from the CPA, did not survive the cryopreservation process [33]. Another study also reported findings that were consistent with those from the previous study where many ice crystals were seen in animal menisci that were cryopreserved using an unspecified concentration of Me_2SO solution (the freezing protocol was not reported) [34].

Moreover, the cryopreservation protocol that was used caused tissue compartmentalization with separation of the cellular and extracellular components, resulting in weakened mechanical properties [34]. The previously reported findings indicate the need for a safer preservation technique that will not jeopardize the structural integrity and cell viability of the meniscus.

Vitrification or ice-free cryopreservation offers a promising means of preservation [35, 36] as it eliminates the problem of ice formation while maintaining the tissue's structural integrity with improved levels of cellular viability [37]. Success of the vitrification technique requires the use of very high concentrations of CPAs to keep the tissue super-cooled below -130 ºC in an amorphous glassy state [36, 38, 39], creating a suspended animation in which tissue can be stored indefinitely. Typically, CPAs are added and removed in a stepwise fashion of increasing concentrations in order to avoid the osmotic damage that can result from single step addition or removal. Designing appropriate tissue incubation times and temperatures, based on understanding the CPA permeation kinetics, is very important to minimize the osmotic damage. A well-planned approach to tissue vitrification looks at understanding the permeation kinetics of the desired CPAs as a first step upon which future studies can be built [40-43]. For the meniscus, the literature is currently lacking any report that can provide basic understanding of the CPA behavior or distribution in this complex tissue. Furthermore, as was mentioned earlier, most reports that attempted to cryopreserve the meniscus used methods that were adopted from articular cartilage. Nonetheless, there is one study [34] in the literature stating to have examined the mechanical properties in pig menisci that were vitrified in Me_2SO using techniques that were adopted from studies performed on different tissues. The authors only cited those articles without specifying the concentration, temperature, exposure times or cooling rate used from any of the cited studies [34]. However, this adaptation lacks accuracy as different tissues exhibit differences

in their permeability to certain CPAs due to variation in the permeability parameters, which need to be determined for the target tissue. Despite of all those limitations, Li et al. [34] reported that vitrified menisci displayed mechanical properties that were slightly lower but comparable with normal menisci.

Successful vitrification of complex, organized tissue is not easy [38, 44] and a comprehensive approach towards tissue vitrification starts with understanding the permeation kinetics of certain CPAs into the target tissue [42, 43, 45, 46]. Permeation kinetics can be accurately determined from applying mathematical modeling to experimental findings [43], including surface dimensions, in order to describe the mass transport of CPA through the extracellular matrix [39, 42, 47]. Our laboratory has published a report that achieved successful vitrification of intact human AC (a tissue that has been very difficult to cryopreserve) with more than 70 % cell viability [36]. This breakthrough was preceded by a series of extensive investigations to understand the CPA permeation and efflux behaviors [41-43, 48, 49]. Interestingly, the existing structural and functional similarities between the AC and the meniscus are expected to help us extrapolate and compare AC results to future findings in meniscus. However, unlike AC, the meniscus is a geometrically complex three-dimensional tissue and to address this complexity we performed an anatomical study that provided us with detailed dimensional measurements for the body of the meniscus (manuscript is being prepared for publishing). Thereby, this study aims to begin the understanding of the loading and unloading behaviors of EG and glycerol by monitoring efflux rate from lateral and medial porcine menisci after incubation with their respective CPA solutions for either 24 or 48 hrs Moreover, to determine the temperature effect, the efflux rate was measured at three different above-zero temperatures.

2.2 Materials and Methods

2.2.1 Solutions and Materials

Pure CPA solutions were purchased from Fisher scientific (Fair Lawn, NJ, USA). CPAs used were ethylene glycol (Certified, E178-1) and glycerol (Spectranalyzed®, G153-1). X-VIVO™10, a serum-free medium (Lonza Walkersville, MD, USA) was used to prepare all CPA solutions in the experiment. To reduce the risk of contamination, especially at higher temperatures, the X-VIVO™10 was supplemented with an iso-osmotic mixture of 100 units/ml penicillin, 100 units/ml streptomycin and 2mM L-glutamine (PSG) (Gibco, Life Technologies Inc.). Herein, the X-VIVO™10/PSG mixture will be referred to as X-VIVO-Plus. In sterile specimen containers, CPA solutions were dissolved to 6M concentrations in X-VIVO-Plus to yield 50 ml of glycerol or EG. Glycerol (mol wt. 92.09) solution was prepared by mixing 27.63 g of pure glycerol with 28.09 g of X-VIVO-Plus (49.5 % w/w). Ethylene glycol (mol wt. 62.07) solution was prepared by adding 18.62 g of pure EG to 33.37 g of X-VIVO-Plus (35.8 % w/w). Despite the miscibility of both CPAs with X-VIVO-Plus, the glycerol solution, due to its viscosity, was manually mixed using a pipettor prior to tissue exposure. All solutions achieved the desired temperature prior to use and the temperature was confirmed using a thermometer (Fisherbrand, Thermo Fisher Scientific Inc., MA). Following completion of tissue incubation in the respective solutions, Kimwipe® (Kimberly-Clark, Rosewell, GA) was used to remove excess fluid from the surface of the meniscus prior to transfer into a different solution. The molality in the washout solutions was determined using a freezing point depression osmometry device "µOSMETTE™" micro-osmometer (Precision Systems Inc., Natick, MA).

2.2.2 Tissue Harvesting and CPA Loading

Intact menisci were harvested from the hind leg of adult male and female Yorkshire pigs that were sacrificed for commercial meat consumption (Parkland Packers, Stony Plain, AB). The menisci were freed from the adherent joint capsule, and bony and ligamentous attachments, and washed in phosphate buffer saline (PBS) to remove excess synovial fluid. Each joint resulted in two menisci, namely, the medial and the lateral menisci. All menisci used in the study were visually intact with no visible tears or degenerative changes.

Each pair of menisci from the same joint were weighed (W_1) individually in a petri dish and assigned randomly to either the control group or one of the two treatment groups. For the treatment groups, menisci were maintained in (1) 50 ml, 6M EG in X-VIVO-Plus; or (2) 50 ml, 6M glycerol in X-VIVO-Plus. Menisci were kept in either glycerol or ethylene glycol solutions for a total duration of 24 hrs and under continuous shaking to insure proper mixing with the solution. Both treatment groups had three temperature sub-groups of 4 ºC, 22 ºC and 37 ºC. Each experimental temperature was maintained constant throughout the loading steps. A group of controls was assigned for each experimental temperature (4, 22 and 37 ºC) and control menisci were maintained in an identical fashion in 50 ml X-VIVO-Plus solution (free of CPA) for the same duration. Each group and specific experimental temperature had two sets: medial and lateral menisci. A total of six replications were performed at each treatment and temperature combination ($n = 6$), resulting in 18 pairs of menisci (lateral and medial) for each of the EG, glycerol and control group. Additionally, in a subsequent experiment due to the slow permeation of glycerol into the meniscus, we added a prolonged incubation period experiment (n = 3) that involved exposing menisci to same concentration of CPAs for 48 hr at 22 ºC only. Nine pairs of

menisci were required in this subsequent experiment. Therefore, a total of 126 menisci were used in this study, 63 medial and 63 lateral menisci.

2.2.3 Determination of Osmolality

At the end of the 24 hr or 48 hr CPA loading period, each meniscus was removed from the respective solution and patted lightly dry with Kimwipe® to remove excess fluid from the surface. To allow the CPAs to move out of the menisci under conditions identical to those used during the respective loading step, CPA-loaded menisci were placed in two consecutive specimen containers with 25 ml of X-VIVO-Plus solution, each for 24 hr. Solutions were homogenized by continuous shaking at the respective experimental temperature. Throughout the CPA unloading, 50 µL samples from the surrounding solution were pipetted at the following time points (1 min, 5 min, 15 min, 30 min, 60 min, 180 min, 360 min, 720 min, and 1440 min), and the sample osmolality was measured using a 'µOSMETE' micro-osmometer. The change in osmolality of the resultant, well-mixed CPA/X-VIVO solution was attributed to the CPA unloading. The osmolality of the washing solution was also determined before each immersion, and this measurement was considered as zero time (0 min). This value provided the baseline osmolality that was used to determine the moles of CPA in the solution. Pipetted samples were put back into the solution before taking the reading for next time point. The iso-osmotic nature of the PSG mixture was confirmed by measuring the osmolality of the X-VIVO™ 10 solution before and after addition of the PSG mixture (data not reported).

2.2.4 Calculations

Ideal dilute solution assumptions were made for all calculations and interpretation of data. To calculate the contribution to the solution osmolality by the effluxed CPA, the baseline osmolality (measured at t = 0 min), m_0, was subtracted from the solution osmolality measurements, m, for each time point. Moreover, to put all data on a comparable basis, since some individual menisci would be larger than others, the molality contribution was multiplied by the volume ratio of the efflux solution to the original solution volume in that particular meniscus based on meniscus weight. In addition, it was assumed that pure water has a density of 1 g/mL. Thus, the effluxed moles of CPA per meniscus solution volume in mmoles/litre is given by:

$$\text{effluxed CPA moles per meniscus solution volume} = (m - m_0) \times \left(\frac{1kg}{1L}\right) \times \frac{25\ mL\ of\ \text{X-VIVO-Plus}}{weight\ of\ the\ meniscus\ \times\ \%H2O\ \text{in the meniscus}} \times \left(\frac{1g}{1mL}\right)$$

where m has units mmolal or millimoles of solute per kg water. The % H_2O in the medial and lateral meniscus was determined from a separate desiccation study (data not shown). The water content of 6 medial and 6 lateral menisci were measured and averaged to yield constant values of 68.45 % and 68.96 %, respectively.

2.2.5 Statistical Analysis and Data Presentation

Data are presented as mean ± standard error of the mean of measurements. All statistical analyses were performed using SPSS version 21 (IBM Corp. Released 2012. IBM SPSS Statistics for Windows, Version 21.0. Armonk, NY: IBM Corp). The different temperature groups were compared using one-way analysis of variance (one way ANOVA) with Bonferroni post hoc tests. Comparisons between 24 hr incubation and 48 hr incubation groups, and between EG and glycerol groups, were made using independent t-tests. All statistical differences were considered to be significant with a p-value of less than 0.05.

2.3 Results

We used nine time points to compare the efflux of EG and glycerol from porcine menisci (lateral and medial) at three different temperatures while using two different incubation periods. Results are presented as figures and tables. All figures have four panels (graphs) presenting efflux data during the respective immersion period. In the graphs, data for the first and second immersions are presented separately in consecutive figures. For simplicity, the figures (1 through 8) can be divided into three sets with each set looking at the data from a certain perspective. The first set of figures (Figures 1 and 2) present the temperature effect after 24 hr incubation period. The second set of figures (Figure 3 and 4) compare the effect of the prolonged incubation period, 48 hr from the secondary experiment, to the 24 hr incubation for both CPAs. Finally, the third set of figures compare the efflux of EG versus glycerol within each experimental condition for lateral meniscus (Figures 5 and 6) and medial meniscus (Figures 7 and 8). Each set of figures is followed by a table comparing the sum of the final achieved millimoles accumulation at t = 1440 min from the two immersions.

The effects of the three different above-zero temperatures on the CPAs (millimoles) efflux from both menisci following a 24 hr incubation period are shown in Figure 2-1 (1^{st} immersion) and Figure 2-2 (2^{nd} immersion). Overall there was an extremely rapid rise in the millimoles of the EG and glycerol in the first 180 min during the first immersion. However, this increase did not show an observable peak as the millimoles of both EG and glycerol continued to rise. The rate then slowed down until it almost levelled off towards the end of the first immersion. Moreover, this marked efflux was more rapid for the higher temperature groups (37 ºC followed by 22 ºC) than the rate at 4 ºC (Figure 2-1). All temperatures showed a similar trend

in the efflux rate with varying levels of significance between individual time points across the three temperatures. In fact, at 37 ºC and 22 ºC there was a statistically significant difference ($p < 0.05$) between t = 60 min and t = 180 min, indicating that the additional millimoles that effluxed over these 2 hrs were sufficient to result in a significant difference. Whereas in the 4 ºC group there was no statistically significant difference ($p = 0.06$, 0.141, 1.0 and 0.9 for EG and glycerol in lateral medial menisci, respectively) between t = 60 min and t = 180 min. During the second immersion (Figure 2-2), the efflux of both CPAs (EG and glycerol) continued from both menisci at all the temperature groups, indicating the presence of some residual CPA within the matrix of the meniscus following the 1st immersion. The majority of this residual CPA appeared to efflux during the first 60 min of the 2nd immersion. Moreover, the final concentration achieved at the end of the 2nd immersion (t =1440 min) represented between 16 -21 % of the same concentration at the end of the 1st immersion for both EG and glycerol. Additionally, there were no statistically significant differences between medial and lateral menisci across the majority of the time points at the three temperatures.

To get an idea about the total millimoles of CPAs that effluxed from the menisci, the sum of the final accumulated concentrations were calculated from the end (t = 1440 min) of the two subsequent immersions (Figures 1 and 2) and averaged for each experimental temperature. This value is termed "average total millimoles of CPA effluxed per solution volume of the meniscus". Table 2-1 shows the total millimoles of CPA effluxed per solution volume of the meniscus where comparisons are made between the experimental temperatures following a 24 hr incubation period for every meniscus-CPA combination. At 37 ºC, the total millimoles of effluxed CPA per solution volume of the meniscus was significantly greater than the same value from both the 22 ºC and 4 ºC groups for almost all meniscus-CPA combinations. However, there was one

exception to the previous statement in the total millimoles of glycerol effluxing from lateral menisci where the 22 ºC group showed no statistically significant difference ($p = 0.07$) compared to that of the 37 ºC group. Furthermore, there was no statistically significant difference between the 22 ºC and the 4 ºC groups across all meniscus-CPA combinations (Table 2-1).

When looking at the results from the secondary experiment where menisci were incubated in the respective CPA solutions at 22 ºC for 48 hr and compared to the corresponding temperatures from the main experiment (Figures 2-3 and 2-4), the efflux rate showed a similar trend to the 24 hr results in both immersions. Despite the use of two incubation periods (24 hr and 48 hr), there was no change in the duration of the immersions (1^{st} and 2^{nd}) after CPA loading as each was performed over 24 hr. Correspondingly, the first 180 min during the 1^{st} immersion exhibited a rapid efflux of CPAs with slowing down in the latter time points (Figure 2-3). Moreover, when comparisons are made between the last time points ($t = 1440$ min) from both groups (24 hr and 48 hr), the millimoles of CPAs effluxed from the menisci showed no statistically significant difference between the two incubation periods ($p > 0.05$). Likewise, the second immersion showed a residual amount of CPA that effluxed during this period. In contrast to the 24 hr period, the sum of the two total millimoles effluxed at the end of the two immersions ($t = 1440$ min) for the 48 hr group had a greater amount of total efflux in almost all meniscus-CPA combinations (Table 2-2). The only exception was in the efflux of glycerol from lateral menisci where the 48 hr results showed a non-significant difference compared to the corresponding meniscus-CPA combination from the 24 hr group ($p = 0.21$).

To illustrate the differences in the efflux rates between EG versus glycerol, the results were plotted individually for each experimental condition (Figures 2-5 through 2-8). EG had a

significantly greater ($p < 0.05$) efflux than glycerol during the 1st immersion (Figure 2-5 and Figure 2-7) in the main experiment. However, during the 1st immersion in the secondary experiment (48 hr incubation period), there was no statistically significant difference between EG and glycerol at 22 ºC (Figure 2-5d and Figure 2-7d). Furthermore, in the main experiment, at the last time point (t = 1440 min) of the 2nd immersion, EG maintained an overall higher efflux than glycerol in three out of four groups. The only difference in the significance level between EG and glycerol at the end of the 2nd immersion in the main experiment was noticed at 37 ºC in the medial meniscus where the difference between EG and glycerol was not statistically significant ($p > 0.05$). Table 2-3 shows the sum of the values at the end of both immersions for each experimental temperature. The average total millimoles of EG (24 hr incubation period) showed a greater efflux than glycerol except in medial menisci at 22 ºC ($p = 0.07$). While following a 48 hr incubation period, glycerol achieved a similar average total millimoles with no statistically significant difference when compared to EG in both medial and lateral menisci (Table 2-3).

2.4 Discussion

This study was undertaken to provide preliminary data on the behavior of two of the most commonly used CPAs (EG and glycerol) with the porcine meniscus for the first time. Due to the lack of any report in the literature investigating CPA behaviors or permeation kinetics into the meniscal tissue, we studied the efflux rate at three different above-zero temperatures while using vitrification relevant concentrations. The CPAs were loaded into the meniscus in a single step and removed in two steps. The loading step was performed over two chosen incubation periods (24 hr and 48 hr). During the unloading steps, menisci were immersed in an isotonic X-VIVO-Plus solution with predetermined osmolality. Ideal solution assumptions were made in the calculations of the millimoles of CPA that effluxed out of lateral and medial porcine menisci. These calculations were based on the changes in the external concentrations of the washout solution (X-VIVO-Plus) over nine chosen time points. Changes in the solution concentrations were attributed to the CPA moving out of the meniscus driven by the concentration gradient. Moreover, due to the observed variation in the meniscus tissue sizes, all measurements were normalized based on the weight and percentage of meniscal water content (studied in the following chapter).

The findings of this study confirmed previously reported differences in the ability of CPA to move in and out of complex tissues [43, 49-52]. Despite the differences in the objectives and methodology used in those studies, this study determined that EG could move faster than glycerol in and out of porcine meniscal tissue (Figure 2-5, 2-6, 2-7, 2-8). However, when menisci were incubated for a longer duration (secondary experiment) glycerol was able to achieve higher concentrations that showed no statistically significant difference to EG. This means that the

initial chosen incubation period (24 hr) was not enough to permit full diffusion of glycerol into the meniscus but when doubled (48 hr) there was enough time for glycerol to load the meniscus (Panel "d" of figures 2-5 through 2-8). However, it is not clear why EG was able to achieve a more rapid efflux. The molecular weight is likely not the reason for this difference as both CPAs have very small molecular weights.

When examining the efflux rate following the two incubation periods, a similar trend was noted in the efflux of both CPAs with the highest rate during the first 180 min following either period. The rates then slowed down until the effluxed amount almost levelled off closer to the end. However, the average total millimoles of effluxed CPAs was statistically higher in the 48 hr group than the 24 hr group in 3 out of 4 meniscus-CPA combinations (Table 2-2). That is to say that more CPA moved into the meniscus when more time was allowed as 24 hours was insufficient. From a practical point of view, the incubation periods used in loading the menisci in this experiment are too long to be applicable in vitrification. However, to develop the vitrification process and minimize damage, a thorough understanding and characterization is needed of the CPAs used in the process.

The thermal effect on the efflux rates during the two immersion periods showed that the 4 ºC group had the slowest rate compared to the higher temperature groups. This confirms the temperature dependence of diffusion reflected by the efflux rate of CPAs used in this study. Moreover, during the second immersion, there was still continual efflux out of the porcine menisci following the 1st immersion, indicating the presence of some residual CPA in the matrix of the meniscus. Given that the incubation period and the washout period (immersion) were both carried out at the same experimental temperature, it would be safe to assume that there was more

CPA uptake (influx) by the menisci at higher temperatures. Since passive diffusion, which is a reversible and spontaneous process, controls the rate of solute diffusion until equilibrium is reached; based on the figures in this study, it is likely that equilibrium was not reached across all temperature groups by the end of the first immersion. However, we cannot say with confidence that at the end of the second immersion equilibrium was reached as no subsequent washout was carried out beyond that point and no spatial distribution of the CPA was determined.

