Optimization of Vitrification for Human Articular Cartilage

by

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ABSTRACT

Osteoarthritis, a disease resulting in the breakdown of cartilage and bone within joints, is a global burden that is growing in scope. There is no cure for osteoarthritis, and the current treatments are all lacking in some form or another. One treatment which attempts to prevent degeneration of cartilage injury into osteoarthritis is osteochondral allografting. This surgery involves the transplantation of healthy bone and cartilage to replace damaged and diseased areas. Osteochondral allografting application is limited primarily by the supply of fresh, healthy tissue and the lack of a long-term storage method that maintains cell viability within cartilage.

Vitrification is a method of cryopreservation that preserves cells and tissues at temperatures low enough to halt all biological activity while maintaining cell health when applied properly. Previous work within this lab resulted in successful vitrification of human articular cartilage, but there is room for improvement. The current research was performed to explore the use of additive compounds as well as the use of a vitrification protocol with altered cryoprotectant exposure criteria in an attempt to improve the post-warmed health of the cryopreserved cartilage tissue.

The use of chondroitin sulphate, tetramethylpyrazine, a combination of these two, ascorbic acid, and glucosamine was investigated in a set of cryoprotectant toxicity mitigation experiments. We found that when evaluating the effect of exposure to these compounds in a toxic cryoprotectant solution coupled with a two-day incubation, that all but the chondroitin sulphate alone were capable of improving tissue health, while there

were no benefits seen when evaluated before the incubation period. The use of additive compounds has been shown to reduce long-term deleterious effects of CPA exposure, indicating that their use may be beneficial to a vitrification application due to the high CPA concentrations involved.

This thesis also experimentally explored an altered cryoprotectant protocol proposed by another student, Nadia Shardt, who used Fick's 1-D law of diffusion to determine the minimum time required for the diffusion of cryoprotectants into articular cartilage in concentrations that were adequate for vitrification. These modifications reduced the protocol length by one and a half hours, but did not result in viability results that were significantly improved over the standard protocol. As the experimental trials in this thesis work all produced a recovery cell viability that is much lower than the previously published results for the standard vitrification protocol, no conclusions can be made regarding which protocol to use based on these data. The experimental groups did not have an obvious deleterious effect on cell viability and, therefore, the reduction in protocol time may be beneficial.

The standard vitrification protocol for articular cartilage has shown good results. The experiments performed here demonstrate that there are two potential avenues that may be exploited to enhance cell recovery and streamline the technical aspects of the protocol. Further research should lead to improvements in the results of this protocol.

PREFACE

This thesis is an original work by Joshua Hahn. The research project, of which this thesis is a part, received research ethics approval from the University of Alberta Research Ethics Board, Project Name “Vitrification of human articular cartilage”, No. Pro00001419, approved 29 Oct. 2008 with annual renewal, renewed until 23 Nov. 2015.

Some of the research conducted for this thesis forms part of a research collaboration, led by Dr. N.M. Jomha, with the collaboration of Dr. J.A.W. Elliott at the University of Alberta. The calculation of cryoprotectant perfusion characteristics into human articular cartilage in chapter 4 was performed by N. Shardt under the supervision of Dr. Elliott. N.M. Jomha and L. Laouar led the experimental design and concept formation for the vitrification experiments in chapter 4 and the immediate trials in chapter 3, and T. Goodine assisted with data collection for a portion of the work in chapter 3 and contributed to manuscript edits. The remainder of the work presented here is my original work. N.M. Jomha, L. Laouar, J.A.W. Elliott, and G.S. Korbutt were involved in thesis editing and/or manuscript composition. Chapter 3 of the thesis is intended to be submitted for publication.

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CHAPTER 1: INTRODUCTION

With the global increase in longevity due to improved medical technology and techniques, diseases normally associated with old age are becoming endemic. One such disease is osteoarthritis, which results in the breakdown of cartilage and bone within joints, with risk factors that include old age, obesity, and cartilage injury. The progression of osteoarthritis can cause pain and limit mobility, resulting in losses to productivity and quality of life. Coupling the increasing prevalence of this disease with its severe impact, the treatment and prevention of osteoarthritis are necessities. One avenue of prevention involves a treatment that is used to treat cartilage lesions in younger patients before they are able to develop into widespread osteoarthritis. The procedure is termed osteochondral allografting and involves the harvest of healthy bone and cartilage from a cadaveric source to be transplanted into individuals that have developed cartilage lesions. Osteochondral allografting can thus be used to repair full-thickness cartilage lesions, which is more beneficial to a patient than replacing the joint with a synthetic implant. Cartilage transplantations are more beneficial due to the ability of the patient to incorporate the transplanted tissue and keep the tissue healthy, giving the potential for transplanted tissue to outlive synthetic implants, which have a finite period of effectiveness.

While osteochondral allografting can be used to successfully treat cartilage lesions, the use of the procedure is limited by graft availability due, in part, to the lack of a long-term storage option. Our work focuses on establishing a cryopreservation protocol that would allow for indefinite storage of cartilage, making tissue banking for transplantation

purposes possible. The current standard for cartilage storage is 4 ^{o}C in media, which is effective for periods of time up to approximately four weeks. As the donor tissue has to be tested for the presence of diseases, it is common that the tissue must be held up to two weeks after harvest. By finding a more effective preservation method, more tissue will be able to be stored and distributed without the difficulties associated with such strict timelines.

Vitrification is a method of cryopreservation that completely avoids the formation of ice, and when properly performed is able to preserve tissue indefinitely. By replacing some of the water in tissues and cells with cryoprotective agents (CPAs) and cooling rapidly, the cells can be preserved in an ice-free environment for an unlimited amount of time. This method of cryopreservation has been applied most notably in the field of assisted reproduction. There is often difficulty in applying vitrification in other fields due to toxicity from the high concentrations of CPAs that are required to eliminate ice formation. There has been some work done with chondrocytes and cartilage, but until Jomha and coworkers published a successful vitrification protocol for intact human articular cartilage there was little success in the preservation of intact cartilage.