2.5 Conclusion

This study aimed to provide basic understanding of the loading and unloading behaviors of EG and glycerol in and out of porcine menisci. Higher temperatures resulted in more rapid efflux of CPAs out of the meniscus. Moreover, EG showed a faster efflux rate than glycerol. Due to the slow influx of glycerol, the 24 hr incubation period was not sufficient to load the meniscus with glycerol. However, when the incubation duration was doubled, glycerol was able to achieve a sufficient level of meniscus loading, similar to that of EG. This indicates the length of time to be permitted in order to achieve optimal loading and unloading from the meniscus when designing future studies aimed at achieving successful vitrification of the meniscus.

2.6 Endnote

A version of this chapter will be submitted for publication, with the following authors: Talal Takroni, Hana Yu, Leila Laouar, Adetola Adesida, Janet A W Elliott, and Nadr Jomha.

Conflict of interest

Each author declares no conflict of interest through commercial association in relation to the submitted article.

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2.8 Tables

Table 2-1. Comparison of the "average total millimoles of CPA effluxed from the solution volume of the meniscus after two washes" comparing three experimental groups that were incubated for 24 hr and washed at three different temperatures.

Meniscus/CPA	Temperature Group	Total CPA (mmol/kg)	Compared with: (p-value)	
Lateral/EG	4 °C	5528 ± 183	22 °C	0.507
			37 ºC	0.000
	22 °C	6168 ± 145	4 °C	0.507
			37 ºC	0.005
	37 °C	7865 ± 489	4 °C	0.000
			22 °C	0.005
Medial/EG	4 °C	5504 ± 297	22 °C	1.000
			37 ºC	0.017
	22 °C	5757 ± 549	4 °C	1.000
			37 ºC	0.041
	37 °C	7383 ± 348	4 °C	0.017
			22 °C	0.041
Lateral/glycerol	4 °C	3969 ± 283	22 °C	0.180
			37 ºC	0.001
	22 °C	4785 ± 219	4 °C	0.180
			37 ºC	0.069
	37 °C	5802 ± 336	4 °C	0.001
			22 °C	0.069
Medial/glycerol	4 °C	3774 ± 359	22 °C	0.191
			37 ºC	0.000
	22 °C	4601 ± 172	4 °C	0.191
			37 ºC	0.020
	37 °C	5903 ± 313	4 °C	0.000
			22 °C	0.020

Table 2-2. Comparison of the average total millimoles of CPA effluxed from the solution volume of the meniscus after two washes between two experimental groups that were incubated (for 24 hr or 48 hrs) and washed at 22 °C.

Meniscus/CPA	Total CPA (mmol/kg)		p-value
	24 hr incubation	48 hrs incubation	
Lateral/EG	6168 ± 146	7259 ± 396	0.014
Medial/EG	5757 ± 549	6921 ± 398	0.006
Lateral/glycerol	4785 ± 219	6223 ± 277	0.211
Medial/glycerol	4601 ± 172	5604 ± 451	0.036

Table 2-3. Comparison of the "average total millimoles" of glycerol versus EG that effluxed per solution volume of meniscus after two washes within each experimental group.

Meniscus	Experimental group	Total CPA (mmol/kg)		p-value
		Glycerol	EG	
Lateral	4 °C/24 hr incubation	3969 ± 283	5528 ± 183	**0.001**
	22 °C/24 hr incubation	4785 ± 219	6168 ± 146	**0.000**
	37 °C/24 hr incubation	5802 ± 336	7865 ± 489	**0.006**
	22 °C/48 hr incubation	6223 ± 277	7259 ± 396	0.099
Medial	4 °C/24 hr incubation	3774 ± 359	5504 ± 297	**0.004**
	22 °C/24 hr incubation	4601 ± 172	5757 ± 549	0.072
	37 °C/24 hr incubation	5903 ± 313	7383 ± 348	**0.010**
	22 °C/48 hr incubation	5604 ± 451	6921 ± 398	0.094

2.9 Figures

Figure 2-1. Comparison of the thermal effect on the average molality (millimoles) of CPAs that effluxed per solution volume of lateral and medial menisci during the 1st immersion in X-VIVO-Plus. Average millimoles are shown for a) EG effluxing from lateral meniscus, b) EG effluxing from medial meniscus, c) Glycerol effluxing from lateral meniscus, and d) Glycerol effluxing from medial meniscus. Each graph displays results obtained from menisci that were incubated with their respective CPA for 24 hr then washed at three different above zero temperatures (i.e., 4 °C, 22 °C, and 37 ºC).

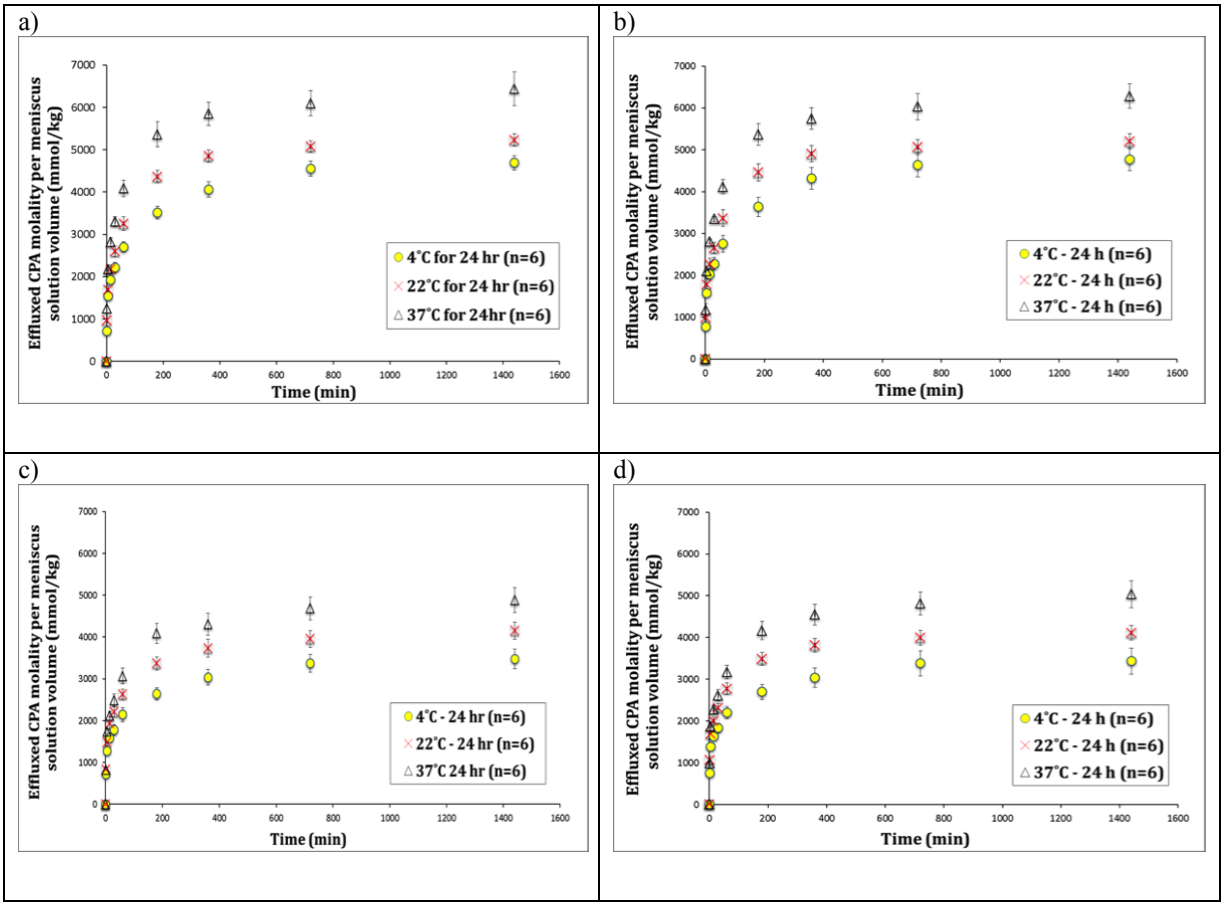


Figure 2-2. Comparison of the thermal effect on the average molality (millimoles) of CPAs that effluxed per solution volume of lateral and medial menisci during the 2nd immersion in X-VIVO-Plus. Average millimoles are shown for a) EG effluxing from lateral meniscus, b) EG effluxing from medial meniscus, c) Glycerol effluxing from lateral meniscus, and d) Glycerol effluxing from medial meniscus. Each graph displays results obtained from menisci that were incubated with their respective CPA for 24 hr then washed at three different above zero temperatures (i.e., 4 °C, 22 °C, and 37 ºC).

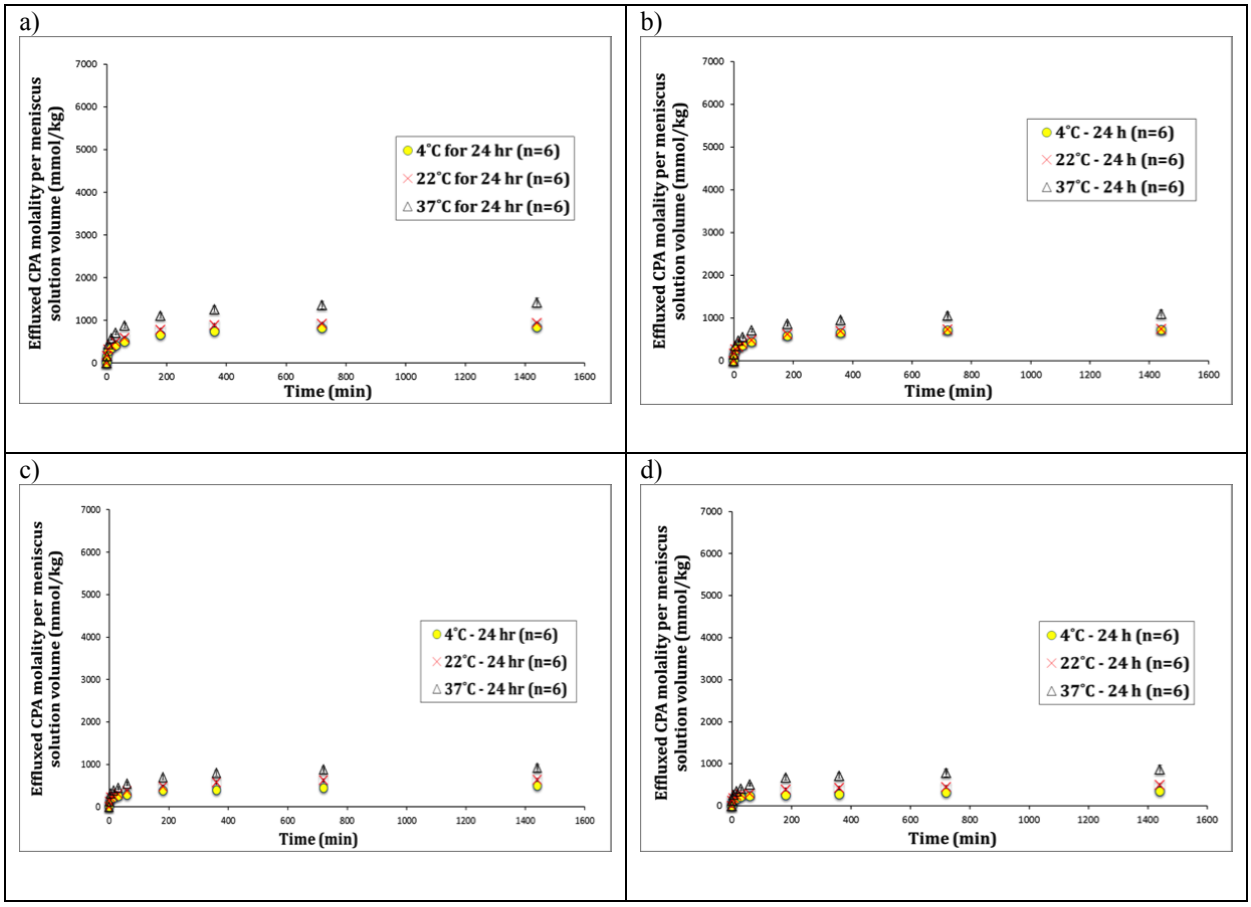


Figure 2-3. Comparing the effect of the two incubation periods (24 hr and 48 hr) at 22 ºC on the average molality (millimoles) of CPA that effluxed per solution volume of lateral and medial menisci during the 1st immersion in X-VIVO-Plus. Average millimoles are shown for a) EG effluxing from lateral meniscus, b) EG effluxing from medial meniscus, c) Glycerol effluxing from lateral meniscus, and d) Glycerol effluxing from medial meniscus. Each graph displays results obtained from menisci that were incubated with their respective CPA for 24 hr or 48 hr then washed at 22 °C.

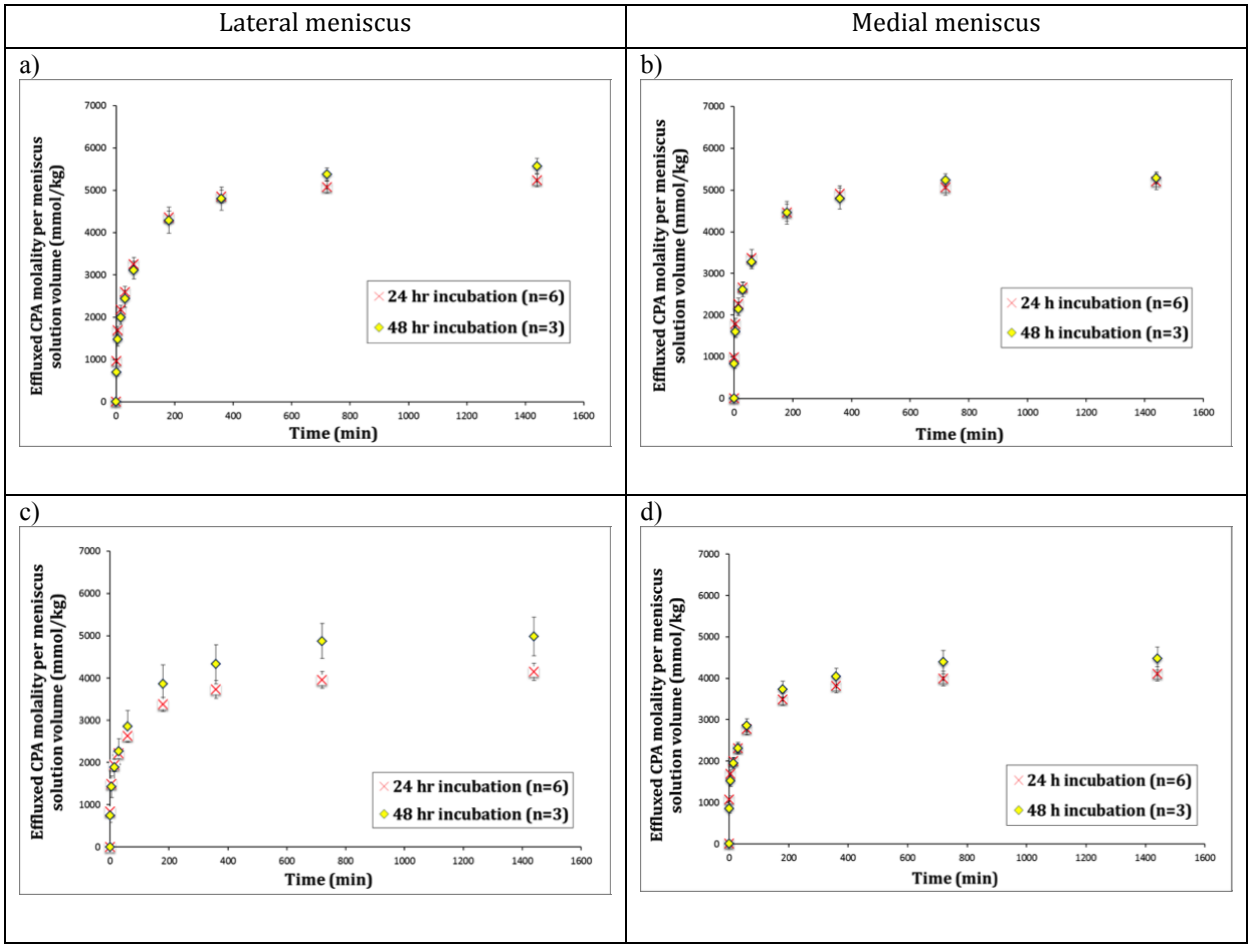


Figure 2-4. Comparing the effect of the two incubation periods (24 hr and 48 hr) at 22 ºC on the average molality (millimoles) of CPA that effluxed per solution volume of lateral and medial menisci during the 2nd immersion in X-VIVO-Plus. Average millimoles are shown for a) EG effluxing from lateral meniscus, b) EG effluxing from medial meniscus, c) Glycerol effluxing from lateral meniscus, and d) Glycerol effluxing from medial meniscus. Each graph displays results obtained from menisci that were incubated with their respective CPA for 24 hr or 48 hr then washed at 22 °C.

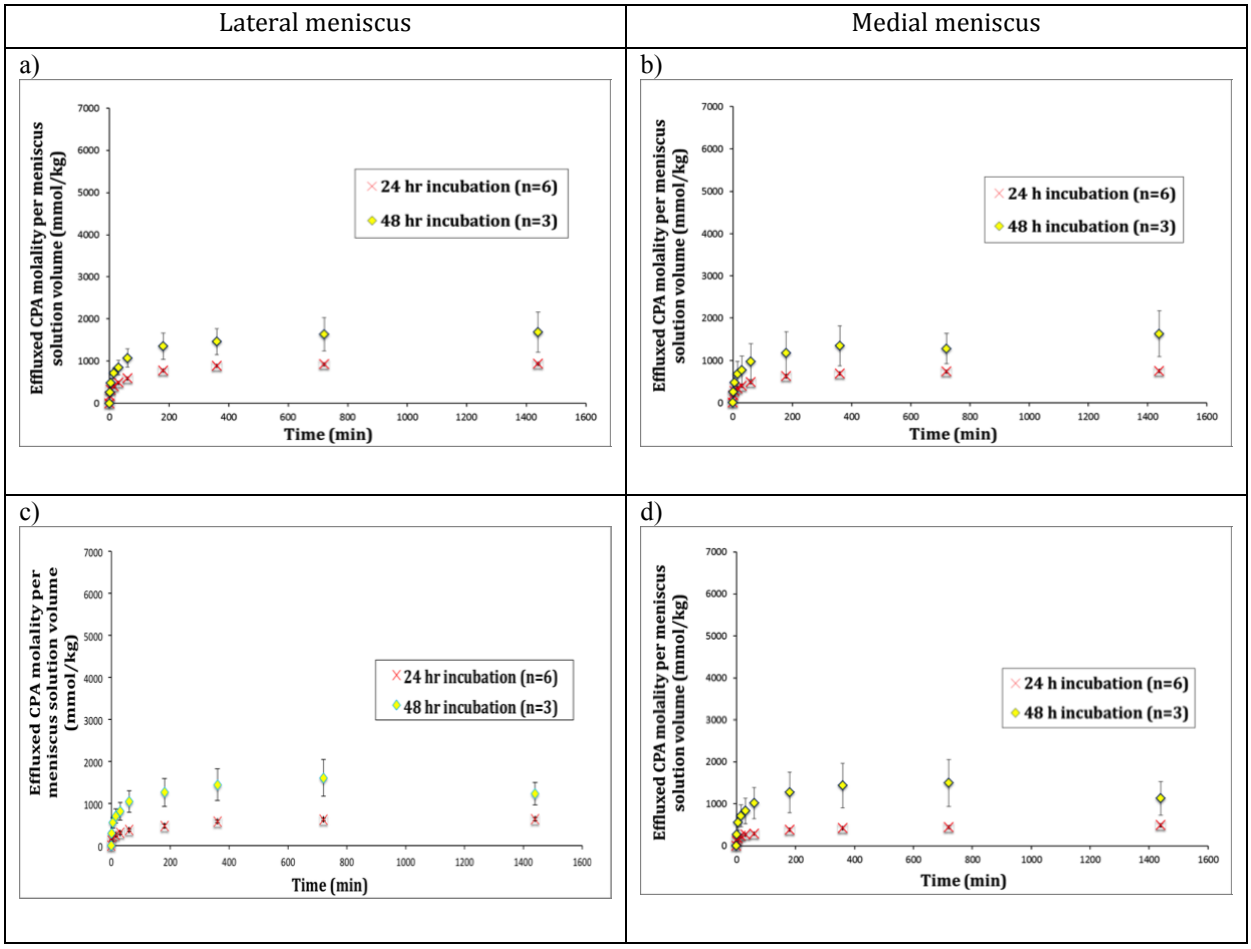


Figure 2-5. Comparing the average millimoles of the two CPAs (Glycerol vs. EG) effluxing per solution volume of the lateral meniscus during the 1st immersion in X-VIVO-Plus. Each panel shows a different experimental temperature: a) 4 °C (24 hr incubation), b) 22 °C (24 hr incubation), c) 37 ºC (24 hr incubation), and d) 22 °C (48 hr incubation) using the lateral menisci.

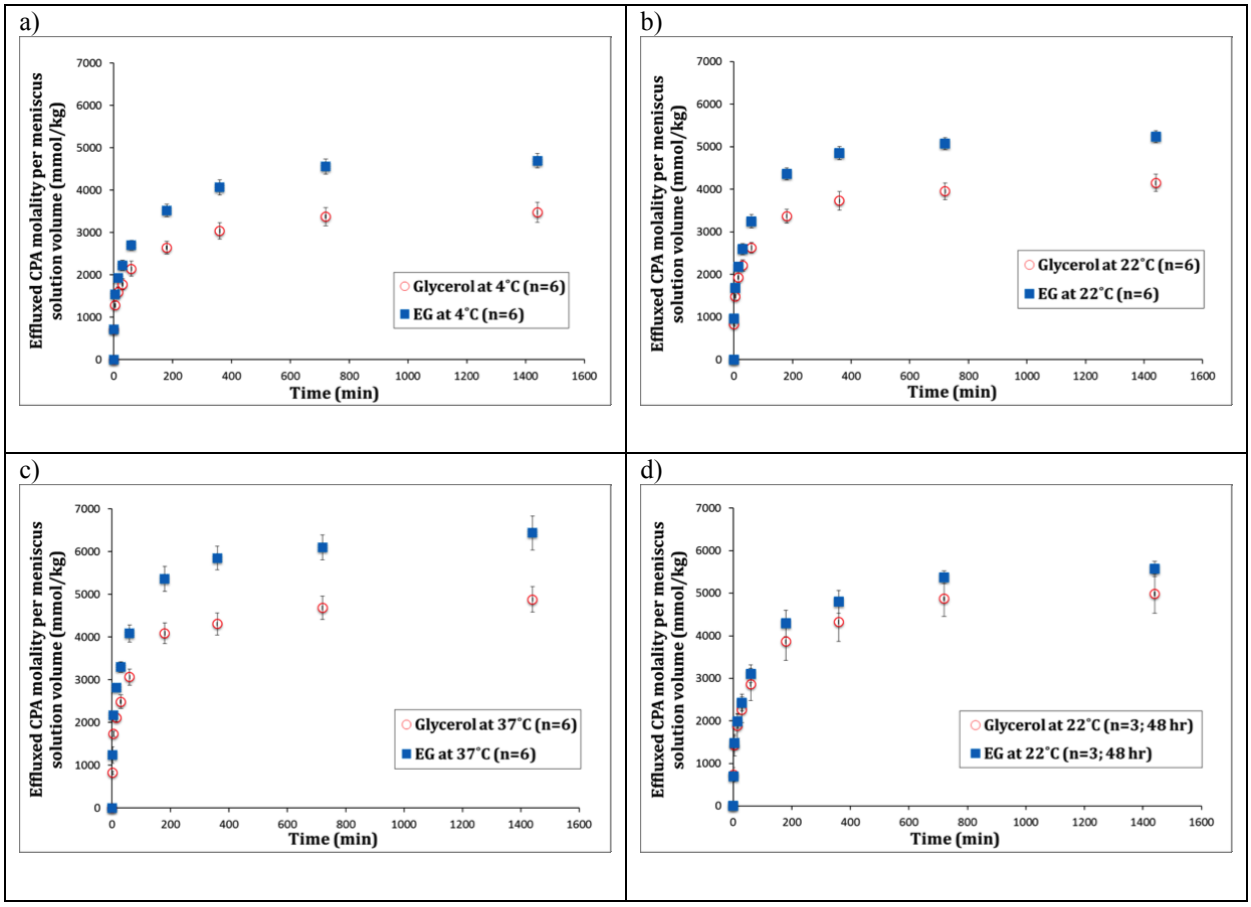


Figure 2-6. Comparing the average millimoles of the two CPAs (Glycerol vs. EG) effluxing per solution volume of the lateral meniscus during the 2nd immersion in X-VIVO-Plus. Each panel shows a different experimental temperature: a) 4 °C (24 hr incubation), b) 22 °C (24 hr incubation), c) 37 ºC (24 hr incubation), and d) 22 °C (48 hr incubation) using the lateral menisci.

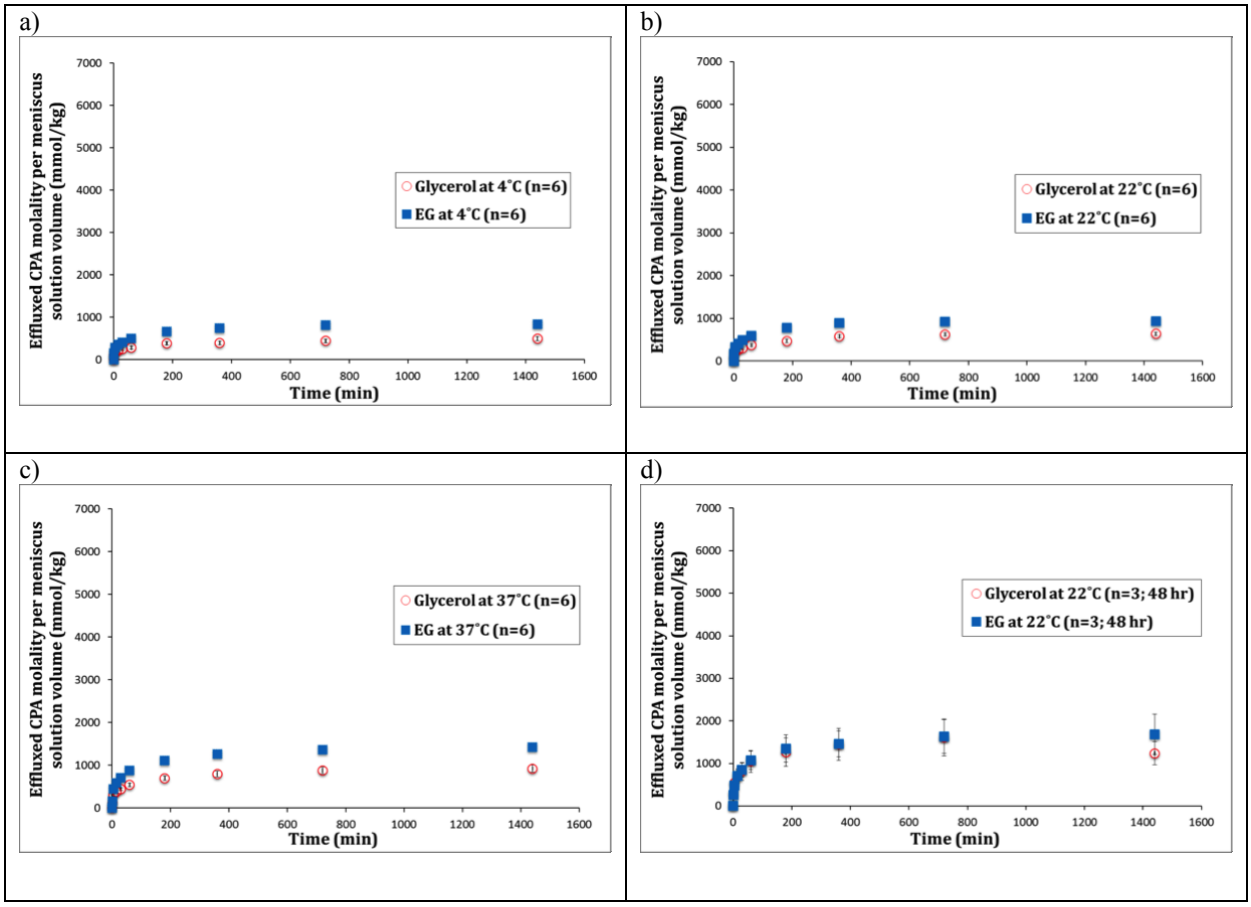


Figure 2-7. Comparing the average millimoles of the two CPAs (Glycerol vs. EG) effluxing per solution volume of the medial meniscus during the 1st immersion in X-VIVO-Plus. Each panel shows a different experimental temperature: a) 4 °C (24 hr incubation), b) 22 °C (24 hr incubation), c) 37 ºC (24 hr incubation), and d) 22 °C (48 hr incubation) using the medial menisci.

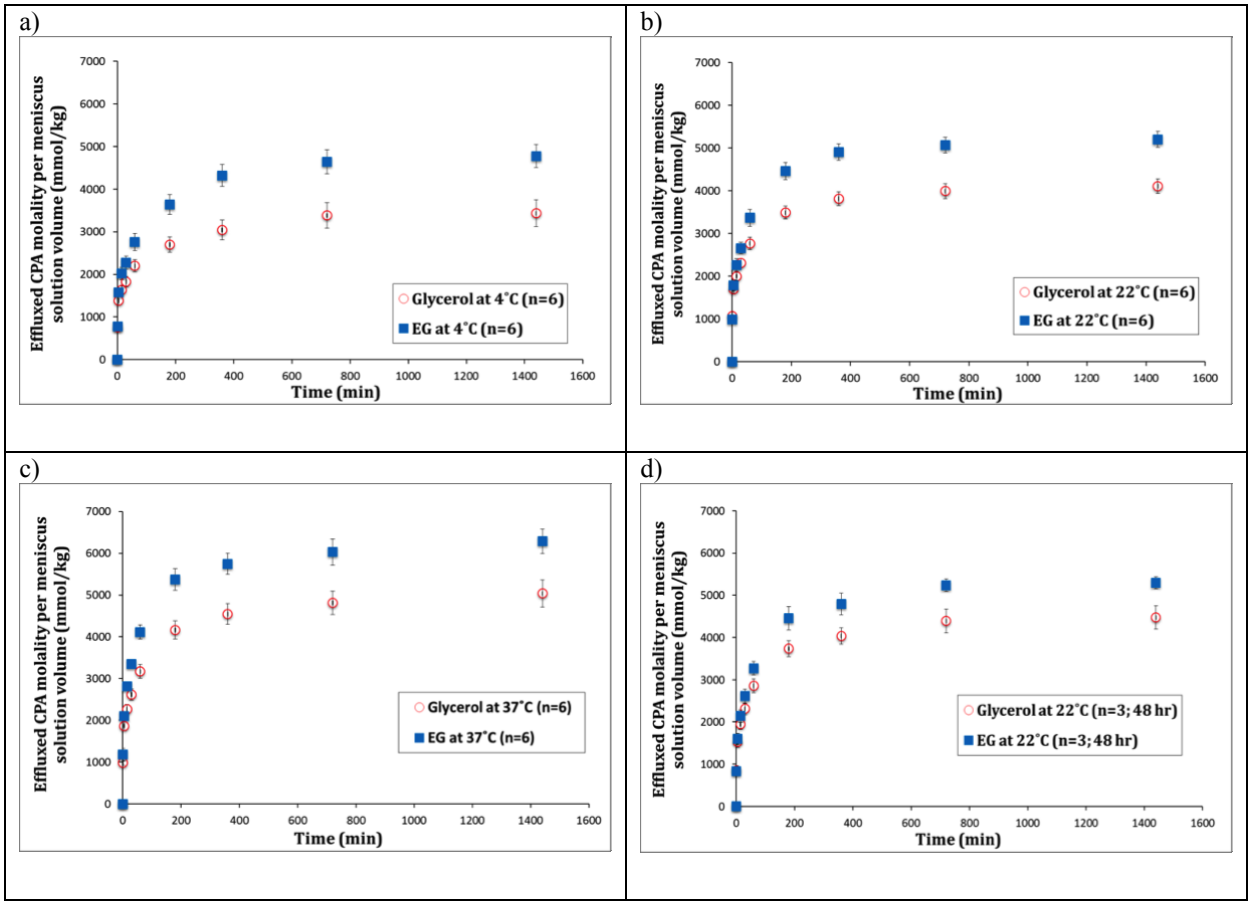
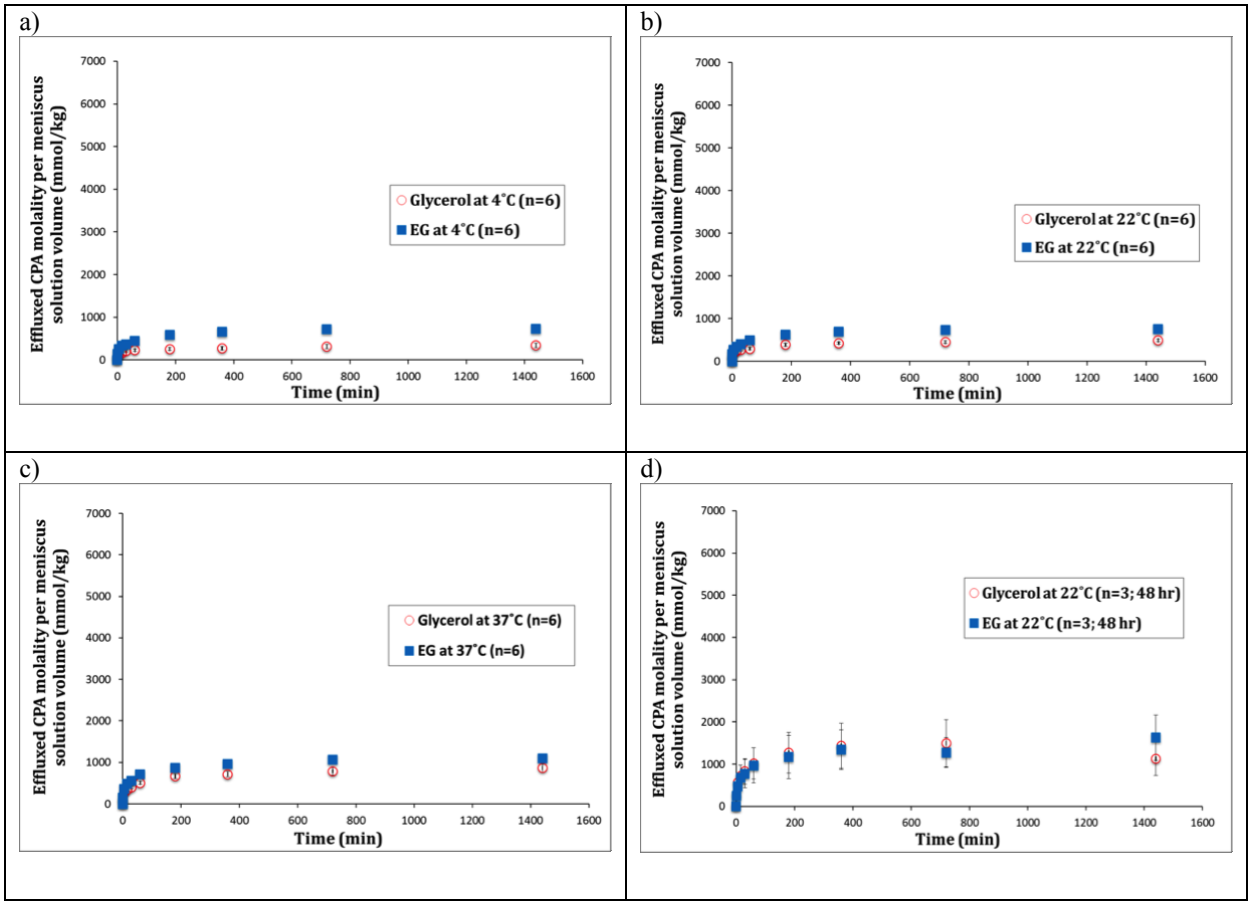


Figure 2-8. Comparing the average millimoles of the two CPAs (Glycerol vs. EG) effluxing per solution volume of the medial meniscus during the 2nd immersion in X-VIVO-Plus. Each panel shows a different experimental temperature: a) 4 °C (24 hr incubation), b) 22 °C (24 hr incubation), c) 37 ºC (24 hr incubation), and d) 22 °C (48 hr incubation) using the medial menisci.



3 Chapter 3. Water Content and Regional Distribution of Sulfated Glycosaminoglycans in Porcine Meniscus

3.1 Introduction

The menisci of the knee joint serve a number of protective functions that are important for normal movement, stability and joint load transmission [1, 2]. Moreover, they are capable of withstanding loads that are several times the body weight [3]. The ability of the meniscus to perform its functions is dependent on the integrity of the extracellular matrix and its components [4]. Structurally, the meniscus is a biphasic tissue composed of fluid and solid matrix interacting with each other to give the tissue its characteristic viscoelastic behavior during periods of physiological and excessive vertical loading [4, 5]. The solid matrix is composed primarily of collagen and a small amount of proteoglycans and DNA [4]. These proteoglycans are macromolecules that contain a core protein with covalently bound glycosaminoglycan (GAG) chains [4]. Different types of GAGs have been detected in the meniscus including chondroitin sulfate (CS), dermatan sulfate (DS), keratan sulfate (KS) and hyaluronic acid (HA) [6]. Compositionally, all GAGs have a characteristic sulfate group except for hyaluronic acid [6]. In tissues with high sulfated GAG content such as articular cartilage and the meniscus, those molecules contribute to the tissue's compressive functions [7, 8]. This contribution of GAGs to the mechanical function of the knee has been confirmed by compressive testing of GAG-depleted meniscal sections, which showed a significant drop in the tissues' ability to withstand compressive loads [7]. Furthermore, the GAG chains embedded within the tissue give it an internally fixed negative charge due to the presence of sulfate (SO^-_3) and carboxyl (COO^-) groups [6]. Thereby, in order for the meniscus to balance this electrical charge, it attracts positively charged molecules

such as cations (Na^+) and polar water into the tissue, leading to a great water holding capacity. This water holding capacity creates a constant swelling pressure within the meniscus [8]. It has been reported that this swelling pressure allows the tissue to return to its normal condition during the off-loading interval of the walking cycle [8-10]. Furthermore, in normal tissue, this swelling pressure is controlled by the elastic forces from the collagen network [8]. In addition to maintaining tissue hydration and elasticity, these GAG molecules also play major roles in cell proliferation, inflammatory response and basement membrane formation [6].