The overall aim of the projects in this study was to focus on the improvement of the cartilage vitrification protocol, to improve the post-warming viability of the cryopreserved tissue and eventually to facilitate tissue banking of articular cartilage. The overall hypothesis was that there was room for improvement in the current vitrification protocol for human articular cartilage and that post-warming cell viability could be improved. The goals of this thesis were to determine if the use of additive compounds could be beneficial to the

viability of cartilage after exposure to a cryoprotectant, either through reducing CPA toxicity or by increasing the ability of chondrocytes to recover from CPA-induced toxicity, as well as to assess a proposed CPA perfusion protocol with altered values for both length of time and concentration of exposure. The experiments performed here demonstrate that there are two potential avenues that may be exploited to enhance cell recovery and streamline the technical aspects of the protocol. The information contained in the following review of the literature will aid the reader in understanding the role that the following experimental procedures take within the larger context of the fields of cryopreservation and the transplantation of articular cartilage, followed by a report of the experimental procedures and their results for each of the sets of experiments.

CHAPTER 2: LITERATURE REVIEW

INTRODUCTION

Osteoarthritis is a disease that affects the cartilage present on joint surfaces, creating defects. Treatments for this vary and will be discussed further, but can include the transplantation of donor tissue to replace damaged cartilage. This procedure has some difficulties associated with it however, as supply and demand of donor tissue is not synchronized, requiring that tissue must often be preserved before use. This preservation, while allowing for less tissue to expire unused, will also reduce the tissue's health and the benefit that it will give to the patient in most situations. As such, there have been several different explorations into new ways to preserve this tissue, including advances in the science of freezing-cryopreservation, and the use of vitrification.

ARTICULAR CARTILAGE

STRUCTURE

Cartilage is a hard yet flexible tissue that exists in places in the body where the stiffness of bone would be inappropriate for the function required. Exploring the morphology of cartilage yields three main subtypes; elastic, hyaline, and fibrocartilage, of which hyaline cartilage is the most pertinent in regards to the treatments for cartilage defects mentioned previously. Hyaline cartilage is found on joint surfaces where it is classified as articular cartilage (AC).

Cartilage grows in a similar fashion to bone, with loosely packed cells producing an extracellular matrix (ECM). These cells, chondrocytes, often form small groups of up to eight cells contained in a single pocket of the matrix known as a lacuna. Articular cartilage is composed mainly of water (~65-80% wet weight), with the remainder being composed of collagen, proteoglycans, and finally chondrocytes, listed by decreasing prevalence in the tissue. The high water content is what allows for the stress-induced deformations of the cartilage during normal use [1].

Collagen constitutes ~10-20% of the wet weight of AC (50-60% dry weight) [2], and is responsible for supplying much of the tensile strength that the tissue displays. The collagen present in AC is primarily type II collagen (90%+) [2], with several other types present in small percentages. Collagen is able to form a large interconnected network of fibrils that provide the cartilage with its tensile strength [3]. This tensile strength of the collagen network is further modified by its interactions and bonding with the proteoglycan network.

Proteoglycans are large polysaccharides formed around a central hyaluronic acid filament. Proteoglycans are responsible for much of the compressive strength found in AC. Aggrecan, one of the larger proteoglycans, is essentially the molecular backbone of cartilage. Through various binding domains it is able to bind hyaluronic acid as well as chondroitin sulfate and keratin sulfate chains (to form a glycosaminoglycan, or GAG), which in turn are responsible for binding water molecules and combine to "*provide a hydrated, viscous gel that absorbs compressive load*" (pg.687) [4].

Chondrocytes are spherical (depending on location in the cartilage) and only constitute ~1-5% of the AC volume. The primary function of the chondrocyte is the maintenance of the extracellular matrix, which in turn is responsible for the strength of AC [3].

An excellent summary of the composition of AC is given by Jerosch [5]:

Proteoglycans are intertwined with the collagen network. Due to the net negative charge of the proteoglycans, a large amount of water is enclosed in the cartilage. The water content is important for the resilience and elasticity of the tissue, as well as for lubrication of the joint system. The proteoglycans of the articular cartilage are large supramolecular complexes, composed of a central hyaluronic acid (HA) filament, to which aggrecan molecules composed of chondroitin sulfate and keratan sulfate are attached by a link protein in a brush-like configuration. The amino sugar glucosamine is a necessary component for the synthesis of many of these proteoglycans, which include hyaluronic acid, heparan sulfate, and keratan sulfate. The production of glucosamine is one of the rate-limiting steps in proteoglycan production.

ORGANIZATION

The organization of the chondrocytes and collagen fibrils within the ECM varies depending on the depth of the tissue, with the result that there are four distinct zones throughout the articular cartilage - the superficial tangential zone, the intermediate or

transition zone, the deep or radial zone, and the calcified cartilage zone, with the last two being separated by the tidemark [1,2].

The top zone within the cartilage is the thin superficial zone, occupying ~10-20% of the cartilage. It is characterized by the chondrocytes having an elongated appearance and lying parallel to the surface, as well as having a high cell density. The matrix in this zone has a higher concentration of collagen and a lower concentration of proteoglycans relative to the other layers. The collagen fibrils in this layer are very thin (~20nm) [2] and also lie parallel to the joint surface, which has the effect of providing more tensile and shear resistance. A further characteristic of the superficial zone is that it has the highest water content of all of the zones, which is due to the low proteoglycan presence in the matrix [1].

The middle zone constitutes ~40-60% of the cartilage. It is characterized by the chondrocytes being more rounded, evenly distributed, and in a lower density than the superficial zone. The matrix has a higher abundance of proteoglycans, with the collagen fibrils being randomly oriented and larger in diameter in this zone [1,2].

The deep zone is also characterized by round chondrocytes and large collagen fibrils (they are largest in the deep zone, 70-120 nm) [2], as well as a further drop in chondrocyte density. The chondrocytes are predominantly aligned perpendicular to the surface and commonly form in columns. This zone also contains the highest concentration of proteoglycans [1,2].

The calcified cartilage zone contains the tidemark at its uppermost limit, the visible border which separates AC from calcified cartilage. Chondrocytes in this zone are few and

hypertrophic, and the predominantly collagen II fibrils from the upper layers have been replaced by collagen X fibrils [1,2]. It has been speculated that the function of this zone is likely to anchor the cartilage to the bone, as collagen fibrils from the deep zone penetrate into the calcified cartilage [2].

Beyond this organization, there are also further divisions within the extracellular matrix of AC. The ECM can be characterized into three subtypes: pericellular, territorial, and inter-territorial. Pericellular matrix is a thin layer of matrix that is in contact with the membranes of the chondrocytes. Territorial matrix immediately surrounds the pericellular region, and can enclose either single cells or clusters, making lacunae easy to visualize under a microscope. The primary function of this type of matrix is the protection of the chondrocytes against mechanical stresses. The inter-territorial matrix forms the majority of the matrix, and is what is generally being referred to when the ECM is mentioned [1].

Articular cartilage is not structurally unique only because of its composition; it is also entirely devoid of any lymphatic system, nerves, or vasculature [6]. This results in relatively slow growth (nourishment comes through diffusion from the synovial fluid) and a limited repair capacity (which is further compounded by the fact that the chondrocytes are bound within the lacunae, and therefore cannot migrate to damaged areas). Articular cartilage is highly dependent on the complex interactions between chondrocytes and the matrix being maintained, making treating or repairing the tissue difficult [7]. However, the lack of lymphatic influences (external immune cells are unable to enter the matrix, as the pores in the matrix are too small) [8] gives AC the status of being “immune-privileged,” which means

that the tissue is unlikely to elicit an immunological reaction, relaxing the need for donor matching when transplanting the tissue.

Articular cartilage is incredibly important in day-to-day functioning as it allows movement with minimal friction within a joint. The importance of this cartilage can be seen most noticeably when it begins to fail, resulting in anything from pain to joint immobility. Once AC begins to break down, it has only limited healing capacity; replacing the damaged hyaline cartilage with fibrocartilage scar tissue, which is incapable of the same durability as hyaline cartilage [6,9]. The failure of cartilage is most often seen in individuals that have been diagnosed with osteoarthritis, a degenerative condition of cartilage that results in its eventual wearing down and failure to protect the bones in a joint from rubbing on each other.

OSTEOARTHRITIS

Osteoarthritis (OA) is a disease that negatively affects AC, with a substantial and growing global presence. According to an epidemiological report by the WHO in 2014, OA accounted for over 18 million YLDs (healthy years lost due to disability, equivalent to 18 million individuals being unhealthy for 12 months each year when calculated using the new metric of number of prevalent cases multiplied by a disability modifier) at a global level, accounting for 2.44% of total global YLDs from all causes in 2012 [10]. This was an increase from 2000 when it was estimated to have caused 13.4 million YLDs, or 2.14% of global YLDs [10]. The loss of productivity associated with OA symptoms accounted for 1-2.5% of the gross

national product of many first world countries, including the USA, Canada, the UK, France and Australia [11].

OSTEOARTHRITIS RISK FACTORS

OA is a disease that can arise from any number of different conditions and with no known explicit cause, but there are known risk factors. Some of the risk factors for OA include advanced age, obesity, genetics, a myriad of diseases or injuries including AC mechanical damage [5,12], along with new evidence indicating a metabolic component to OA development [13]. While the potential causes of the disease are many, the effects of the disease remain fundamentally the same, being defined by the degradation of articular cartilage.

OSTEOARTHRITIS SYMPTOMS

As the cartilage becomes damaged the collagen network is altered, resulting in an increase in the water content of the cartilage. Coupled with a loss of proteoglycans this reduces the elasticity of the cartilage and makes additional damage more likely [5]. Further, once the cartilage loses elasticity, "*the bone beneath the cartilage changes and develops bony overgrowth. The tissue that lines the joint can become inflamed, the ligaments can loosen, and the muscles around the joint can weaken."* This results in pain and movement limitations when using the joint [14]. Once the cartilage has deteriorated, and as pain and inflammation become increasingly problematic, treatment options are considered.

ARTICULAR CARTILAGE INJURY TREATMENT

There are no non-operative treatments that have proven to restore normal AC but there are several surgical options for treatment that have had some positive effect. These

can be divided generally into replacement, bone marrow stimulation, and regenerative measures [15]. The treatments that will be most affected by improved cryopreservation methods are the regenerative and replacement techniques. The treatments that would be aided involve the transplantation of healthy AC (or chondrocytes) into an AC lesion. This can be accomplished in several ways, using either autologously retrieved tissue/cells or by harvesting the tissue/cells from a donor. Focus will be on the treatment best served by cryopreservation – AC allograft transplantation.