Meniscus fibrochondrocytes are responsible for the extracellular matrix synthesis [11-13]. These cells are a very small fraction of the tissue constituents leading to difficulty in determining their count [13]. The number of cells changes during different physiological and pathological conditions, including growth, aging, injury and repair, and this change is usually determined by measuring the DNA content in the tissue [14]. The role that these cells play in tissue degradation, healing and remodeling is not clearly understood [13]. The determination of cellularity in the native meniscus is valuable for quantitative studies aimed at preserving tissue cellularity for allograft tissue banking [15-18] or those aimed at utilizing decellularized meniscal scaffolds as transplant constructs [19-21].

Unlike many tissues in the human body, blood vessels penetrate only the outer peripheral 10 - 30 % border of the meniscus width in human [22, 23], dog [24] and pig [25], meaning that the majority of the tissue is avascular. The presence of these blood vessels allows the vascular-derived pro-inflammatory mediators and growth factors to stimulate the repair process following injury

[26, 27]. Thereby, injuries to the outer peripheral region of the meniscus are more likely to heal than those in the inner region [28-32]. The inner two-thirds and the outer one-third regions of the meniscus also differ in their cellular morphology and extracellular matrix composition [33]. However, the healing capacity was found to have no link to the intrinsic difference between cells of the inner and outer regions but rather the healing ability is a pure function of the additional vascularity [34]. From a surgical and reparative perspective, the meniscus is usually considered with this distinction between the avascular inner two-thirds and the vascular outer one-third. Thereby, it would be beneficial to investigate those two regions separately.

With that distinction in mind, assessment of the structural feature of animal menisci such as GAG and DNA count is important prior to developing models for human meniscus studies. Meniscal investigations on animal models show preponderance of the canine model [35-38]. However, this model is getting less popular due to increasing pressure from animal interest groups opposing the use of dogs in biomedical research [39]. On the other hand, the porcine model, probably due to its common use in commercial meat production, is a readily available and cost-effective option for meniscal investigations. This model has been utilized in a wide range of meniscal studies [21, 34, 40-44], which have contributed to the understanding of different aspects of the meniscus. Although studies on the composition of the meniscus in this model have covered different aspects of the extracellular matrix, the literature is currently lacking any quantitative report on the DNA content in this model.

In our laboratory, we are interested in orthopaedic tissue preservation (human and porcine) using vitrification instead of the conventional cryopreservation technique with the aim of banking donor tissues, mainly articular cartilage and meniscus, for future allograft transplantation. The vitrification technique involves tissue exposure to very high concentrations of cryoprotectant agents (CPAs) that need to achieve sufficient permeation into the tissue before cooling to very low subzero temperatures while maintaining the structural integrity of the tissue and cellular viability [17]. The CPA permeation process is affected by many factors, one of which is the fixed charge density inherent to hydrated tissues such as the meniscus [45]. This fixed charge density due to the presence of negatively charged molecules on the surface of GAGs, has been reported to make the permeation process more difficult in articular cartilage samples [46]. Thereby, this study was designed to provide baseline quantitative information about the GAG content and the DNA content in the porcine meniscus that can be used for future studies. Total and regional water contents were determined as well. Since, GAGs provide the meniscus with its great water holding capacity, this raises the question whether regional differences in the sulfated GAG content will affect the regional water content. Therefore, another objective was to determine the correlation between sulfated GAG content and the regional water content.

3.2 Materials and Methods

3.2.1 Tissue Harvesting

Stifle joints from the hind leg of adult Yorkshire pigs of both sexes were obtained from a local abattoir (Stony Plain, AB). A total of 12, randomly selected right and left stifle joints were removed and stored at 4 °C for up to 48 hours until dissected. The attached menisci were freed from adherent joint capsule, bony and ligamentous attachments, and washed in phosphate buffer saline (PBS) to remove excess synovial fluid. The menisci were then inspected for physical signs of tears or articular cartilage degeneration in the joint. All menisci used in the study were visually intact with no visible tears or degenerative changes. Six pairs of medial and lateral menisci were used for the total water content determination; the remaining six pairs of menisci were used for the quantification of GAGs, DNA and regional water content.

3.2.2 Total Water Content Determination

Twelve fresh, wet weighed lateral ($n=6$) and medial menisci ($n=6$) were placed, partially covered, in a desiccation cabinet (NALGE®) under negative pressure (15 pounds of pressure, 103.41 kPa) at room temperature for 8 days. Daily weight readings (g) were recorded until equilibrium (constant weight) was reached *i.e.,* the difference between three successive weightings was smaller than 0.01g. Assuming a density of 1g/ml, tissue water content was calculated by subtracting the dry weight from the initial wet weight of the tissue, and expressed as a mean volume (ml) ± SEM and percentage of the original wet weight.

3.2.3 Tissue Sample Preparation for Biochemical Analysis

Six medial (n=6) and six lateral (n=6) menisci were assigned for the harvesting of the cylindrical explants and regional water content determination. The harvesting of the cylindrical explants was based on visual estimation of the sites before physical cuts were made. Each meniscus was visually divided into two regions: outer third and inner two-thirds, and each region was further divided into three different sites along the surface: anterior, central and posterior. This resulted in six sampling sites from the body of each meniscus. Using a dermal punch device (Sklar Instrument, 889 S Matlack St. West Chester, PA), 4 mm diameter full thickness cylindrical explants were taken from the meniscal body at each sampling site (Figure 3-1a,b). Thereafter, each meniscal explant was washed twice in PBS and diced into small pieces to increase the surface area for enzymatic digestion, then dried in a desiccator until no weight decrease was observed, and the dry weight was recorded. The dried meniscal explants were subsequently digested in 1 ml of proteinase K solution (1 mg/ml in 50 mM Tris with 1 mM EDTA, 1 mM iodoacetamide and 10 mg/ml pepstatin A; all purchased from Sigma-Aldrich) at 56 ºC for 23 hr or until no solid substance remained. After enzymatic digestion, tissue samples were vortexed and stored at -80 ºC until experimental use. Prior to measurements, samples were allowed to thaw completely at room temperature and vortexed to ensure proper homogenization of the digest.

3.2.4 Biochemical Analysis: Quantification of Sulfated GAG and DNA Content

Digested cylindrical explants were used for the quantification of sulfated GAGs and DNA as per protocol used in our laboratory [47, 48]. Briefly, the sulfated GAG content was quantified

spectrophotmetrically by a 1,9-dimethylmethylene blue dye DMMB (Sigma-Aldrich, Oakville, Canada) binding assay using chondroitin sulfate (CS) as a standard [49, 50]. The chondroitin sulfate standard was in 100 μg/ml (in μl) prepared in PBE/Cysteine at 8 different concentrations (0, 5, 10, 25, 30, 50, 80 and 100 %). A standard curve for the CS spectrophotometric reaction was then created and tissue samples were normalized to this curve. Samples were diluted at 1:5000 dilutions and 2 μL aliquots were taken from each digested sample and placed in a 96-well plate in triplicates. Two hundred and fifty microliters (250 μL) of DMMB were added to each well containing either CS or tissue aliquots. Spectrophotometric quantification of the GAG-DMMB mixture, in each sample, was done immediately versus milli-Q water using an MRX Mircoplate Reader (Dynatech laboratories Inc., Virginia, USA) with absorbance level set at 525 nm. Following the reading, the amount of GAG in every well was determined by subtracting the absorbance value of water from the absorbance value of the GAG-DMMB mixture. All measurements from the same sample were performed in triplicates and the average was used for the analysis. The GAG amount was then normalized against the CS standard curve, multiplied by the dilution factor of the respective aliquot and the average of the three measurements per sample was taken as the working value for sulfated GAG. Additionally, since cylindrical explants were digested after drying, the working value for the GAG content was divided by the dry weight of the respective explant to estimate the concentration of the sulfated GAG in each digested sample. Explants from all six sampling sites were analyzed individually then grouped by the region of origin, either inner or outer, to determine the total GAG and DNA content in the respective region.

DNA content was quantified using the CyQUANT® cell proliferation assay kit (Invitrogen), with supplied bacteriophage λ-DNAase as standard. Fluorescence was measured using a black 96-well plate in triplicate using a Cytofluor II microplate reader (PerSeptive Biosystems, Massachusetts, USA), with a 450/50 nm excitation wavelengths and emission wavelength of 530/25 nm. Both sulfated GAG and DNA contents were reported separately in micro-grams (μg) per dry weight of the sample and then the value of the GAG per DNA was also determined.

3.2.5 Regional Water Content

Following removal of the explants, the remaining intact tissue including the meniscal body and the horns was sectioned along the lines of division. First, the outer third and inner two-thirds were separated from each other and the circumferential cut was extended to involve the horns at the anterior and posterior ends. The cutting landmarks were based on the gross appearance of the meniscus (Figure 3-1c, d). The outer region was thicker than the inner region. Then both the outer and inner portions of the meniscus were cut into three approximately equal sections, representative of the sampling sites: anterior, central and posterior (Figure 3-1e, f). The thinnest section was the inner central section in both medial and lateral menisci; the outer central section was the thickest from lateral menisci while from medial menisci the outer posterior section was slightly thicker than the central section. Each section was weighed (wet weight), desiccated and reweighed again (dry weight). The regional water content was determined from the difference between the wet and dry weight. Sections were analyzed individually and then grouped to calculate the mean water content for each region: inner two-

thirds or outer one-third. Moreover, the percentage of water was used to determine the nature of the relationship between the regional water content and the GAG content.

For clarity in the remainder of the chapter (results and discussion), the term "site" indicates the origin of the cylindrical explants used for the GAG and DNA content. For these sites: anterior, central or posterior portions from the inner 2/3 were individually compared to the corresponding sites from the outer 1/3 of the same meniscus. While the term "region" indicates that all three sampling sites from either the inner 2/3 or outer 1/3 were all grouped and analyzed together, resulting in 18 replicates, to determine the overall value for either region. In the reporting of the regional water content the term "section" was used instead of the word site. When indicated, differences between medial and lateral menisci involved same-site and same-region comparisons only.

3.2.6 Statistical Analysis

Statistical analysis for differences in GAG, DNA and regional water content between sampling sites and regions was performed using one-way ANOVA with Tukey's post-hoc test. p-values less than 0.05 were considered statistically significant. An independent sample t-test was performed to determine the difference in the total water content between medial and lateral menisci. Results are presented as mean ± standard error of the mean (SEM) or as percentage ± SEM when indicated. A simple linear regression analysis was performed to test the nature of a linear relationship between the regional water content and sulfated GAG content from the respective region in both medial and lateral menisci separately. The sulfated GAG content was used as a predictor and the water content was considered as the outcome. In this analysis, when the p-value was below 0.05, the regression was considered significant. The significance level of R was taken from an abridged table of cut off for statistical significance of correlation coefficient that is based on the sample size in this study. For R^2 value that is above 0.25, the regression analysis will be considered as having a good linear relationship. But when the R^2 value is between 0.25 and 0.1, it will be considered as having a weak linear correlation and the regression analysis is considered as having no linear correlation when the R^2 value was < 0.1. All statistical tests were performed using SPSS version 21.0 (IBM Corp. Released 2012. IBM SPSS Statistics for Windows, Version 21.0. Armonk, NY: IBM Corp).

3.3 Results

3.3.1 Total and Regional Water Content

The porcine meniscus is a hydrous tissue and water constituted 68.45 % (SEM ± 0.67 %) and 68.96 % (SEM ± 0.40 %) of the total wet weight in medial and lateral porcine menisci, respectively. Assuming a density of 1g/ml, the water volume was also determined for all menisci. The average water volume was found to be 3.45 ml (SEM ± 0.20 ml) in the medial meniscus and 4.58 ml (SEM ± 0.23 ml) in the lateral meniscus. The difference in meniscal water volume between medial and lateral menisci was statistically significant ($p = 0.005$). Under the desiccation conditions specified in the subsection of the methods above, intact porcine menisci lost most of their water in the first few days, with 66 % of the total water loss having taken place over the first 72 hours with the rate of water loss gradually slowing until it became zero. It took 6 days for the porcine menisci to achieve a constant weight, indicating the duration needed for complete dehydration.

The regional water content showed no statistically significant difference ($p > 0.05$) between the inner and outer regions in both medial and lateral menisci; nor among the three sections (anterior, central, posterior) of each region (Table 1). On average, water accounted for 66.31 % (SEM ± 1.00 %) and 65.19 % (SEM ± 1.09 %) of the wet weight of the inner two-thirds regions in medial and lateral menisci, respectively. In the outer third regions, water accounted for 63.08 % (SEM ± 0.70 %) and 63.88 % (SEM ± 1.14 %) of the wet weight in medial and lateral menisci, respectively.

3.3.2 Sulfated GAG and DNA content

The results of sulfated GAG content and DNA content determined by spectrophotometry and fluorescence are summarized in Table 1.

Spectrophotometric quantification showed a marked concentration of the sulfated GAG in the inner two-thirds regions of both medial and lateral porcine menisci ($p < 0.001$). Correspondingly, the mean sulfated GAG content in the inner region was four-fold and three-fold greater than the outer region in both medial and lateral porcine menisci, respectively. When each sampling site (anterior, central, posterior) from the inner two-thirds region was compared with the corresponding site in the outer third region from the same meniscus, the GAG content showed a statistically significant difference ($p < 0.02$) as shown in Figure 3-2a,b. However, there was no statistically significant difference between medial and lateral menisci for all corresponding sampling sites and regions ($p > 0.05$).

The DNA content showed variability between similar regions in medial and lateral menisci (Figure 3-2c,d). The overall DNA content in the inner two-thirds region of lateral menisci was significantly greater ($p = 0.001$) than that of the outer one-third region. Whereas, in the medial meniscus the overall DNA content in the outer one-third region was significantly greater ($p = 0.02$) than the inner two-thirds, the opposite of what was found in the lateral meniscus. Within the medial meniscus, individual sites (anterior, central, posterior) showed no significant difference between the inner and outer regions, indicating similar DNA content within these two regions. While for the lateral meniscus only the posterior site demonstrated a

significantly greater DNA content in the inner two-thirds region versus the outer one-third region, while the anterior and central sites showed no statistically significant difference between the inner and outer regions, as shown in Table 1.

The ability of meniscal cells to synthesize matrix GAG was determined as GAG per DNA content. For the two main regions, the overall GAG per DNA content of the inner two-thirds region exceeded that of the outer one-third region in both medial and lateral menisci ($p < 0.002$). Moreover, the inner two-thirds region in the medial meniscus had a higher GAG/DNA concentration than the same region in the lateral meniscus ($p = 0.03$). However, when looking at individual sites, this significance level in the synthesis rate was seen only within corresponding sites of the medial meniscus but not within the lateral meniscus. Within the medial meniscus, all of the sampling sites showed statistically significant differences between the inner and outer regions ($p < 0.012$). While within the lateral meniscus, all of the sites showed no significant difference between the two regions (Figure 3-2e,f). This discrepancy could be attributed to the DNA content, which was used as the denominator. As described above, the overall DNA content was higher in the outer region of the medial meniscus even though individual sites had no significant differences. Cross comparison of the similar sites between the medial and lateral menisci of the GAG/DNA content showed no statistically significant difference for both inner and outer sites ($p > 0.6$). Interestingly, when looking at the sampling sites individually, within the same meniscus there is an observable pattern in the concentration. In fact, within the same meniscus specimen, the GAG per DNA seemed to be highest in the central site followed by the anterior then the posterior site of the same region in both medial and lateral menisci (Figure 3-2e,f).

3.3.3 Correlation between regional sulfated GAG and regional water content

The sulfated GAG content from different regions showed no significant correlation (p = 0.52) with the water content from the respective region in both the medial and lateral menisci. Due to the lack of significance, the correlation coefficient was not reported.

3.4 Discussion

This study using a surgically relevant anatomical sampling that was based on the difference in healing capacity showed regional variation in the sulfated GAG distribution indicative of the physiological differences along with the determination of the DNA content and water content.

The total meniscus water content was measured by desiccation, which is a reliable and simple method to carry out. The porcine meniscus is a well-hydrated tissue with water constituting 68 % of its wet weight. Our reported results indicate that the total water content of porcine meniscus is slightly higher than canine meniscus [37] and lower than the human meniscus [33]. Adams et al. [51] reported that water constituted 66.65 % (SD, ± 1.64 %) and 65.09 % (SD, ± 1.52 %) of the total wet weight in medial and lateral canine menisci, respectively. Based on our results it is clear that porcine menisci contained about 2-5 % less water than human meniscus [33]. Nonetheless, in contrast to normal menisci, in a study to assess the biochemical changes in the meniscus using an experimentally induced osteoarthritic canine model, the total water content was found to increase and remain elevated throughout the course of the disease [51]. The authors attributed this increase to the inability of the damaged collagen fiber network to counteract the swelling pressure exerted by the GAGs in the meniscus [8, 51]. Interestingly, in an observational side part of our experiment, a similar swelling effect was seen in fully dried menisci. This side part was carried out due to curiosity to see what happens to the desiccated menisci when immersed in PBS. At the end of the desiccation period, two fully dried, whole medial and lateral menisci, were immersed in PBS at room temperature. For instance, the two

fully dried medial menisci (1.87 g and 1.4 g) were able to achieve more than 50 % (3.08 g and 2.13 g, respectively) of their initial fresh wet weights (5.8 g and 3.6 g, respectively) after only one hour in PBS and they continued to swell afterwards. After 48 hours in the PBS we found that they had swelled beyond (6.57 g and 4.81 g, respectively) their initial fresh wet weights. Moreover, the rate of weight gain was faster in medial menisci compared to the lateral ones. Although, it is outside the scope of our study, if acellularization can be confirmed in fully desiccated menisci, they can be used as scaffolds for meniscal tissue engineering [21, 52].

Our values for the regional water content are in general agreement with some of the previously published reports on animal menisci with little variation in the content and landmarks used for tissue dissection [37, 53]. The dissection method utilized in most studies divided the meniscus into three different areas using either longitudinally or radially oriented cutting lines [35, 37, 42, 53] whereas in this study we divided each meniscus used for the regional water content determination, into inner two-thirds and outer one-third then further divided each region into three sections as described in the methods above. Adams and Muir [35] determined the water content in the menisci of four foxhound dogs that were divided transversely into three areas: anterior, central and posterior without distinction between the inner and outer zones. Despite the difference in tissue dissection, they reported total water content of 63.8 % (SD, ± 0.9 %) for the lateral anterior and posterior regions (pooled together to give one value), and 63.1 % (SD, ± 1.2 %) for the lateral central region; similarly, medial meniscus areas had slightly less water content but the difference was not statistically different. In like manner, using the same anatomical dissection [35], Stephan et al. [37] determined the water content in the menisci of 52 adult dogs and reported the water content of the lateral anterior and posterior areas (grouped together and

termed lateral pole) as 65.0 % (SD, ± 0.2 %) and 65.3 % (SD, ± 0.3 %). Regional variation in the water content was also determined in the menisci of growing swine after they were divided radially into inner, middle and outer thirds [53]. In this study, the authors reported the water content in lateral menisci as 75.8 % (SD, ± 1.8 %), 70.1 % (SD, ± 1.0 %) and 66.0 % (SD, ± 0.3 %) for the inner, middle and outer one-third, respectively; medial menisci showed the same pattern of variation with slightly less water content [53]. Moreover, the authors also reported statistically significant differences between the those regions [53], a feature that is different from our findings (Table 1). However, in the latter study [53], the studied menisci were from growing, 20-week-old pigs when the tissue is expected to be more hydrous compared to adult meniscal tissue [4, 12].