Osteochondral allograft transplantation is a treatment method that would benefit from successful cryopreservation of AC; it is a process whereby bone and full-thickness cartilage is removed from a tissue donor and transplanted into the AC lesion [16]. If performed autologously there are limitations, as the cartilage is removed from another area within the diseased joint, thereby limiting the size of the lesion that can be treated as well as introducing new lesions in the form of the donor sites within the joint (see figure 2.1) [17,18]. This indicates that the use of allogeneic donor tissue may be preferable, as the deficiencies of the autologous procedure are no longer present. The use of allografting is indicated most strongly in young individuals that require a transplantation from excessive degradation within the joint. This is in contrast to a synthetic knee replacement, or total knee arthroplasty, which is the replacement of the joint with a synthetic implant and is the current gold standard for end-stage OA treatment. An allograft is preferred in these cases due to a perception that younger individuals are more active and will exert more stresses on an artificial implant, and are more likely to outlive their implant. One study on the survivorship of femoral condyle allografts found that transplants were successful post-

transplant (defined as not having to be replaced with a synthetic implant) at rates of 88.9% at 10 years, 71.4% at 15 years, and falling to 23.8% at 20 years, with associated qualities of life all showing improvement over pre-transplant baseline in all time periods [19].

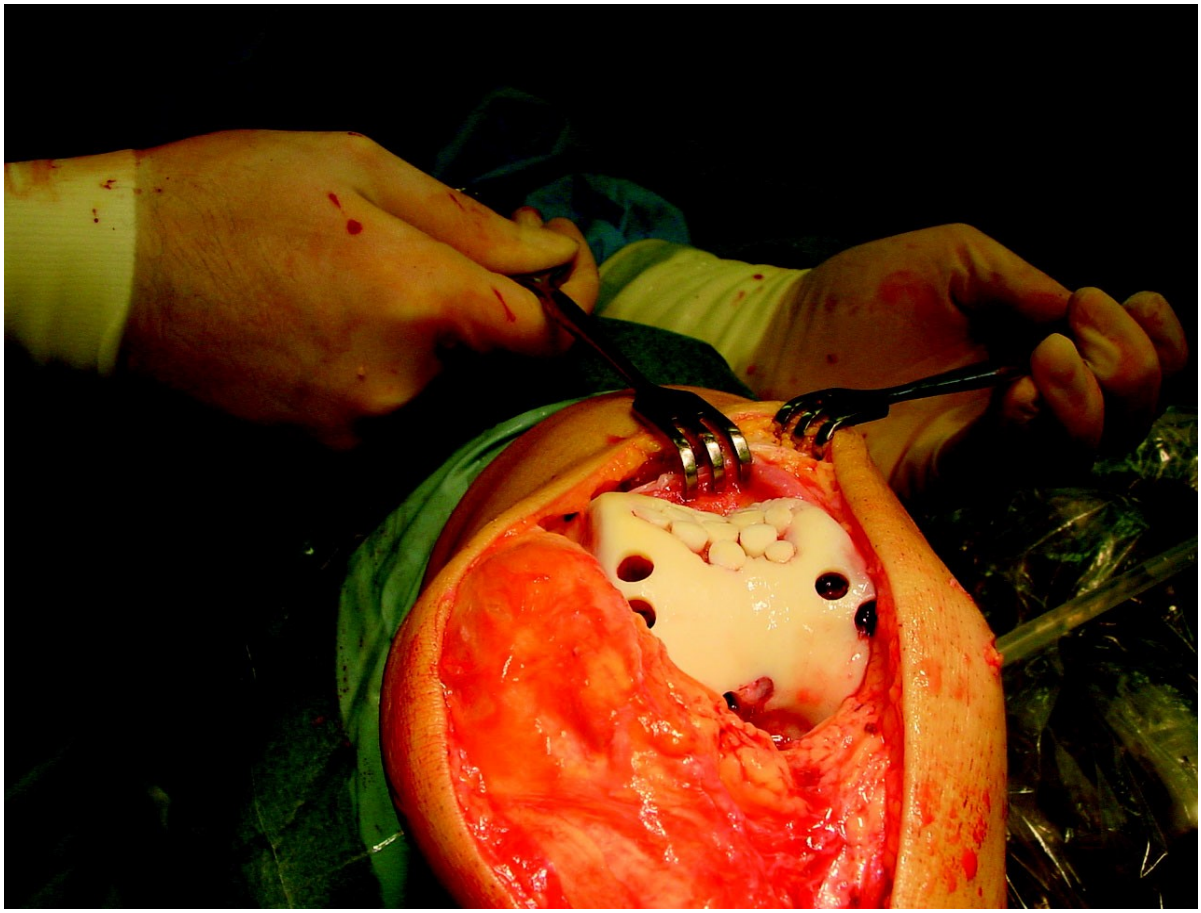

Figure 2.1. Open moscaicplasty on the femoral trochlea. This figure is reused from Hangody and Fules [17], Figure 7, Page 29, with permission from the Journal of Bone and Joint Surgery [American].

Cartilage Transplantation

Cartilage is a tissue that can be donated for re-use upon death. Recovery of AC from tissue donors enables osteochondral allografting to be performed, but special consideration must be made to ensure that the AC is both safe to implant as well as healthy enough to be beneficial to the patient. Due to the fact that the biphasic nature of AC must be maintained

for proper functioning [7], paired with its limited healing capacity, it is important that only cartilage with healthy chondrocytes is used for transplantation. As fresh tissue is not always available at times of demand, and due to the stringent requirements of tissue health, it must be preserved until a patient is prepared to undergo surgery. This is a limiting factor in the use of donor AC, as AC stored at 4^{o}C begins to degrade beyond two weeks [20,21], with the percent viable cells dropping as low as 68% after 4 weeks [22]. It should also be mentioned that while fresh tissue is more challenging to obtain in a timely manner, it has historically been more viable than frozen samples (however it will incite a slightly more elevated immune response from the bone being transplanted than will the frozen sample) [23]. The cut-off cell viability percentage below which transplanted cartilage will be unable to maintain itself is unknown, but good surgical practice would dictate using the best possible tissue. Transplanted frozen tissue has been shown to deteriorate more rapidly than transplanted fresh tissue [24,25], likely due to lack of viable cells and matrix distortion caused by ice formation [26]; therefore, freezing is not a good method for tissue banking of AC. For these reasons, a storage technique that maintains cell viability and matrix integrity has been sought.