Proteoglycans and their related glycosaminoglycans, despite their small concentration in the extracellular matrix of human meniscus [33], play an important role in the mechanical function of human and animal tissue [54]. Regional differences in GAG and DNA may provide an understanding of the variation in the compressive properties and cell biosynthetic activity. In this study, the GAG content was determined from dried meniscal explants that underwent enzymatic digestion using proteinase K. Moreover, drying the diced tissue explants at room temperature minimized the possibility of releasing volatile organic substances with water that can happen if higher temperatures are used for tissue drying [55]. GAG quantification was done using DMMB, which is a common, rapid and reliable assay for the sulfated GAG quantification that depends on the color change when this cationic dye binds to the sulfate group of the GAG chains [56]. The reported quantitative data on the sulfated GAGs in this study confirmed and extended on previously reported descriptive and quantitative findings indicating higher concentrations of

sulfated GAGs in the inner region of the meniscus when measured in different species [23, 35, 37, 42, 57-59]. This regional concentration of sulfated GAGs is consistent with the higher compressive loading sustained by the inner aspect of the meniscus [7]. As only the sulfated GAG portion was determined, this study cannot predict the hyaluronic acid contribution to the overall GAG content in the meniscus [6, 50]; however, it has been reported that the presence of HA in the sample does not interfere with the detection of the sulfated GAGs due to the lack of interaction with the dye and difference in the absorption spectrum [49, 60]. The majority of the sulfated GAG content in the porcine meniscus can be attributed to the overall predominance of chondroitin sulfate throughout the tissue [42]. Although some studies have looked into the characterization of individual GAGs, it was outside the scope of the current study. Determining the distribution of GAGs in the porcine meniscus adds to our understanding of the structural similarities between the morphologically similar menisci in human and animal models commonly used in meniscal investigations. The human meniscal body has been reported to have a higher concentration of GAGs than the horns [57]. In a study of human and canine menisci, Adams and Ho [57] reported that the human meniscus was less intensely stained with Safranin-O than was the canine meniscus, suggesting a higher GAG content in the canine meniscus compared to the human counterpart. Nakano et al. [42] studied the regional differences of GAGs in three zones of four porcine menisci. They reported a gradual decline in the overall GAG content from the inner zone toward the outer zone. Our measured values for the sulfated GAGs were higher than those reported by Nakano et al. [42]. In their study [42], they determined only the differential fraction of chondroitin sulfate and dermatan sulfate, which are not representative of the overall sulfated GAG content. Nonetheless, for comparative purposes, we have combined the reported differential fraction of chondroitin sulfate and dermatan sulfate from the previous study, mg/g dry weight, for the inner region only and then converted the resultant value into μg/g dry weight to allow for

same unit comparison with our findings from the inner two-thirds region. Although this value is not representative of the total sulfated GAG content in their samples, our findings were 13-fold greater than what was determined by Nakano et al. [42]. This difference might be due to the fact that we quantified the total sulfated GAG content while Nakano et al. [42] reported only a portion of the sulfated GAGs (CS and DS) that were used in the comparison. Another possible explanation for this variation may be partly explained by the difference in tissue dissection and the age of the pigs used in the previous study. However, the previous study [42] showed the GAG quantity in the medial meniscus, which is more prone to injury [61], to be higher in value than that in the lateral meniscus but in our study the difference was not great enough to result in statistical significance. In contrast to the structure of the normal meniscus, osteoarthritic changes have been found to result in alteration in the GAG concentration in damaged menisci [51, 62]. Adams et al. [51], in an experimentally induced osteoarthritic model in dogs, reported a decrease in the hexosamine content in menisci taken from osteoarthritic knees when compared to those taken from normal knees. Hexosamine content indicates the presence of both GAGs and proteoglycans [42]. Nakano and Aherne [53] reported loss of the GAG staining with Safranin-O in damaged porcine menisci. Similar findings were also reported in human meniscus cells cultured with IL-1β, a pro-inflammatory mediator commonly found in osteoarthritic or injured joints [47, 63]. Chowdhury et al. [47] reported reduced GAG staining intensity with Safranin-O in human meniscal cells cultured in the presence of IL-1β, suggesting that the cells might have lost part of their GAG synthesizing ability [47]. Based on these studies on arthritic or damaged menisci, it is expected that the GAG content will be lower in damaged porcine menisci. However, the current study cannot confirm this similarity as the GAG content was only measured in normal porcine menisci.

Tissue hydration is a dominant variable in controlling permeability and compressive stiffness [41]. The water holding capacity of GAGs have been reported in a number of in vitro studies [53, 58]. Nakano et al., in two separate studies [53, 58] on the porcine meniscus, reported greater water content in the inner one-third and this was linked to the findings from one of their earlier studies that showed higher GAG content in the same region, but no correlation analysis was performed in their second study. Our correlation analysis failed to confirm an association between the sulfated GAG and water content regionally. This may suggest that the sulfated GAGs may not have somehow an important role in the determination of water content in normal porcine meniscus tissue. However, this assumption may not be true due to the inclusion of parts of the meniscal horns, especially, with the outer anterior and posterior sections used in the regional water content determination leading to probably higher water content that is not reflective of the GAG differential between the inner and outer regions. The meniscal horns resemble ligaments and contain blood vessels; thereby, they should have been excluded during the dissection [35, 51, 64]. Additionally, the arbitrary dissection used in dividing the meniscus is another possible factor that could have led to variation in the sizes of tissue sections used for the regional water content determination. This indicates the importance of using clear anatomical landmarks, preferably based on anatomical measurements, in menisci used for water content determination and biochemical analysis. Likewise, in a different tissue, Oh et al. [65] measured the sulfated GAG content and water content in human dermal samples, where the GAGs have been reported to maintain skin hydration and elasticity. The author reported no correlation between the total sulfated GAG and water content in the human dermis [65]. Furthermore, Maroudas in his studies on the articular cartilage stated that the water content seems to be little affected by the local GAG content in healthy cartilage [8].

To our knowledge this is the first study to quantify the cellularity in the porcine meniscus. We determined the DNA content from digested meniscal explants using proteinase K, which provided a simple one-step digestion of the tissue [66]. The presence of EDTA, in the proteinase K solution, inactivates the DNase enzyme due to removal of Ca^{2+} ions, which act as stabilizers for the DNase activity [67, 68], thereby preserving the DNA in a pure form during the digestion process. Moreover, the CyQUANT GR dye binding gives a fluorescence enhancement that is highly specific for DNA and no other tissue component displays such significant fluorescence making it very accurate [69]. As cells in the tissue are usually embedded within other extracellular matrix components, the DNA quantification can become difficult due to interference by these components [66, 70]. However, in the CyQUANT® cell proliferation assay kit, the presence of cell lysis buffer, allows the dye solution to access the cellular DNA [69]. In our study, we found a greater DNA content in the inner region of the lateral meniscus, whereas in the medial meniscus the outer region had greater DNA content. Due to the lack of quantitative DNA reports in the literature, we were not able to confirm this difference indicating the need for future studies addressing this aspect of the meniscus.

The GAG/DNA value, indicative of the amount of matrix GAGs per cellular DNA, showed increased synthesis of sulfated GAGs in the inner 2/3 regions of both medial and lateral porcine menisci. McAlinden et al. [14] studied the proteoglycan synthesis in human meniscal explants (harvested from the central region of the meniscus) by measuring the rate of (^{35}S)-sulfate incorporation per mg DNA of tissue and reported no difference in the rate of synthesis between the medial and lateral menisci taken form the same specimen [14]. Moreover, McAlinden et al. used meniscal explants that were harvested only from the central area of the meniscus based on

their preliminary experiments (data not reported) that showed variation in the rate of synthesis between the anterior, central and posterior regions of the meniscus [14]. In our study the GAG per DNA was also high in the central inner sites despite the variability in the significance level within medial or lateral menisci. However, our finding of non-significant difference between the sites of the inner 2/3 region with their corresponding ones from the outer region in the lateral meniscus is in agreement with what has been reported by Croutze et al. [48] for cultured human meniscus fibrochondrocytes (MFC). Although all MFCs came from osteoarthritic knees of patients undergoing total knee replacement, the authors [48] reported that in culture conditions human MFCs isolated from the inner and outer regions had similar GAG/DNA level.

Combining our interest in designing future studies aimed at meniscus vitrification with findings from this study, the following theoretical points can be expected when designing CPA addition and removal protocols. First, as the movement of the CPAs into the tissue relies heavily on the tissue water mobility, the high GAG content in the meniscus will give it a high swelling pressure and low hydraulic permeability (L_p) making it difficult for the cryoprotectant agents to move into the tissue [45, 46]. Second, when using our DNA count from the lateral meniscus as an example, the above mentioned differential cellularity could mean that the inner more populated regions of the tissue are at much higher chance of cellular injury compared to the least populated outer region where plasma isotonicity, from blood vessels, could possibly play a role in the outer cells osmotic response. All of these points, combined with the geometric complexity of the tissue, suggest that efforts at achieving successful vitrification of the meniscal tissue are not going to be straightforward and further work is needed to determine the best approach to vitrify the meniscus for the purpose of banking vitrified donor menisci.

3.5 Conclusion

Taken together, our results provided insight to the fluid content and part of the organic matter in porcine meniscus. The intact porcine meniscus is almost as hydrous as the human meniscus. Our data also provided supporting evidence for the previously reported regional variation in the GAG content between the inner and outer regions, corresponding with the increased compressive demand in the inner region. Medial and lateral menisci have similar GAG content. For the overall DNA content there was a discrepancy in the concentration between inner and outer regions for the meniscal regions of the medial and lateral menisci. The inner two-thirds regions of both medial and lateral menisci demonstrated higher GAG/DNA content than the outer region. However, there was variation in the synthetic index within different sites of the medial meniscus but not within the lateral meniscus sites. Due to the lack of quantitative reports on the regional variation of the native meniscus cell count further studies are needed to investigate this difference between the medial and lateral menisci.

3.6 Endnote

A version of this chapter will be submitted for publication, with the following authors: Talal Takroni, Leila Laouar, Adetola Adesida, Janet A W Elliott, and Nadr Jomha.

Conflict of interest

Each author declares no conflict of interest through commercial association in relation to the submitted article.

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3.8 Tables

Table 3-1. Average quantities of sulfated GAG content (in thousands µg/g dry weight of explant), DNA content (µg/g dry weight of explants), GAG/DNA (µg/µg) and percentage of regional water content in porcine menisci. Aliquots for the GAG and DNA came from the same digested tissue sample. Values are presented for every site (n=6) as mean ± standard error of the mean. The whole region column indicates the overall result of the three sites after they were pooled and analyzed together. Differences are determined in rows between the two major sides of the table: the inner 2/3 and outer 1/3; three sites and whole region of the inner 2/3 were compared with the same sites and whole region of the outer 1/3. Statistically significant differences ($p < 0.05$) between the inner and outer regions are in bold. No medial versus lateral differences are indicated in this table.

	Inner 2/3				**Outer 1/3**			
	Mean (± SEM)				Mean (± SEM)			
	Anterior (n=6)	Central (n=6)	Posterior (n=6)	**Whole Region**	Anterior (n=6)	Central (n=6)	Posterior (n=6)	**Whole Region**
Medial:								
Sulfated GAGs (1×10^3 µg/g dry weight)	**272.74 (± 36.47)**	**256.11 (± 50.40)**	**220.49 (± 30.63)**	**249.78 (± 22.40)**	70.87 (± 12.10)	53.24 (± 4.51)	46.43 (± 9.14)	56.85 (± 5.55)
DNA (µg/g dry weight)	429.46 (± 54.30)	361.62 (± 73.10)	423.45 (± 43.00)	404.84 (± 32.38)	501.14 (± 50.36)	570.41 (± 40.05)	558.20 (± 49.00)	**543.25 (± 26.36)**
GAG/DNA (µg/µg)	**716.92 (± 152.86)**	**792.67 (± 162.57)**	**536.73 (± 71.80)**	**682.11 (± 77.88)**	169.91 (± 61.74)	95.00 (± 9.66)	83.86 (± 16.32)	116.25 (± 22.25)
Regional Water (%)	68.41 (± 1.31)	63.07 (± 1.80)	67.44 (± 1.50)	66.31 (± 1.01)	64.26 (± 1.41)	61.02 (± 1.22)	63.98 (± 0.49)	63.09 (± 0.70)
Lateral:								
Sulfated GAGs (1×10^3 µg/g dry weight)	**268.29 (± 40.81)**	**240.25 (± 32.51)**	**276.13 (± 52.37)**	**261.56 (± 23.45)**	93.44 (± 15.69)	81.85 (± 3.50)	66.68 (± 10.23)	80.66 (± 6.53)
DNA (µg/g dry weight)	487.13 (± 47.83)	425.51 (± 40.00)	**791.20 (± 57.74)**	**567.94 (± 47.02)**	398.25 (± 30.82)	321.58 (± 33.48)	407.87 (± 66.71)	375.90 (± 27.00)

GAG/DNA (μg/μg)	552.72 (± 59.00)	572.71 (± 71.77)	356.91 (± 78.92)	**494.12 (± 44.90)**	241.00 (± 40.70)	281.75 (± 53.36)	194.38 (± 52.36)	239.04 (± 27.96)
Regional Water (%)	66.18 (± 1.53)	62.00 (±1.87)	67.38 (±1.81)	65.19 (±1.10)	63.77 (± 0.82)	65.38 (± 3.27)	62.49 (± 1.10)	63.88 (± 1.15)

3.9 Figures

Figure 3-1. Sites of meniscal sampling in menisci used for harvesting the cylindrical explants and sections used for regional water content. *Top row:* Shows the six sampling sites following removal of the cylindrical explants in both lateral (a) and medial (b) menisci. *Middle row:* shows lateral (c) and medial (d) menisci following separation of the inner two thirds and outer one-third regions from each another. Cuts were extended to involve the meniscal horns *Bottom row:* Shows each region in both lateral (e) and medial (f) cut into three sections, representative of the sampling sites: anterior, central and posterior

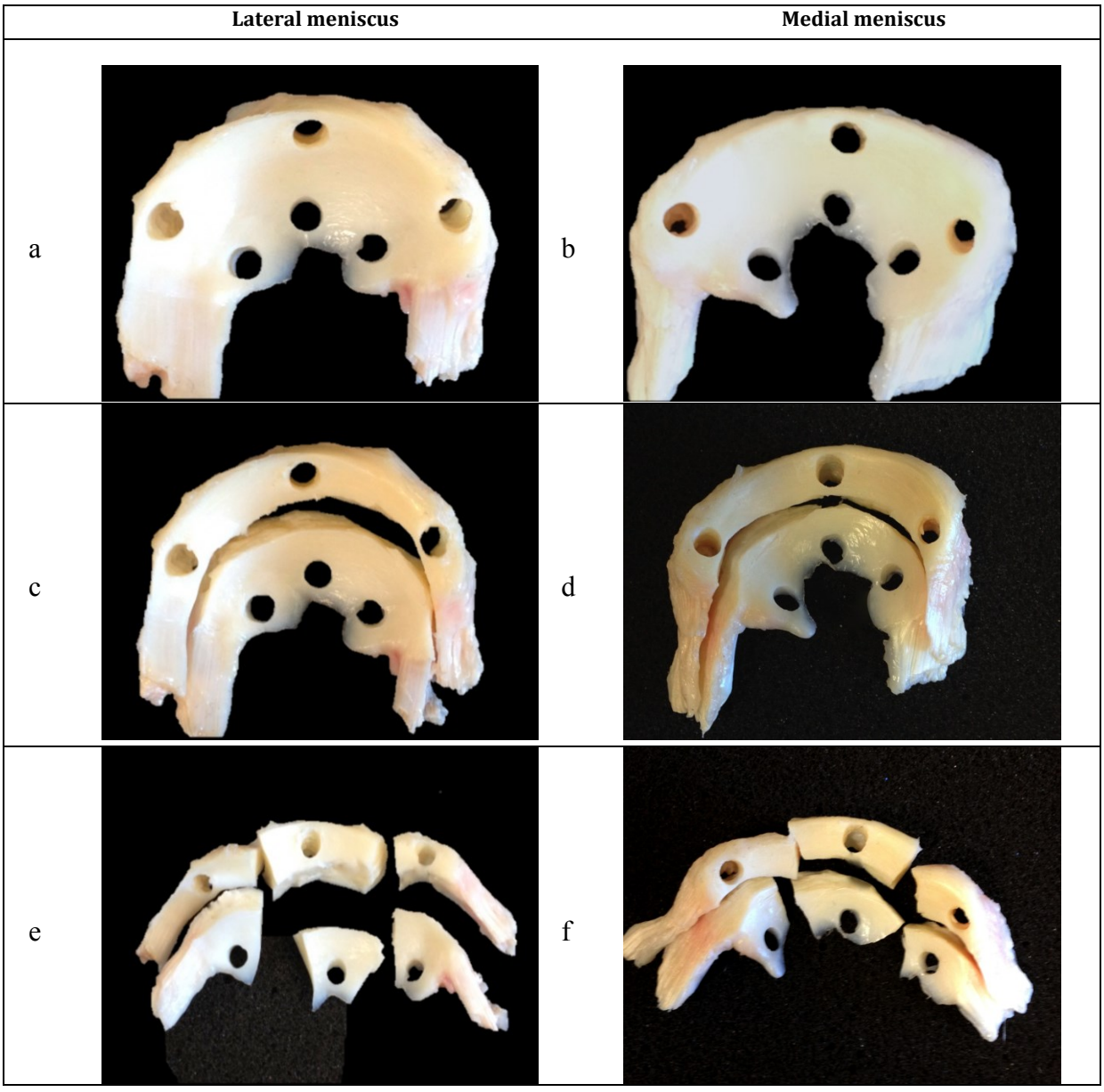
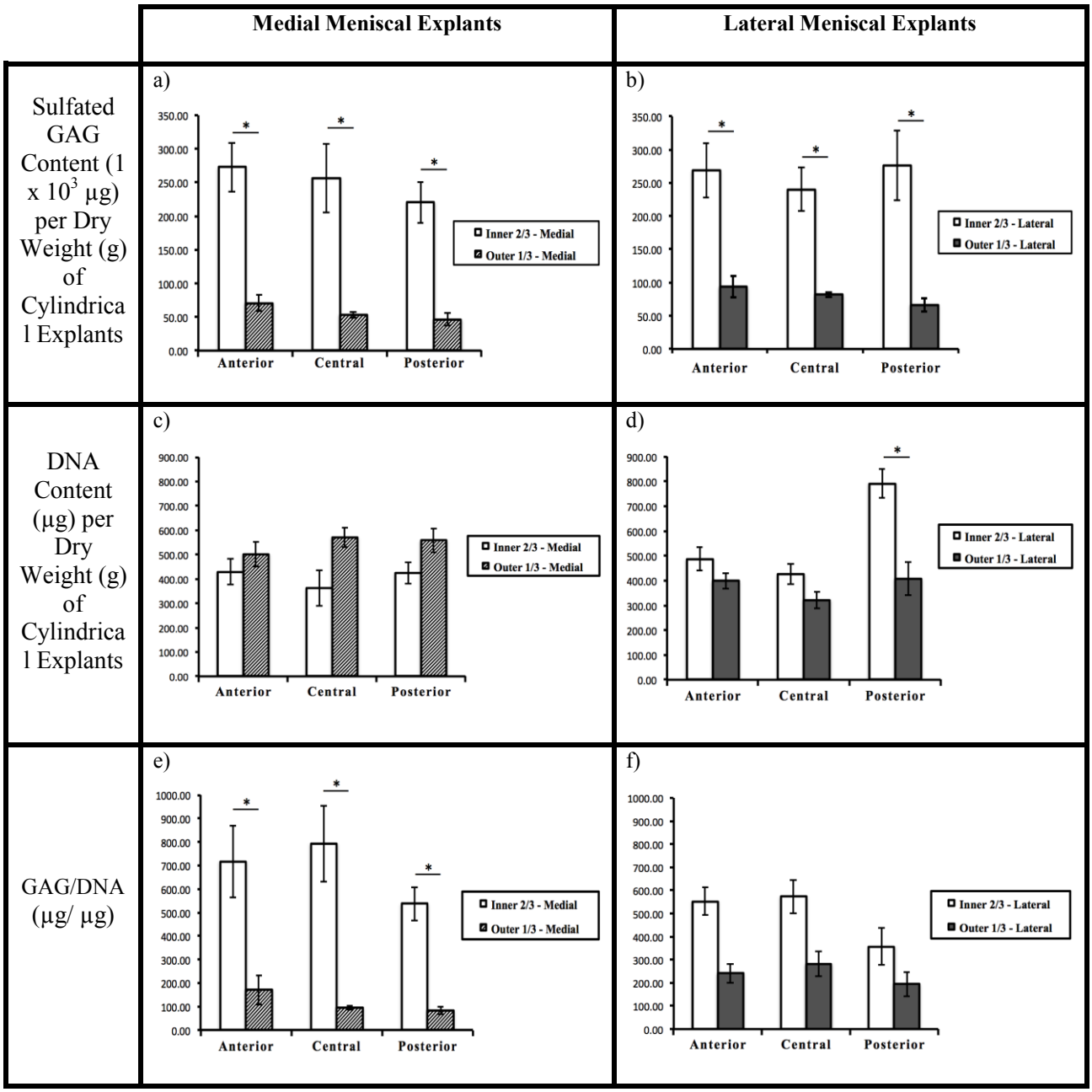


Figure 3-2. Comparison of sulfated GAG, DNA and GAG/DNA contents from different sites within medial and lateral porcine meniscus. Statistically significant differences ($p < 0.05$) between the inner and outer sites are indicated with underlined asterisks over the bars comparing those two regions from medial and lateral meniscal explants. The first column from the left indicates the features being compared with the measurement unit. Sulfated GAG content values are expressed in thousands (1×10^3) µg/g dry weight.



4 Chapter 4. Anatomical Study: Comparing The Human, Sheep and Pig Knee Meniscus

4.1 Introduction

The meniscus holds considerable clinical significance in the human knee and the anatomical features demonstrate propensity for injury [1]. The tendency of human menisci to injury has motivated scientists to look for effective options to treat patients with meniscal injury. However, before a new treatment option can be translated into clinical practice in humans, it has to go through a testing phase in an animal model. The menisci of all mammals, regardless of the walking style or size have the same semilunar shape [2]. Various animal models, especially large domestic quadrupeds, have been utilized in the development of new and successful treatment options for meniscal injury. However, the ability to predict responses in human menisci has not been determined for any model.

Historically, the first study of animal models dates back to the fourth and fifth centuries B.C. when Aristotle dissected various animals in an effort to determine the anatomical differences between them [3, 4]. The modern time concept of using animal models in human research has been linked to August Krogh's famous statement made in 1929, which states "*For a large number of problems there will be some animal choice, or few such animals, in which it can be conveniently studied*" [5]. Ideas similar to the Krogh principle have been widely accepted in relation to plants [6] and have led to the discovery of large numbers of medicinal plants [5]. Prudent application of this principle recognizes species-related differences (anatomy, physiology, nutrition, gait and reproductive differences) that can affect the response of certain animal species

to experimental investigations [5, 7]. In addition, availability and ease of obtaining a particular animal, ethical implication and cost are among the factors to be taken into consideration when choosing an animal model for human research [7].

In meniscal research, various animal models have been utilized including dog, pig, sheep, goat, cow and rabbit [8-12]. Despite the contribution of these models to the overall understanding of meniscus biology, no single animal model has been identified as the most suitable for meniscal research [7]. The dog model has been the focus of many reports in the literature [11, 13-15]. Consequently, this model seems to have the highest amount of information available on identifying and comparing meniscal injuries in dog to human. The popularity of this model among meniscal investigators has been linked to the frequent veterinary hospital presentations and the ease of postoperative handling due to animal compliance, especially in trained dogs [7, 16]. However, the use of this model is beginning to decrease due to the increasing pressure from animal interest groups in society [17]. The sheep model, on the other hand, is getting more attention as a potential model for meniscal research [7]. This could possibly be attributed to the mentioned pressure from animal interest groups opposing the use of dogs in research and the common use of sheep as a source for commercial meat production. Studies comparing the sheep meniscus with other animals and with human will increase our understanding of the degree of resemblance that the sheep model has to human meniscus. The pig model is another practical and economically feasible model, commonly utilized in studies investigating meniscal biomechanics, ultrastructure, transplantation and repair techniques [8, 12, 18, 19]. The cow meniscus has been utilized in a limited number of reports in the literature; however, based on anatomical

measurements the cow meniscus was found to be significantly bigger than the human meniscus [20] and thus cow will not be considered in this work.

The non-uniform shape and complex geometry of the meniscus requires the availability of detailed knowledge of meniscus size and dimensions in human and other species commonly used in meniscal studies. Such information will be useful to investigators in different realms of science and can serve as a guide or a baseline to build upon in designing studies that depend on the geometry of the meniscus. For instance, meniscal transplantation is a promising and emerging procedure that provides an alternative treatment option to patients who have had total meniscectomy [21]. The advantages and potential benefits of meniscal transplantation have been proposed and supported by many clinical [22-26] and experimental studies performed in animal models [27-34]. However, the existing difficulty in obtaining fresh grafts due to the limited number of human donors and the need for size matching between the donor and the recipient demand the development of an effective preservation and storage method that can be used to bank donor meniscal tissue. Vitrification or ice-free cryopreservation is a promising tissue preservation technique that can provide indefinite storage of orthopaedic tissues while maintaining their structural properties and cellular viability as was recently demonstrated for intact human knee articular cartilage [35]. Tissue vitrification requires high concentrations of cryoprotectant agents (CPAs) and rapid cooling to achieve an amorphous glassy state [36]. However, the process to achieving successful vitrification is an extremely difficult with many potentially laborious steps especially, for complex tissue such as the meniscus. For instance, CPA permeation kinetics [37-41] need to be mathematically modeled [42] to minimize CPA exposure and subsequent risk of cellular toxicity. At this stage, anatomical dimensions will be a critical

input to the mathematical models for meniscal cryopreservation development. Moreover, the existing difficulty in obtaining intact human meniscal tissue for such investigations makes the use of animal models very vital for ex vivo meniscal studies.

To date, there is little data on meniscus volume, weight and physical dimensions. If there were sufficient data, it could be used to select an appropriate animal model to advance meniscal surgical repair, or in mathematical models to advance meniscal preservation and transplantation. In major textbooks and reports in the literature, the anatomical description of the meniscus is limited to the general appearance, vascularity pattern and attachments [1, 43-45]. Proffen et al. [20], in an anatomical study compared the human knee and its structures with six animal species: pig, sheep, cow, goat, rabbit and dog. In this study [20], dimensions of the meniscus were determined in all species but were limited only to the width and anterior-posterior length. Their findings [20] indicated that the sheep and goat stifle joints had the closest resemblance to the human knee and that these would likely make good models for meniscal injury treatment research. Based on such findings, and given that our laboratory had used the pig stifle joint as a model for musculoskeletal research, we performed a more detailed and extensive anatomical study comparing the human menisci with those of sheep and pig. Therefore, the purpose of the current study was to compare the weight, volume and detailed dimensions of the body of the human medial and lateral menisci with sheep and pig menisci. Comparison of the size and dimensions is focused on the meniscal body, the area extending between the anterior and posterior horns.

4.2 Materials and Methods

4.2.1 Tissue Collection

Six medial and six lateral healthy menisci (3 paired and 3 unpaired) were harvested from refrigerated knees of 9 deceased human male donors with an average age of 47 (range, 20 to 61 years), sourced from a tissue banking facility (Comprehensive Tissue Centre, Edmonton, AB). Approval of the Research Ethics Board of the University of Alberta, Edmonton, Canada was obtained and institutional safety and ethical guidelines were followed. All specimens came from deceased, unidentified organ and tissue donors. When both menisci were taken from the same joint (n = 3), they were considered as separate samples not as a pair of samples, unless indicated in the study. Sheep menisci (n = 6 medial and lateral) were obtained from adult Columbia and Clun Forest sheep (SunGold Specialty Meats Ltd., Innisfail, AB). Pig menisci (n = 22 medial and lateral) were obtained from adult Yorkshire pigs (Parkland Packers, Stony Plain, AB). All animal joints used in this study were from the hind leg (stifle joint) of sexually mature, male and female animals that were sacrificed for commercial meat consumption at two local abattoirs. Following death, all animal joints were freed from skin and attached muscles, sectioned from mid-thigh to the lower third of the hind leg, placed in plastic bags and refrigerated for up to 48-hrs until dissected.

4.2.2 Tissue Dissection

Human knee joints were dissected by cutting the collateral ligaments to expose the joint followed by careful removal of the cruciate ligaments and adherent joint capsule. Tissue hydration was maintained with phosphate buffered saline (PBS) irrigation of exposed surfaces inside the joint to prevent water evaporation. Only menisci without signs of injury or degenerative changes were included in the study. Intact medial and lateral menisci were removed en bloc and were further freed from synovial attachments, washed in PBS to remove excess synovial fluid, patted dry with sterile gauze and weighed using a standard scale.

In stifle joints, the dissection started anteriorly with a supra-patellar incision that was extended inferiorly on both sides to remove the patella, patellar tendon and surrounding fat pad. The collateral ligaments on both sides were excised to expose the periphery of both menisci. Removal of the patella allowed for clear visualization of the anterior joint structures and provided stability when the joint was turned over to expose the posterior joint structures. Then, the dissection was carried out posteriorly to remove the popliteal blood vessels, posterior joint capsule and all adherent soft tissue. Subsequently, the posterior horn of lateral meniscus was carefully excised from the base of its bony attachment to the posteromedial corner of the medial femoral condyle and the posterior cruciate ligament (PCL) was severed from its posterior tibial insertion site(s). Next, stifle joints were put into flexion and the anterior cruciate ligament (ACL) was severed either from the base of its anterior tibial insertion or mid-way along its course between the femur and tibia. At this point, the femoral condyles were separated from the rest of the joint to provide a clear top view of the menisci. Finally, cutting from the peripheral border to

the anterior and then posterior, each meniscus was separated from the remaining adherent membrane and the horns excised at the bony attachments followed by complete removal of the menisci. Intact medial and lateral menisci were washed in PBS to remove excess synovial fluid. Menisci were then patted dry and weighed prior to measurements.

4.2.3 Volume Determination

Volume displacement was performed based on Archimedes principle. Each medial and lateral meniscus included in the study was fully immersed in a 25mL calibrated glass cylinder, graded with 0.5mL increments, prefilled with 15mL of PBS, ensuring that the upper edge of the meniscus was sitting below the fluid line. The difference in volume before and after placement was attributed to the total volume of the body of the meniscus with the horns. Herein, the meniscal horns were included in the volume determination to allow for comparison with similar findings in the literature.

4.2.4 Meniscal Body Dimension Measurements

Numerous dimensions were measured directly on the surface of each meniscus. Dimensions included circumference, width, peripheral height, height of the articulating surfaces from base to top and the length of the superior articular surface that accommodates the femoral condyle (Fig 4-1). Two circumference measurements were quantified using a flexible, plastic tape measure that was molded: first, around the peripheral rim of the body of each meniscus from

the junction of the fiber bundles of the anterior horn with the body to the posterior horn junction with the body; the second circumferential measurement included the body and both horns, from the tip of the anterior horn to the end of the posterior horn (Fig. 4-1a). A plastic Vernier caliper was used to measure width and peripheral height of the body of both medial and lateral menisci at three different locations, moving from front to the back: anterior third, middle third and posterior third. Width was recorded horizontally from the outer border to the innermost border (Fig. 4-1a). Peripheral height was measured vertically at the periphery from base to top (Fig. 4-1b). Length of the concave, sloped curvature of the superior articular surface facing the femoral condyle was measured with a tape measure. The tape measure was contoured from the tip of the highest point (peripherally) to the lowest point of the innermost edge (Fig. 4-1c) at three locations for each meniscus: anterior, middle and posterior. Height of the articulating surface, which represents a vertical measurement of the mass of the meniscus located between the two opposing articular surfaces, the upper one facing the femoral and the bottom one that is sitting on the tibial bones, was then measured. This measurement was recorded only in the middle third of the body, at four equally distanced points: a, b, c and d, starting from a point that was 2mm away from the peripheral border until the innermost thin edge (Fig. 4-1c). Here, one jaw of the Vernier caliper was placed on the upper femoral surface while the other jaw was placed below the flat bottom surface. Both jaws were moved in a stepwise manner to the four locations at equally calculated distances.

4.2.5 Statistical Analysis

Descriptive and comparative statistical analysis was performed to determine differences between species using one-way ANOVA with Bonferroni's post-hoc testing. *P*-values < 0.05 were considered statistically significant. Values are reported as mean ± standard error of the mean (SEM). Additionally, human menisci went through further analysis to determine differences between medial and lateral menisci. Differences between human medial and lateral menisci were determined for all human menisci included in the study using a paired two-sample t-test. All statistical tests were performed using SPSS version 21.0 (IBM Corp. Released 2012. IBM SPSS Statistics for Windows, Version 21.0. Armonk, NY: IBM Corp).

4.3 Results

4.3.1 Observations

All observations presented herein describe major differences noticed visually during dissection of animal joints as they relate to the human knee with emphasis on the overall shape of the meniscal body in all species. Photographs of human knee and menisci are shown in Fig. 4-2; photographs of pig and sheep stifle joints and, menisci are shown in Fig. 4-3.

The body of both medial and lateral menisci looked smooth and glistening. In pig and sheep, similar to human medial and lateral meniscus, the body of the medial meniscus was more tightly attached to the capsule throughout its peripheral border leading to restricted mobility, while the body of the lateral meniscus was slightly more mobile with a small area located at the middle third that was devoid of capsular attachment, permitting the tip of the scalpel to be passed underneath it. The body of the lateral meniscus had a more uniform width from front to back, while the body of the medial meniscus became broader toward the back. The medial meniscal body in both animals had a noticeable curve towards the back making it have a boomerang-like shape at the posterior third. The pig meniscal body was subjectively stiffer than human and sheep meniscal bodies. Moreover, the lateral meniscal body in pig had a small, notched protrusion at the junction of the body with the posterior horn.

The horns in both sheep and pig were loose and more mobile than the body of the meniscus with visible penetrating blood vessels. Grossly, the horns were arranged in small bundles, in a fashion resembling the cruciate ligaments, running horizontally from the body to their insertion sites on the tibial plateau with thin layers of transparent septa surrounding the bundles. The anterior horn of the lateral meniscus separated the two bundles of the anterior cruciate ligament, which lay side by side in the sheep and pig joints (Fig. 4-4). In human knees the anterior and posterior horns of the lateral meniscus attached centrally at the anterior and posterior intercondylar fossae respectively, while the horns of the medial meniscus had broader insertion sites on the edge of the tibial plateau. Sheep and pig anterior horn attachments on the tibial plateau were broader, away from the center, and the posterior horn of the lateral menisci passed behind the PCL to attach to the lateral aspect of the medial femoral condyle posteriorly. The sheep PCL had a wide posterior tibial insertion such that it almost looked as if it had two insertion sites.

4.3.2 Direct Measurements

Values presented include measurements taken from refrigerated (4 ˚C) medial and lateral menisci in human (n = 6), sheep (n = 6) and pig (n = 22).

4.3.2.1 Weight and Volume

Meniscal wet weights and volumes are presented in Table 4-1. The size of the meniscus varied among the species included in the study. The average wet weights and volumes of human

menisci were larger than sheep (at a ratio of sheep:human of 0.5:1 for both medial and lateral meniscus) and smaller than pig menisci at a ratio of pig:human of 1.3:1 for the lateral and 1.2:1 for the medial menisci (Table 4-1). Compared to human meniscus, the wet weight and volume of lateral menisci in both animals showed statistically significant differences, whereas for the medial menisci only the sheep medial meniscus had weight and volume that were significantly smaller than the human medial meniscus. Across species, the weight and volume of the lateral meniscus were greater than the medial meniscus. In addition, there were strong correlations between the meniscal weight and volume for all species with a correlation coefficient (R^2) of 0.97 and 0.93 for the human medial and lateral, respectively; for pig $R^2 = 0.92$ and 0.90 for the medial and lateral, respectively; and for sheep $R^2 = 0.96$ and 0.76 for the medial and lateral, respectively.

4.3.2.2 Lateral Meniscal Body Dimensions

Measurement (mean ± SEM) and statistical significance of the results of the sheep and pig in comparison to the human lateral meniscus are summarized in Table 4-2. Dimensions of the lateral meniscal body were generally largest in pig menisci, with the exception of the circumference measurements. Human lateral menisci had the longest circumference for both the body and the whole circumference, which included the horns. Statistically significant differences ($p < 0.001$) from the human circumference were found in sheep (for the body and body with horns); in pig only the circumference of the body was significantly smaller. The whole circumference in the pig lateral meniscus was not significantly different ($p = 0.21$) from that of the human lateral meniscus, and that could be attributed to the longer horns in pig. Generally, for all dimensions other than circumference the values of the dimensions of the sheep lateral

meniscus showed no statistically difference when compared to the human lateral meiscus. Whereas, the pig lateral meniscus, with the exception of the circumference, had dimensions that were significantly larger than the human lateral meniscus with an average ratio of pig:human of 1.4:1 (range, 0.8:1 – 2.0:1). Comparing measurements taken from the three specified locations within every species, the horizontal width and peripheral height remained fairly consistent in human from front to back, a feature that was different from the animal menisci. In contrast to the human meniscus, the width and peripheral height of the lateral meniscus in the sheep and the pig were fairly similar in the anterior and posterior thirds while they always decreased in the middle third. Similarly, the sloped superior articular length in human lateral meniscus was greatest in the anterior third while it was greatest in the posterior third of pig and sheep lateral meniscus. The articulating height in all species, which was a parallel continuation to the peripheral height taken at the middle third, decreased gradually toward the innermost edge.

4.3.2.3 Medial Meniscal Body Dimensions

Medial meniscus dimensions and statistical significance in comparison to the human medial meniscus are summarized in Table 4-3. The circumference of the human medial meniscus was the largest and was significantly different from that of the sheep and the pig, for both measurements ($p < 0.001$). For the remaining dimensions measured, the sheep medial meniscus for the most part, was not significantly different from the human ($p > 0.1$); while the pig medial meniscus was significantly larger ($p < 0.001$) than the human medial meniscus with an average ratio of pig:human of 1.3:1. The width in all species increased gradually from anterior to posterior. Sheep meniscal body width showed no statistically significant difference from the

human meniscus other than the measurement at the posterior third of the sheep medial meniscus, which was significantly smaller the same measurement from the human counterpart ($p < 0.001$). The greater width of the pig medial meniscus was significantly different from the human meniscus except for the posterior third, which showed no statistically significant difference from the human meniscus at the same location. In contrasting with the human medial meniscus, the sheep and the pig medial meniscus had a peripheral height that was almost identical at the anterior and posterior thirds, and greater than the middle third measurement. The length of the superior articular surface was greatest in the posterior third of the medial meniscus in all species. The articulating height was found to decrease gradually towards the inner edge across all species.