CRYOBIOLOGY

MECHANISMS OF CRYO-DAMAGE

When attempting to cryopreserve cells or tissues care must be taken, as there are several mechanisms by which cooling and sub-zero temperatures can cause damage to that

which is being preserved. Most of these damage mechanisms fall into two main categories; those caused by slow-freezing and those caused by rapid-freezing.

SOLUTE EFFECTS

In classical cryopreservation (defined here as cryopreservation involving the formation of ice), particularly in slow-freezing, the primary means of cellular damage is due to high solute concentrations, caused by two separate mechanisms. High solute concentrations naturally occur during freezing, as the formation of extracellular ice removes pure water from the extracellular solution, thereby increasing the concentration of the remaining solutes. Initially this has a beneficial effect for the cell, as the hypertonic extracellular environment causes an osmotic gradient to form, which removes water from the cell interior and reduces the likelihood of intracellular ice formation (which is damaging to cells) [27]. However, as the gradient increases it becomes dangerous for the cell to the point that it can dehydrate beyond its ability to recover. Indeed, with a large enough internal-external solute concentration difference, it has been suggested that the gradient can become such that the osmotic force generated is enough to damage the cell membrane resulting in cell death [28].

The reverse can also damage cells. During dehydration, the intracellular solute concentration increases which presents a challenge when rewarming the sample. As the extracellular ice melts it creates a hypotonic environment. A cell can adjust to this environment through the influx of water to dilute the internal solute concentration, as well as the expulsion of internal solutes. Of these two means of adjusting tonicity, the transport of solutes across the membrane requires pumps or channels while osmosis is a passive

process, with osmosis being faster. This has the effect of allowing water to enter more rapidly than ions can exit. If the extracellular tonicity falls too low, these processes will be unbalanced and the cell will take in excess water until lysis occurs [29]. For this reason, it is important to tailor cooling and warming protocols to each cell type to prevent these excessive osmotic forces, as each cell type has unique diffusion and active transport processes.

The second means by which solute concentrations can damage cells was described by Lovelock [30], who found that the solute concentrations during freezing could be detrimental to the cell regardless of dehydration. In his work on red blood cells, he found that simple exposure to high concentrations of salts (4 M NaCl, 5 minutes exposure) was enough to damage the cells significantly (resulting in ~81% of cells perishing). Farrant and Morris [31] refined the notion of damage due to solute concentration, eventually being termed "solution effects" by Mazur [27]. As suggested by Farrant and Morris, a high extracellular electrolyte concentration alters the cellular membrane, which makes the cell susceptible to leaking out normally non-permeable solutes when an additional stress is applied (such as the dramatic temperature changes encountered in cryopreservation or the rapid osmotic changes during thawing). This effect is most pronounced during slow freezing, as during rapid freezing the cell membranes are not exposed to the high solute concentration for long enough to alter them significantly [32]. The modulation of tonicity was one way that Farrant postulated that cryoprotective agents were effective in preventing cell death during cryopreservation [33,34].

ICE FORMATION

In addition to the damage done by solute concentrations, damage can also occur simply due to ice formation. Ice formation that is directly dangerous to cells can form either intracellularly or extracellularly, of which intracellular is the more damaging. The dangers of ice formation generally increase during rapid freezing procedures, for a number of reasons.

During rapid freezing, the cells have less time to equilibrate to the increasing tonicity of their surroundings, which results in intracellular supercooling taking place (the reduction of temperature below the freezing point without ice formation). A supercooled cell is able to remain liquid until the homogenous water nucleation temperature at -40 ^{o}C [35], the lowest temperature to which pure water can be cooled before nucleating in the absence of an external nucleating agent, allowing for reduced ice crystal formation with rapid cooling.

Intracellular ice crystals vary in size and their danger to the cell on warming. The ice crystals that form during rapid cooling may not be large enough to damage the cell (this varies with how rapidly the cooling takes place) [36]. However, if rewarming is not performed at a high enough rate, these crystals are able to grow to the point that they can eventually destroy the cells. If warming occurs quickly enough, the crystals reach the melting point before they are able to grow substantially, thus preventing further damage to the cell. This effect has been demonstrated in several different species and at several different experimental conditions, showing that the survival of cells after cryopreservation can be altered by the warming rate in rapidly cooled samples (warming rate has little effect in slow-cooled samples, due to the absence of intracellular ice) [37].

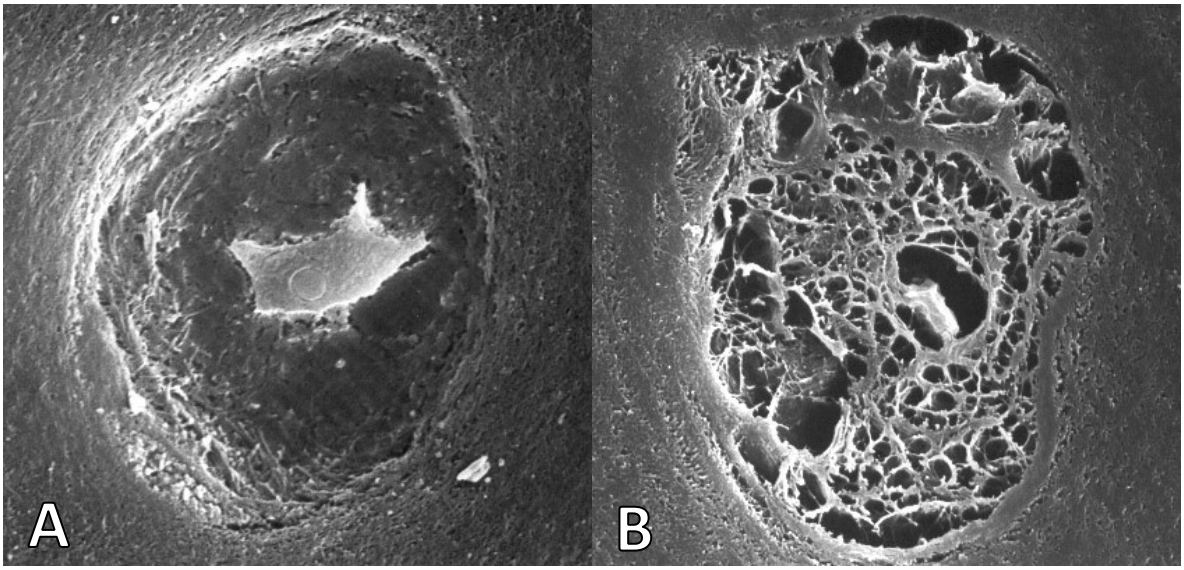

Figure 2.2. Scanning electron microscope images of chondrocytes at 2500X magnification.

A – Scanning electron micrograph of fresh control in PBS solution

B – Negative control of sample in PBS plunged directly into LN [liquid nitrogen] from room temperature.

This figure was created using figures 5.1 and 5.3 with modification from Jomha [38], pages 141 and 143, used with permission from the author.

Intracellular ice formation is dangerous mainly because of the mechanical damage that it will do during freezing and expansion, rupturing the cellular membrane as well as damaging internal organelles [38,39] (see figure 2.2 for electron microscopy of ice damaged cells). When intracellular ice occurs in a tissue, it has the added danger of being able to spread to adjacent cells and destroy them as well. An experiment by Acker et al. [40] demonstrated that once intracellular ice formation occurred in a cell, it could spread to neighbouring cells (hypothesized to occur via cell-cell junctions, and supported by later experiments) [41]. The experiment involved four different cellular models; single cells in suspension, cells attached to glass, colonies of cells attached to glass, and multicellular spheroids. The experimenters observed that single cells in suspension were able to reach a lower temperature before intracellular ice nucleation and that when cells were in contact

with other cells and formed intracellular ice, the ice quickly spread to adjacent cells. The conclusion of the paper was generalized to the statement that, "*it is insufficient to simply assume that techniques to successfully cryopreserve cells in suspension can be applied to the same cells in an organized tissue*" (pg. 370) [40].

Alternatively, extracellular ice is not normally as harmful to cells. It is generally accepted that extracellular ice does not directly damage cells [27] (although some argue that mechanical crushing can still occur) [42]. The primary means by which extracellular ice is damaging to cells is through the osmotic effects that it generates during freezing, as previously described.

OTHER MECHANISMS

Other mechanisms of cold damage not directly related to the rate of temperature change include "chilling injury" and "thermal shock." Chilling injury is reported as a decrease in cellular health (depending on what is being measured - metabolism, etc.) after exposure to a relatively cold temperature (the exact temperature and exposure time varies by cell type), whereas thermal shock is damage that is believed to be caused by cooling and incubation in a specific temperature range, often paired with a high cooling rate.

CHILLING INJURY

The exact mechanism of chilling injury is currently unknown, and the temperature at which it will occur varies by cell/tissue, with some types being much more susceptible to chilling injury than others [43,44]. Two prominent mechanisms thought to be the cause of chilling injury have been supported by experiments in various tissues. One theory posits that chilling injury is caused by a phase change of the lipid bilayer membrane from a liquid

state to a gel, complicating the normal actions of the cell [45]. Another theory states that when cooled, the cellular membrane contracts more rapidly than the intracellular contents, creating abnormal pressures and leading to membrane rupture [46,47]. Chilling injury is a complex phenomenon, as it can be modulated by the tonicity of the solution, the rate of cooling, and the type of cell [29].

One method of avoiding chilling injury is through vitrification, with the use of very high cooling rates, preserving the cell before damage can occur. There is also evidence that cooling the cell in a hypertonic solution can prevent injury, as some cellular dehydration appears to have a protective effect against chilling injury [47].

Of the different tissues that have been examined, brain slices have shown evidence of being quite resistant to chilling injury, while kidney and some liver tissues are very susceptible [29,44,48].

THERMAL SHOCK

Thermal shock is a multifactorial mechanism of damage, depending on time of exposure, temperature, and molarity of holding solutions, as well as the rate of cooling [49]. The mechanism by which thermal shock is dangerous to a cell closely relates to the effects of hypertonic solution damage as mentioned previously; however, there appears to be a limited set of exposure criteria for thermal shock to be the dominant mechanism. Takahashi and Williams [49] evaluated the mechanisms of thermal shock through a series of experiments designed to try to separate the damage caused by temperature effects from those caused by hypertonic effects. They found, in agreement with Lovelock [30], that an external molarity

of 4 Osm NaCl or greater caused damage exclusively by solution effects. However, they found that in red blood cells exposed to >1.4 Osm NaCl while above 12.5 ^{o}C, and subsequently cooled to below this temperature, thermal shock hemolysis would occur (as concentration is increased the damage mechanism shifts to increased solution effect damage). Further, through several experiments they concluded that incubation time and temperature, solute concentration, and rate of temperature change all had both independent and interconnected effects on the degree of thermal shock injury during cooling. In practice, thermal shock and solution effects damage are closely related, making it difficult to determine which is responsible for cell damages. Thus, damage from thermal shock is often attributed to solute effects [29].