4.3.3 Human Meniscal Parameters

4.3.3.1 Lateral versus Medial Menisci

No statistically significant differences were found between lateral and medial menisci ($p > 0.05$). Furthermore, as three medial and lateral menisci were paired, belonging to the same donors while the remaining three sets were from different donors, human menisci were further stratified into two groups to determine differences within and between paired and unpaired samples. In the paired sample comparison, only the meniscal width showed a statistically significant difference ($p = 0.03$) between medial and lateral menisci. Moreover, there were no statistically significant differences between the paired and the unpaired groups.

4.4 Discussion

The purpose of this descriptive laboratory study was to develop an understanding of the human meniscus weight, volume and anatomical dimensions, and to compare those parameters with sheep and pig menisci.

It was observed that both sheep and pig joints are grossly similar to their human counterpart but some differences do exist. The data showed interspecies and intra-group variability in some of the parameters measured. Both animal models are cost-effective and relatively easy to obtain options that can be used for experimentation. The average cost of each joint was $4.80 (Canadian dollars) for sheep and $3.30 (Canadian dollars) for pig. Pig joints had a large amount of anterior fat pad, subjectively it was felt that this could lead to difficulty with arthroscopic device insertion into the joint [46]. Moreover, pig menisci demonstrated increased stiffness and the sheep meniscus was softer, making it easier to create surgical lesions for meniscal research using the sheep model [47].

For the direct dimensional measurements, the body of each meniscus was divided into three cross sectional locations: anterior third, middle third and posterior third. Dimensions were measured individually at these three locations. Results are shown in tables summarizing individual measurements from each location and the average of all three locations was also included. Circumference, width, peripheral height, articulating height and length of the superior articular surface were measured from the body of every meniscus included in the study. Data

reported herein should help to establish an understanding of the anatomical measurements in the human menisci and to provide direct evidence that the sheep model, from a comparative anatomy point of view, is a suitable and readily-available model that can be used to evaluate meniscal investigations with the aim of applying findings to human meniscus.

Human menisci have been the focus of the majority of reports found in the literature. Most reports have looked specifically into certain, but not all, aspects related to studying the human meniscus anatomically. Magnetic resonance imaging (MRI) has been used to quantify meniscal sizing and dimensional parameters [48, 49]. Our findings for the human meniscal volume differ from previously published studies that used similar volume determination techniques. In a study of 21 fresh frozen cadaver knees, Stone et al. [48] determined the volume of human menisci using 3D MRI and compared it with volume determined by the water displacement method. They reported the water displacement volume as 2.5 ml (SEM, ± 0.3 ml) for the medial meniscus and 2.5 ml (SEM, ± 0.2 ml) for the lateral meniscus in human. Furthermore, the authors reported that the MRI technique consistently underestimated the true volume of the meniscus [48]. Bowers et al. [49] determined the volume of human menisci taken from cadaveric knees using MRI and compared each finding for the same meniscus to its respective water displacement volume. They reported mean volume of 3.04 ml (SD, ± 0.04 ml) for the medial menisci and 3.07 ml (SD, ± 0.07 ml) for the lateral menisci. In our study, we found the volume to be larger at 4.5 ml (SD, ± 1.4 ml) and 5 ml (SD, ± 1.3 ml) for the medial and lateral menisci, respectively. Differences observed between our findings and previous reports could be attributed to our use of refrigerated, male menisci while Stone et al. [48] and Bowers et al. [49] used frozen samples; freezing can cause tissue dehydration, leading to a post-thawing

volume that may be lower than the initial volume. Moreover, in our study adequate tissue hydration was maintained throughout the dissection process by irrigating exposed surfaces inside the joint with PBS to decrease water evaporation during dissection, which was not mentioned in the other studies. Perhaps more importantly, the genders of the cadaver menisci studied by both Stone et al. [48] and Bowers et al. [49], that were not mentioned in their reports could have been another contributing factor. This factor could also explain the observed differences as we used only male menisci in our study.

Dimensional parameters similar to those reported in this study for human menisci were found in a small number of studies [50-58]. In some of those studies, despite the complexity of their experimental protocols, the dimensional measurements did not focus on the body of the meniscus that has the greatest contribution to the knee mechanics. The width, height and anterior-posterior length were the most commonly investigated dimensional parameters. Moreover, the terminology used in describing some of the dimensional parameters could also be confusing. For instance, the width of the meniscus was usually measured from the peripheral edge to a line connecting the anterior and posterior horns while the menisci were still attached to the tibial plateau [54, 55, 57, 58]. This width does not describe the portion of the meniscus itself that undertakes the biomechanical functions. Instead, it also includes the area of the tibial plateau enclosed by the meniscus, extending from the most peripheral border of the meniscus at the meniscosynovial junction to the horns insertions. This is more of a clinical approach to meniscal width as opposed to our more anatomical approach. In this study, we measured the width of the body of human menisci at three different locations: anterior third, middle third and posterior third to account for the widening observed in the medial meniscus from front to back, and determined

the average of the three measurements. Erbagci et al. [51] in a dimensional MRI study of normal human menisci in 174 male and female subjects (mean age 29, range 18 – 60) reported the mid-body width at 7.4 mm (SD, ± 2.65 mm) and 8.4 mm (SD, ± 0.83 mm) for the medial and lateral menisci, respectively. McDermott et al. [53] in an anatomical study of meniscal allograft sizing, used a digital Vernier caliper to determine the width of the meniscal body at the mid-portion of 44 lateral and 44 medial menisci. They reported a meniscal body width of 9.3 mm (SD, ± 1.3 mm) and 10.9 mm (SD, ±1.3 mm) for the medial and lateral menisci, respectively. In our study, we found the width at the middle third of human menisci to be 8.3 mm (SD, ± 1.2 mm) for the medial meniscus and 11.6 mm (SD, ± 1.3 mm) for the lateral meniscus. Bloecker et al. [50] and Wirth et al. [56] used 3-dimensional reconstruction of MRI images to measure meniscal width at different locations and reported only the average of all measurements. Wirth et al. [56] studied intact medial menisci of 11 female subjects (mean age 55.3 ± 6.0 years) and reported the mean width of the female medial meniscus as 8.96 mm (SD, ± 0.5 mm), while Bloecker et al. [50] who studied healthy medial and lateral menisci of 47 male subjects (mean age 57 ± 9 years, range 45-79) reported the mean width as 9.92 mm (SD, ± 1.0 mm) for the medial meniscus and 10.1 mm (SD, ± 1.2 mm) for the lateral meniscus. We found the mean width in male menisci to be 10.55 mm (SD, ± 3.4 mm) and 11.59 mm (SD, ± 1.0 mm) for the medial and lateral menisci, respectively. These differences suggest the need for gender stratification of meniscal dimensional measurements.

The peripheral height of intact human meniscal body, also termed “maximal thickness” by some authors [50, 56], was found in a limited number of studies. Erbagci et al. [51] reported the height at mid-body as 5.03 mm (SD, ± 0.91 mm) for the medial meniscus and 4.94 mm (SD, ±

0.9 mm) for the lateral meniscus. Wirth et al. [56] determined this measurement at the highest point of the medial meniscal body, which they termed maximal thickness, in 11 female subjects and found it to be 6.72 mm (SD, ± 1.45 mm). Bloecker et al. [50] reported the height, described as maximal thickness, as 7.7 mm (SD, ± 1.13 mm) and 7.2 mm (SD, ± 0.97 mm) for the medial and lateral meniscus, respectively. Values consistent with these are reported herein, and we found that the peripheral height was greatest posteriorly with a value of 7.0 mm (SD, ± 1.6 mm) and 6.2 mm (SD, ± 1.8 mm) for the medial and lateral menisci, respectively. Interestingly, contrary to healthy menisci, the height of menisci from 257 symptomatic osteoarthritis in female knees was found to be lower with an average value of 2.9 mm (SD, ± 2.0 mm) for the medial meniscus and 5.4 mm (SD, ± 2.5 mm) for the lateral meniscus [52].

The ability of the meniscus to function as a load distributor is dependent on the robustness of the peripheral border to withstand axial loading and convert it into circumferential hoop stress within the collagen fibers [1]. The circumference length has been quantified by Kohn et al. [59] and McDermott et al. [53]. Kohn et al. [59] in a cadaveric study used a non-elastic polyester thread that was placed along the periphery of 56 medial and lateral human menisci, extending to their horn insertions. They stratified menisci into left knee and right knee groups. Their report showed the circumferential length to be 110.86 mm (SD, ± 13.18 mm) and 111.15 mm (SD, ± 11.07 mm) for the medial and lateral menisci of the right knee joints; and there was no significant difference between menisci taken from the right and left knees of the same donors. McDermott et al. [53] in an anatomical study measured the circumference in 44 menisci using a thin steel wire that was molded around the peripheral rim of each meniscus from the anterior to the posterior bony insertion. They reported the circumference length as 99.0 mm (SD, ± 9.3 mm) and 91.7 mm

(SD, ± 9.6 mm) for the medial and lateral menisci, respectively; noting that the medial meniscus was longer than the lateral meniscus. Those measurements are comparable to those reported in the current study as 101.17 mm (SD, ± 7.7 mm) and 103.5 mm (SD, ± 3.9 mm) for the medial meniscus and lateral meniscus, respectively. In addition, we have included another measurement that was focused only on the meniscal body, excluding the horns resulting in a measurement of the meniscal body circumference, which was not found in any report obtained (Tables 4-2 and 4-3).

Limitations in this study were the small sample sizes in the human and sheep groups, and the inclusion of only male donors in the human group. Although the latter helped in reducing the variability in the parameters measured, it precluded the determination of the gender-based differences in the human samples. Another limitation was not measuring the antero-posterior (A-P) length of all menisci included in the study. However, as our measurements were performed after specimens were completely separated from the tibial plateau, this could change the arc or the curve at which the menisci turn to insert into the tibial plateau contributing to inaccurate lengths, especially in the human lateral meniscus, which has more central anterior and posterior insertions. Nonetheless, in a small subset of six medial and lateral menisci in pig (not reported), this length was measured and was compared to the circumference of the respective sample. In the same subset, we found that the A-P length in pig menisci represented an average of 46.63 % (range, 42.22 – 51.90) of the whole circumference in lateral menisci and 46.31 % (range, 46.18 – 51.67) of the whole circumference in medial menisci. When compared with the circumference of the body alone, the percentage increased to 57.13 % in the pig lateral meniscus and to 55.21 % in the pig medial meniscus.

Despite the cross-species similarities in the shape of the knee and meniscus, quadruped knees are loaded in the flexed position, while human knees are fully loaded in extension indicating differences in the range of motion at which animal menisci perform their biomechanical functions [15]. Despite the positional difference, the sheep model has been reported by others to be a suitable model for meniscal repair studies. Chevrier et al. [9], using histology and scanning electron microscopy, performed a study comparing the sheep and rabbit meniscus to the human meniscus. They reported the sheep meniscus to have greater structural similarity to the human meniscus in terms of vascularization patterns, cell density and ECM collagen ultrastructure [9]. Burger et al. [60] who investigated the effect of sutured and unsutured radial meniscal tears on the articular cartilage degeneration in 20 sheep, reported that their meniscus displayed a pattern of articular cartilage degeneration that was similar, although more rapid, to what has been reported [61, 62] for the human meniscus. In support of these reports, our study found that the human medial and lateral meniscal dimensions were most similar to the sheep medial and lateral menisci. However, the weight and volume of the human medial meniscus was more comparable only to the pig medial meniscus.

4.5 Conclusion

Evaluation and development of new treatment options or products to repair human meniscus requires testing in an appropriate animal model to allow for clinical translation. Furthermore, tissue dimensions control CPA permeation directly affecting outcomes of cryopreservation protocols, meaning that closely matching dimensions would be important for selecting an animal model for cryopreservation studies. In this study, with respect to physical anatomy, we found that sheep lateral and medial meniscal dimensions more closely matched the human meniscal dimensions when compared to those of pig. Based on observational and dimensional features of the two animal models reported in this study, our data indicated that the main dimensional parameters of the sheep model would make it a more suitable animal proxy, supporting other reports advocating the use of the sheep model in meniscal research related to physical dimensions. Furthermore, these detailed measurements can be used in mathematical models of cryoprotectant agent permeation into the meniscus in future studies aimed at vitrification of the meniscus for storage and transplantation.

4.6 Endnote

A version of this chapter will be submitted for publication, with the following authors: Talal Takroni, Leila Laouar, Janet A W Elliott, Adetola Adesida and Nadr Jomha.

Conflict of interest statement

Each author declares no conflict of interest through commercial association in relation to the submitted article.

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4.8 Tables

Table 4-1. The average meniscal weight (g) and volume (ml) in human, sheep and pig. Human values are in bold. Statistically significant differences to the human menisci are indicated with asterisks where (*) marks significance levels with p-values < 0.05 and (**) marks values that had significance level with p-values < 0.001. Pig menisci had the largest values while the human menisci were larger than the sheep and smaller than the pig menisci

	Mean ± Standard Error of the Mean (S.E.M)			
	Lateral Meniscus		Medial Meniscus	
	Weight (g)	Volume (ml)	Weight (g)	Volume (ml)
Human (n=6)	**4.95 (± 0.44)**	**5 (± 0.53)**	**4.43 (± 0.46)**	**4.5 (± 0.58)**
Sheep (n=6)	2.5 (± 0.19)**	2.33 (± 0.17)**	2.21 (± 0.22)*	2.22 (± 0.22)*
Pig (n=22)	6.44 (± 0.26)*	6.52 (± 0.27)*	5.02 (± 0.17)	5.09 (± 0.18)

Table 4-2. Dimensions, in millimeters, of the lateral meniscus in the human, sheep and pig. Human dimensions are in bold. Statistically significant differences from the human lateral meniscus are marked with asterisks where (*) indicates significance levels with *p*-values < 0.05 and (**) indicates significance level with *p*-value < 0.001.

		Lateral Meniscus		
		Mean ± Standard Error of the Mean (S.E.M)		
		Human (n=6)	**Sheep** (n=6)	**Pig** (n=22)
Circumference				
	Body	**84 (± 1.73)**	50.5 (± 0.96) **	66.77 (± 0.80) **
	Body with horns	**103.5 (± 1.6)**	73.83 (± 2.27) **	94.54 (± 2.17)
Width				
	Anterior	**11.5 (± 0.43)**	11.67 (± 0.33)	15.95 (± 0.23) **
	Middle	**11.62 (± 0.53)**	9.83 (± 0.31)	14.63 (± 0.35) **
	Posterior	**11.67 (± 0.33)**	11.33 (± 0.42)	16.63 (± 0.30) **
	Average	**11.59 (± 0.24)**	10.94 (± 0.27)	15.74 (± 0.2) **
Peripheral height				
	Anterior	**6.41 (± 0.89)**	7.08 (± 0.49)	11.23 (± 0.28) **
	Middle	**6.33 (± 0.49)**	4.83 (± 0.31)	8.04 (± 0.30) *
	Posterior	**6.17 (± 0.75)**	8.5 (± 0.22)	13.13 (± 0.29) **
	Average	**6.3 (± 0.39)**	6.8 (± 0.41)	10.80 (± 0.31) **
Articulating height				
	Point - a	**4.0 (± 0.45)**	3.25 (± 0.17)	5.72 (± 0.16) *
	Point - b	**2.5 (± 0.22)**	1.67 (± 0.21)	3.5 (± 0.17) *
	Point - c	**1.13 (± 0.13)**	0.93 (± 0.03)	1.82 (± 0.12) *
	Point - d	**0.67 (± 0.03)**	0.55 (± 0.05)	0.93 (± 0.02) **
Superior articular length				
	Anterior	**13.5 (± 0.43)**	10.67 (± 0.42)	16.54 (± 0.37) **
	Middle	**12.5 (± 0.72)**	10.33 (± 0.49)	16.91 (± 0.39) **

	Posterior	**12.5 (± 0.43)**	13.0 (± 0.36)	19.45 (± 0.31) **
	Average	**12.83 (± 0.32)**	11.33 (± 0.37)	17.63 (± 0.26) **

Table 4-3. Dimensions, in millimeters, of the medial meniscus in the human, sheep and pig. Human dimensions are in bold. Statistically significant differences from the human lateral meniscus are marked with asterisks where (*) indicates significance levels with *p*-values < 0.05 and (**) indicates significance level with *p*-value < 0.001.

		Medial Meniscus		
		Mean ± Standard Error of the Mean (S.E.M)		
		Human (n=6)	**Sheep** (n=6)	**Pig** (n=22)
Circumference				
	Body	**88.67 (± 2.13)**	55.5 (± 1.33)**	64.95 (± 1.04)**
	Body with horns	**101.17 (± 3.15)**	71.83 (± 1.74)**	89.04 (± 1.86)**
Width				
	Anterior	**8.5 (± 0.62)**	9.83 (± 0.30)	14.23 (± 0.32)**
	Middle	**8.33 (± 0.49)**	7.67 (± 0.49)	12.23 (± 0.30)**
	Posterior	**14.83 (± 0.79)**	10.83 (± 0.48)**	16.00 (± 0.37)
	Average	**10.55 (± 0.81)**	9.44 (± 0.39)	14.15 (± 0.27)**
Peripheral height				
	Anterior	**5.5 (± 0.34)**	5.83 (± 0.31)	10.18 (± 0.34)**
	Middle	**5.0 (± 0.45)**	4.42 (± 0.37)	6.23 (± 0.22)
	Posterior	**7.0 (± 0.68)**	5.83 (± 0.47)	10.04 (± 0.37)*
	Average	**5.83 (± 0.34)**	5.36 (± 0.27)	8.82 (± 0.29) **
Articulating height				
	Point - a	**3.0 (± 0.00)**	3.25 (± 0.36)	4.45 (± 0.14)**
	Point - b	**1.58 (± 0.20)**	1.5 (± 0.34)	2.45 (± 0.14) *
	Point - c	**0.95 (± 0.02)**	0.86 (± 0.05)	1.13 (± 0.07)
	Point - d	**0.72 (± 0.04)**	0.55 (± 0.05)*	0.77 (± 0.03)
Superior articular length				
	Anterior	**9.67 (± 0.80)**	10.5 (± 0.72)	13.91 (± 0.36)**

Middle	**9.15 (± 1.8)**	10.33 (± 0.49)	13.77 (± 0.37)**
Posterior	**15.67 (± 0.84)**	12.0 (± 0.58) *	17.59 (± 0.43)
Average	**11.49 (± 0.98)**	10.94 (± 0.37)	15.09 (± 0.31)**

4.9 Figures

Figure 4-1. Meniscal dimensional measurements in a pig stifle joint representative of all measurements recorded across species. Pane (a) shows the two circumference measurements along the periphery of the meniscal body (*solid semi-circular line*) and the whole periphery (*dotted semi-circular line*); the width of the meniscal body as measured from the three locations, indicated with blue arrowed lines. Pane (b) shows the three locations for the peripheral height, arranged from right to left: anterior, middle and posterior third. Pane (c) shows a cross-sectional view at mid-point in a pig meniscus demonstrating the superior articular length (*solid curved red line*) and the articulating height (*black vertical lines*) at the four points (a, b, c and d).

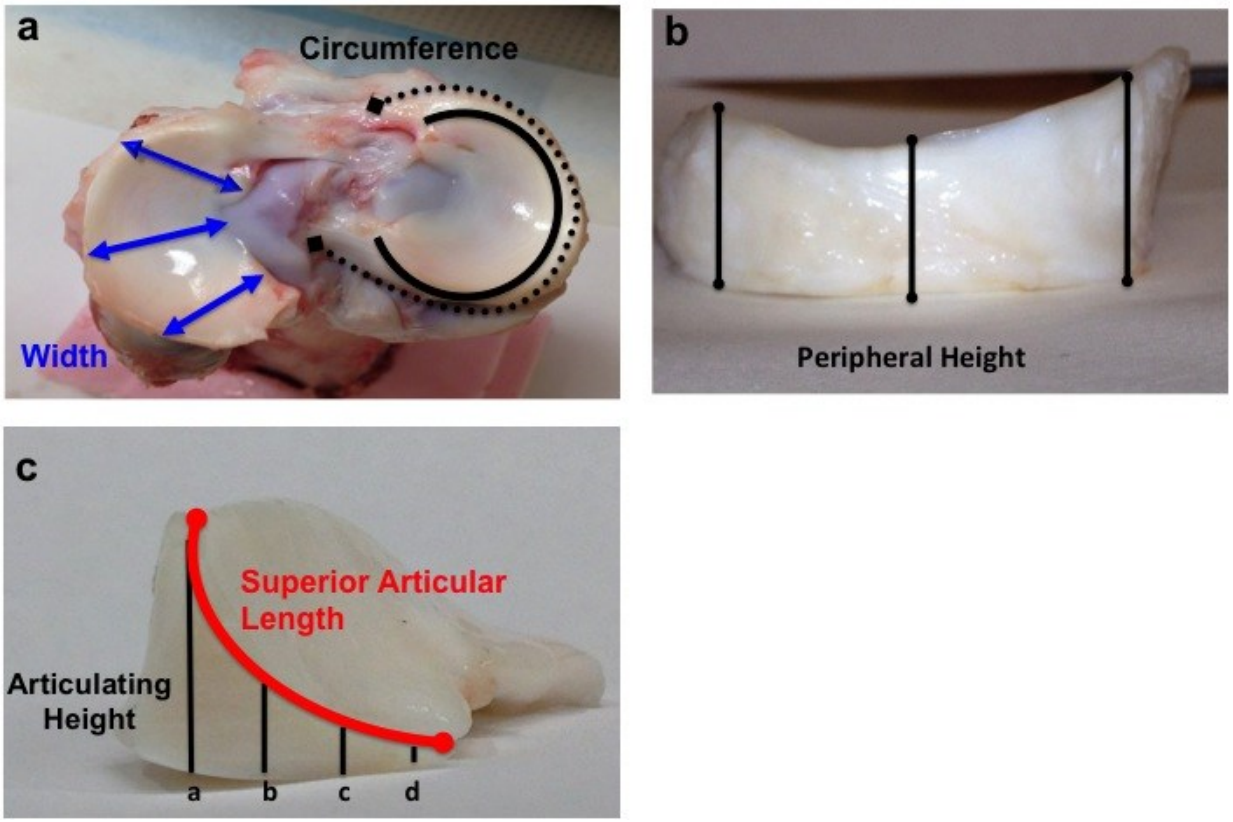


Figure 4-2. Gross anatomy of the human knee joint at different phases during dissection. Pane (a) shows the knee joint partially opened after severing the anterior cruciate ligament (ACL). Pane (b) shows the superior view after removal of the femoral condyles showing the meniscal insertion sites. Pane (c) shows menisci following complete separation from the joint surface placed on a sterile green sheet. *Left,* lateral meniscus with symmetrical width from front to back. *Right,* medial meniscus widens gradually towards the back.

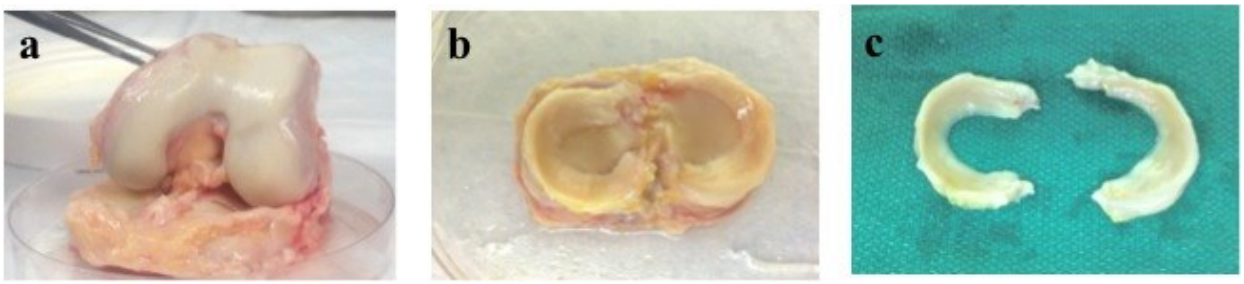
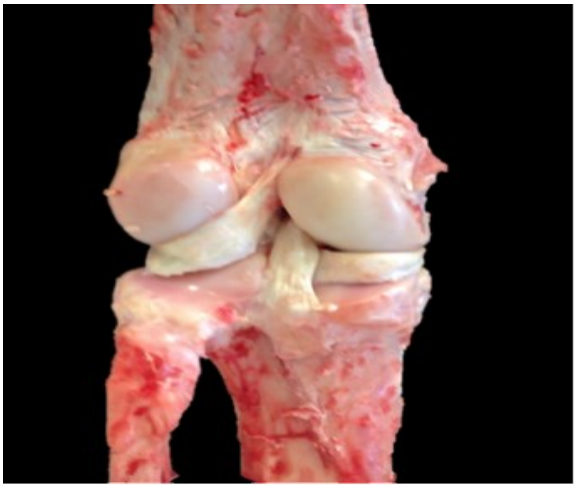
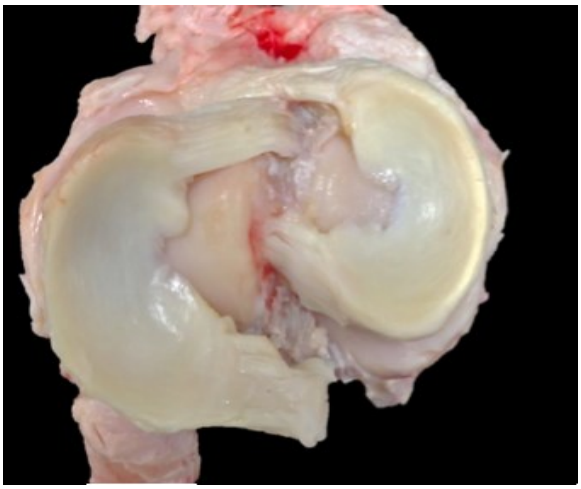
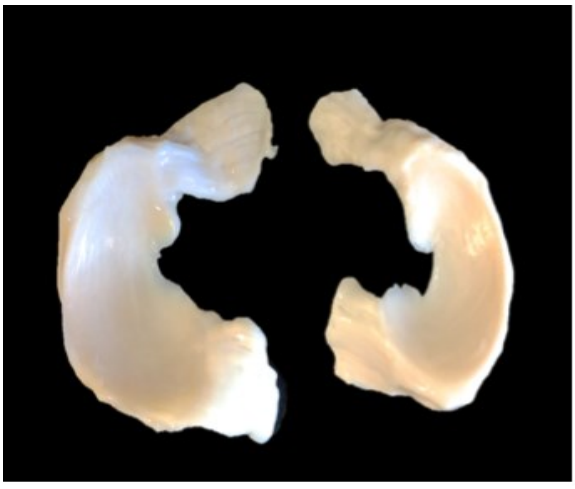


Figure 4-3. Different views of a left stifle joint in pig (left column) and a right stifle joint in sheep (right column). *Top row*: shows posterior view of the femoral and tibial parts of the joints after removal of the surrounding fat. This back view demonstrates the attachment site of the posterior horn of the lateral meniscus into the postero-lateral corner of the medial femoral condyle and below there is the PCL insertion site into the tibia. Noticeable is the absence of the fibula in the sheep joint. *Middle row*: shows top view of the medial and lateral menisci with their tibial insertion sites after removal of the femoral condyles and cutting the cruciate ligaments. The medial meniscus shows both horns attached to the tibial plateau and the lateral meniscus shows only the anterior horn attachment to the tibial plateau. The posterior horn of the lateral meniscus is hanging free after being separated from its insertion to the medial femoral condyle. The anterior horn of the medial meniscus was the most anterior structure in the sheep and pig stifle joint. *Bottom row*: shows the morphology of the medial and lateral menisci after complete separation from the joint surface. Left bottom: the pig lateral meniscus is to the left and the medial meniscus is to the right. Right bottom: the sheep medial meniscus is to the left and the lateral meniscus is to the right.

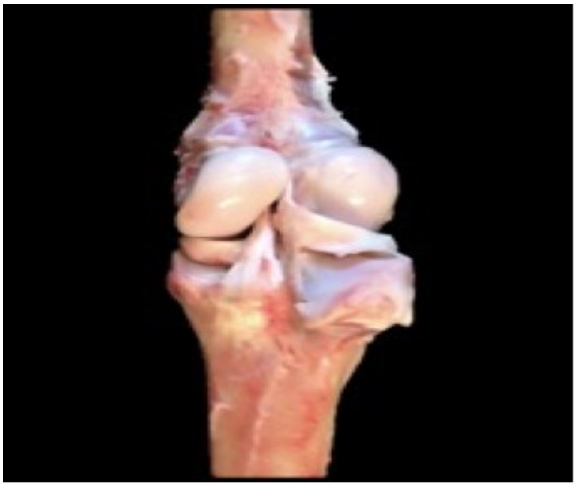
Pig stifle joint

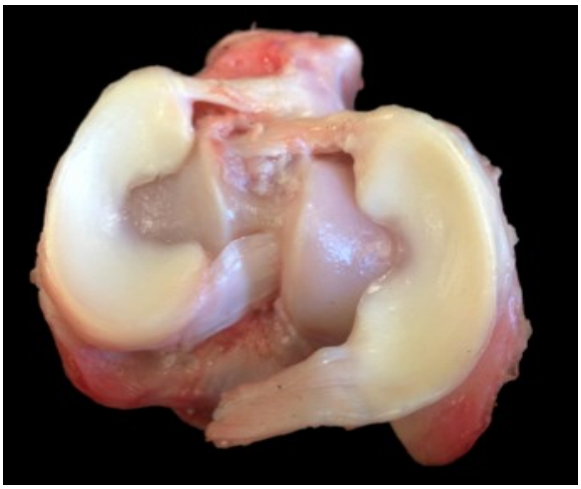






Sheep stifle joint





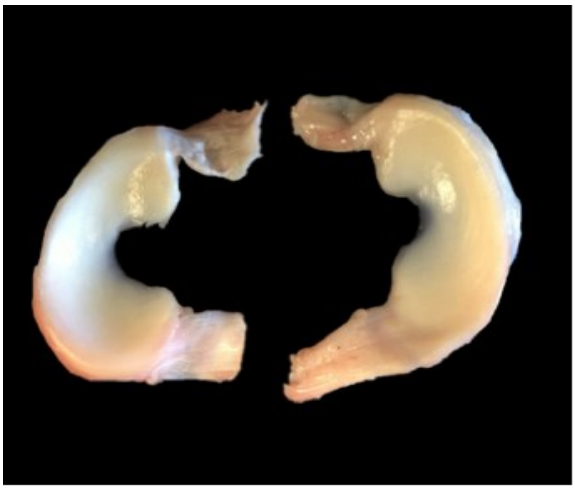
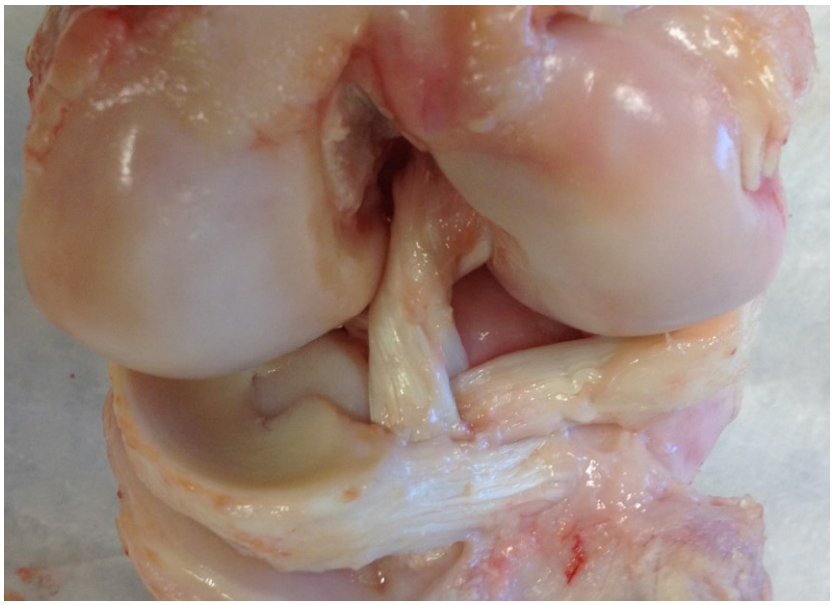


Figure 4-4. Pig joint with the two bundles of the anterior cruciate ligament (ACL) separated by the anterior horn of the lateral meniscus.



5 Chapter 5. Conclusion

5.1 Concluding Remarks and Future Directions

Studies presented in this thesis were designed to investigate the cryoprotectant agent behavior, using two CPAs (ethylene glycol and glycerol), with porcine meniscal tissue. Additionally, this work examined microstructural and anatomical characteristics of the porcine meniscus. The theoretical literature is currently lacking any report on the CPA behaviour with meniscal tissue, a factor that has continued to result in failure of all previous attempts to cryopreserve the meniscus. Nonetheless, this study confirmed earlier findings and answered our questions regarding the biochemical composition and anatomical features of the meniscus. Herein, a brief background will provide the importance of the subject in discussion and the rationale for carrying out this work and future directions based on the findings from the performed studies.

The menisci fulfill important, protective biomechanical functions in the knee joint during normal and excessive loading conditions. Meniscal injuries lead to a decrease in the protective role of the meniscus in the knee joint leaving the articular cartilage exposed to increased static loading. Loss of meniscal function is associated with an increased risk for knee pain and osteoarthritis (OA) [1]. These injuries have been found to be more common in the 3rd, 4th and 5th decades of life, especially in athletically active individuals. In 2007-2008, 4.2 million Canadians (16 % of the population) aged 15 years or older reported having arthritis, that

consequently resulted in huge burden on the heath care system with an estimated cost close to $6.4 billion [2]. These numbers are expected to almost double by 2031 [2].

Currently, irreparable meniscal injuries are treated with arthroscopic partial meniscectomy. This procedure leads to excellent short-term results with knee deterioration seen at long-term follow-up as it create an area inside the knee joint that is devoid from protective meniscal tissue. Moreover, arthroscopic partial meniscectomy has not resulted in a remarkable reduction in the overall incidence of OA, meaning that partial meniscectomy only provides a temporary solution for meniscal injuries [3]. Additionally, non-salvageable injuries always require total removal of the meniscus, leaving the articular surfaces exposed to rub against each other during loading. The resultant wear and tear accelerates degenerative changes [3].

The lack of satisfactory results following meniscectomy merited the search for alternative treatment options, such as allograft meniscal transplantation. Several clinical studies have been carried out examining the feasibility and efficacy of human meniscus transplantation [4]. Patients involved in several published studies reported pain relief and overall functional improvement [4]. However, meniscal allograft transplantation is not widely applied due to the limited number of available donors' grafts and the need for size matching and infectious diseases testing to ensure the recipient's safety. Moreover, the current absence of an effective tissue storage technique makes it difficult to mitigate those limitations that are hindering the wide application of meniscal transplantation [5]. The development of a long-term storage method for menisci would permit the creation of a meniscus tissue bank where donor menisci could be stored after screening and it

would allow surgeons to choose size-matched allografts from a pool of available shapes and sizes [5].

Cryopreservation uses cryoprotectant agents (CPAs) including ethylene glycol (EG) and glycerol to preserve a variety of live tissues, and understanding of the CPA behaviour in relation to the meniscus will be critical in designing a vitrification protocol for meniscus. Moreover, successful vitrification would preserve viable cells to maintain the biosynthetic activity and biomechanical properties of the meniscus [6]. Within this work, we investigated the post-incubation efflux behaviours of EG and glycerol out of the porcine meniscal tissue by virtue of passive diffusion. Using changes in the concentrations of two consecutive washout solutions, we evaluated the efflux rate, temperature effect and the difference between EG and glycerol following two selected incubation periods. Results from this work confirmed our anticipated effects and differences. These preliminary data will facilitate future design of experiments aimed at achieving meniscus vitrification.

Many advances in meniscal clinical practice have been based on basic science investigations using translational animal models [7]. Even with the ability to use cadaveric human specimens in certain branches of meniscal investigations, it would be very difficult to thoroughly examine particular aspects within the living human such as function, response to injury and efficacy of a new experimental procedure [8]. On the other hand, animal models can allow for in-vivo and ex-vivo examinations, and provide easily obtainable and cost effective methods for studying the meniscus. However, despite the anatomical and structural similarities, the ability of these animal models to accurately represent actual responses in the human meniscus

is still unknown [8]. While no specific animal model has been found to be the most suitable for all aspects of meniscal investigations, the porcine model is commonly used in our laboratory for Orthopaedic tissue experimentation. Before the porcine meniscus can be regarded as a suitable model for human meniscal investigations, it has to be examined from a physiologic, anatomical and functional prospective. Moreover, any relevant findings from the porcine model need to be compared to commonly used animal species and to the human meniscus.

Additionally, through this work, anatomical and structural features of the porcine meniscal tissue were examined. Structurally, through desiccation and biochemical analysis, the water content, glycosaminoglycans (GAGs) content and DNA content provided evidence for physiological features of the porcine meniscus that can be compared to the human meniscus. However, the DNA content showed an unanticipated variation between medial and lateral menisci that merit future investigations to confirm this finding.

Anatomically, the weight, volume and dimensions of the porcine meniscus body were compared to corresponding measurements from the human and sheep menisci. However, based on anatomical features, the sheep meniscus showed more resemblance to the human meniscus than the porcine meniscus, suggesting that it might be a more suitable model than the current model that was used.

Findings from this work will serve as baselines upon which to build in designing future studies aimed at achieving successful vitrification of animal menisci that can be later applied to the human meniscus. Based on previously published work in our laboratory on the articular cartilage, we know that all of these cumulative findings will be of relevance in studying the permeation of certain CPAs into the porcine meniscal tissue. The preliminary data of the behavior of EG and glycerol will aid in determining the duration of the incubation period required to achieve sufficient loading of the porcine meniscus. Structurally, the water content indicates that CPA will have enough water molecules to bind to. Moreover, based on prior evidence, the high concentration of the sulphated GAGs in the porcine meniscus is expected to have a negative effect, leading to slower rates of permeation. Moreover, anatomical dimensions will be of critical significance to apply mathematical modelling aimed at describing the CPA permeation kinetics.

There are still many unanswered questions and the effort to achieve meniscus vitrification will be an extensive, multi-experimental effort. We can look at the current work as a first step toward designing future studies that focus on investigating the CPA permeation kinetics. In further research, different types of CPAs can be studied to provide a wide range of times needed to achieve sufficient meniscus loading. Once those times have been determined, the spatial distribution of CPA within the meniscus can determined using magnetic resonance imaging.

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