Slow vs. Rapid Cooling

An understanding of the physical processes that govern slow and rapid cooling is required to design protocols to avoid the damage that each cooling rate is capable of producing. Of the various means by which cells can undergo damage during cryopreservation, those caused by slow cooling and those caused by rapid cooling are to some degree opposed. Mazur et al. proposed a two-factor hypothesis in 1972 [36], which was the first formal characterization of cell damage during freezing being attributable to separate mechanisms linked to either slow or rapid cooling. This insight led to new techniques and advances in cryopreservation including the improvement of the controlled rate cooling procedure and the introduction of the two-step cooling procedure, both of which have been shown through experiments carried out by a range of scientists to be more effective than the use of either rapid cooling or slow cooling alone.

CONTROLLED-RATE COOLING

Controlled rate cooling is relatively straightforward concept; by cooling at an optimum rate between slow and rapid cooling, the majority of damages associated with each rate can be avoided. The protocol had already been found to be beneficial, with theories to explain why [50], but the formalization of the damage mechanisms allowed for the optimum cooling rate to be understood. Controlled-rate cooling was incorporated as the first step in the two-step cooling technique, as explained below.

TWO-STAGE COOLING

The two-factor hypothesis, once formulated into cryopreservation applications (also referred to as two-step freezing) [37,51,52], incorporates the proper control of cooling rates during freezing to prevent the most intensive damages caused by slow or by rapid cooling. In practice, this begins with the initial controlled-rate slow cooling stage, in which a sample is cooled to a predetermined holding temperature for a scheduled amount of time to allow proper exosmosis without intracellular supercooling or ice formation. This is followed by the rapid stage, wherein the sample is rapidly cooled (often to below -100^{o}C) [53-56] and can be stored for long periods of time at this temperature. The two-stage freezing technique is generally accepted to have been formulated by Farrant, McGann, and their associates, with several works focusing on finding optimum values for use in two-stage cooling [37,52,53]. Work on optimizing two-stage freezing was continued by Farrant and McGann [37,52,57,58] and the technique has become the predominant method for carrying out freezing cryopreservation [51,59,60].

While the cooling method is an important facet of a cryopreservation protocol, there are other factors that will play a major role in the success of the protocol. One of these factors is the use of cryoprotective agents. Cryoprotective agents (CPAs) are beneficial to slow-cooling applications and have been in use since the work of Polge et al. in 1949 [61]. The experiment by Polge et al. involved the reported vitrification of multiple samples of semen from several species at -79 ^{o}C (in a glycerol solution - a common CPA), thawing, and evaluating the samples based on the motility of a control sample. The results varied by the species from which the spermatozoa originated, but the best results found that after rapid-freezing in glycerol near complete motility was recovered upon thawing, as opposed to essentially no motility recovery in the control without glycerol (note: the article refers to the procedure as vitrification, but the reported temperature of -79 ^{o}C and CPA concentration of only 5-20% is insufficient to raise the glass transition temperature sufficiently from ~-130 ^{o}C, making true vitrification unlikely in this case). This experiment opened an important avenue of research in cryopreservation, and it has evolved into a multi-faceted and complex field with great potential.

Cryoprotective Agents

Cryoprotective agents will be discussed further in relation to vitrification; but, it should be mentioned that CPAs have also shown to be of use in more traditional forms of cryopreservation (freezing, through the use of slow, rapid, or two-stage cooling). With more detail on CPAs to follow, it is nonetheless beneficial to mention the mechanisms by which they aid in traditional cryopreservation.

TYPES OF CRYOPROTECTIVE AGENT

There are two main types of CPA, penetrating and non-penetrating, based on their interaction with cellular membranes. An important characteristic of a CPA is freezing point depression, which is an effect that both types of CPA will exert, however, how that affects the cells can vary slightly. A penetrating CPA is capable of both reducing intracellular ice formation by freezing point depression, and acting as an osmotic buffer as extracellular ice begins to form (by keeping more of the extracellular environment liquid and preventing the concentration of solutes). Non-penetrating CPAs act primarily as an osmotic buffer in a similar manner to penetrating CPAs, but because they do not enter the cell they can be used in a slightly different manner. Non-penetrating CPAs can be used in conjunction with penetrating CPAs to ease the creation of the osmotic gradient by increasing external tonicity and further helping to prevent ice formation. Non-penetrating CPAs also have the advantage of usually being less toxic to cells [29].

CRYOPRESERVATION APPLICATION

To this point, an effort has been made to detail the bulk of the important theory and discoveries relevant to cryopreservation; this is only important however, if it can be put into the context of a useful application. Detailed below is a brief account of some of the applications, both successful and failed, of cryopreservation theory.

CRYOPRESERVATION OF CELLS

Attempts at cryopreservation have been extremely varied in their methodology, choice of specimen (be it cells, tissues, or even whole organs), and in their success. One of the most prominent examples is assisted reproduction; specifically the cryopreservation of

spermatozoa, eggs, or embryos. The previously mentioned study by Polge et al. [61] is indicative of how long cryopreservation has been applied to reproductive cells, with the field progressing to the point of live human birth from cryopreserved samples in 1986 [62]. Although assisted reproduction has a long history in cryopreservation it still has difficulties, as observed in a meta-analysis by Oktay, Cil, and Bang [63] regarding the success of cryopreserved oocytes. When comparing cryopreserved specimens to fresh ones, the fresh specimens result in a clinical pregnancy notably more often. However, this is not to say that cryopreserved oocytes are not viable, with the reported live birth rate per injected oocyte only 1.9 times better in fresh than in cryopreserved samples (and even then only successful at a rate of 6.6%/oocyte, equating to a 3.2% difference) [63].

Another cell type that has a long and varied past in cryopreservation is the chondrocyte. The first success with chondrocyte cryopreservation came from Smith in 1965 [64] with isolated chondrocytes from human and animal hyaline and fibrocartilage. Smith's experiment involved the recovery of chondrocytes from mature cartilage, followed by the suspension of the cells in a dimethyl sulphoxide (DMSO, a common cryoprotectant) solution with slow cooling to -79^{o}C. This was followed by thawing and evaluating, by microscopic examination, activity and ability to re-grow into cartilage upon homografting to cancellous bone. Smith found that the majority of the thawed samples were normal under the microscope and capable of re-growing cartilage when transplanted, although if they were left to incubate they would remain active for less time than controls before dying. AC in particular is arguably more useful if the entire tissue structure can be preserved [65] and, as demonstrated recently by Kahn, Les, and Xia [66], the use of freezing on articular cartilage will

have an adverse effect on that structure, thus necessitating a more sophisticated cryopreservation method. Unfortunately, as concluded by Acker et al. [40], what works for cells cannot necessarily be applied to tissues. Indeed, using traditional cryopreservation methods there has been little success in attempts to cryopreserve intact AC [12,54,67].

CRYOPRESERVATION OF INTACT TISSUES

While intact human articular cartilage has proven difficult to cryopreserve, there are other tissues that provide examples of success when being cryopreserved. One example of a tissue that can be successfully preserved is the cornea. One method of many, established by Hagenah and Bohnke [68], is able to cryopreserve corneal tissue with 71% recovery compared to unfrozen controls. This example also serves to illustrate some of the difficulties encountered in cryopreservation as the endothelial cell density, an important practical feature, in these "successfully" preserved corneas is still well below that of a fresh control (2430 cells/mm^2 vs. 3395 cells/mm^2 respectively).

An examination of the success of cryopreserving cartilage reveals that there has been some success; fibrocartilage in particular has proven better suited to cryopreservation. Fibrocartilage meniscal tissue has been demonstrated to be able to withstand cryopreservation and transplant, continuing to survive and function after a year [69]. Sheep articular cartilage has also been able to be successfully cryopreserved using traditional techniques [70].

The transition to cryopreserving human articular cartilage has not been readily successful however, with attempts to cryopreserve human AC using traditional methods

being disappointing. The experiments giving the best results were unsatisfactory, with live cells present only in either the superficial or the deep layers after freezing, making it far from functional as a tissue. Indeed, as seen in an experiment conducted by Ohlendorf, Tomford, and Mankin [67] comparing the effects of slow-freezing (with and without a cryoprotective agent) and rapid-freezing on bovine AC, it was found that only the superficial layer resulted in any live cells after a freeze-thaw cycle. This was observed in both of the slow-freezing trials, with greater numbers of live cells observed in the trials with a cryoprotectant, and no cells surviving the rapid-freezing procedure. It was postulated that this may be due to morphological differences in the intermediate layer, which was later examined and discounted by Muldrew et al. [12].

Further advances in cryopreservation resulted from the use of vitrification, which allows for the avoidance of many of the different freezing damage mechanisms that can occur during conventional cryopreservation.

VITRIFICATION AS A MEANS OF CRYOPRESERVATION

Vitrification is a physical process whereby a liquid (for use in cryopreservation, this will be an aqueous solution) forms an amorphous (non-crystalline) solid (glass). As stated by Fahy et al. [29], "*A glass is simply a liquid that is so cold that it is unable to change its structure significantly over time scales that are of interest to the observer,"* which is indicative of the method by which a glass is formed. Vitrification has taken place when a liquid is cooled beyond the glass transition temperature, at which point the viscosity increases rapidly (by approximately 1000 times) [71] to the point of solidification. This is in contrast in many ways

to freezing, the nearest physical analogue to vitrification, which is a phase change where a liquid abruptly forms a crystalline solid. The distinction between the two is quite important, particularly in regards to cryopreservation, as there are differing molecular organizations and physical properties between a vitrified and a frozen solid. For example, at the point of freezing the volume of a sample will increase, leading to the mechanical dangers effected by intracellular ice formation, while during cooling a vitrifying sample will continue to reduce its volume [72].

While crystalline solids have different physical and chemical properties than do the liquid forms, vitrified solids have properties similar to the supercooled liquid, resembling an unmoving liquid. An early vitrification researcher defined a vitrified solid as, *"a form of matter which maintains the structure, energy, and volume of a liquid, but for which the changes in energy and volume with temperature are similar in magnitude to those of a crystalline solid*" (pg. 227) [72].

Vitrification Mechanics

It is worth mentioning that while much of the current literature discussing the mechanics of vitrification centres on the formation of plastic or elemental glasses, the principles remain the same regardless of application. Further, as the discussion below primarily concerns vitrification as a means of cryopreservation, "ice" will be used to describe the crystalline solid form of a solution, even though this would not necessarily be appropriate when discussing vitrification in general (such as the formation of some plastics through vitrification).

When attempting to form a glass, there are a number of things to consider. Firstly, the formation of glass and ice are mutually exclusive, individual molecules cannot be arranged in both a crystalline and non-crystalline form (although a larger sample can have areas of each), so care has to be taken when vitrifying to avoid ice formation. This is complicated by the fact that the glass transition temperature (T_g) for a compound is below the freezing temperature (T_m) of the same compound (generally, $T_g \approx 0.66\ T_m$, with the coefficient varying from 0.45-1.0, measured in K) [73]. While this makes preferentially forming glass as opposed to ice difficult, there are several factors that can increase T_g or that can help to prevent ice formation. As listed by Jadhav et al. [74], many different factors can affect the T_g of a given compound. Those most pertinent to cryopreservation include; molecular weight, molecular structure (with many different modifiable features, such as molecular bonds, side-groups, etc.), moisture content, cooling rate (a high cooling rate is doubly effective, because it increases T_g as well as reducing time for ice formation), and pressure. Upon evaluating these factors it should be obvious that some compounds are naturally more inclined to vitrify than others, and that modifications can be made that will improve the glass-forming ability of a compound.

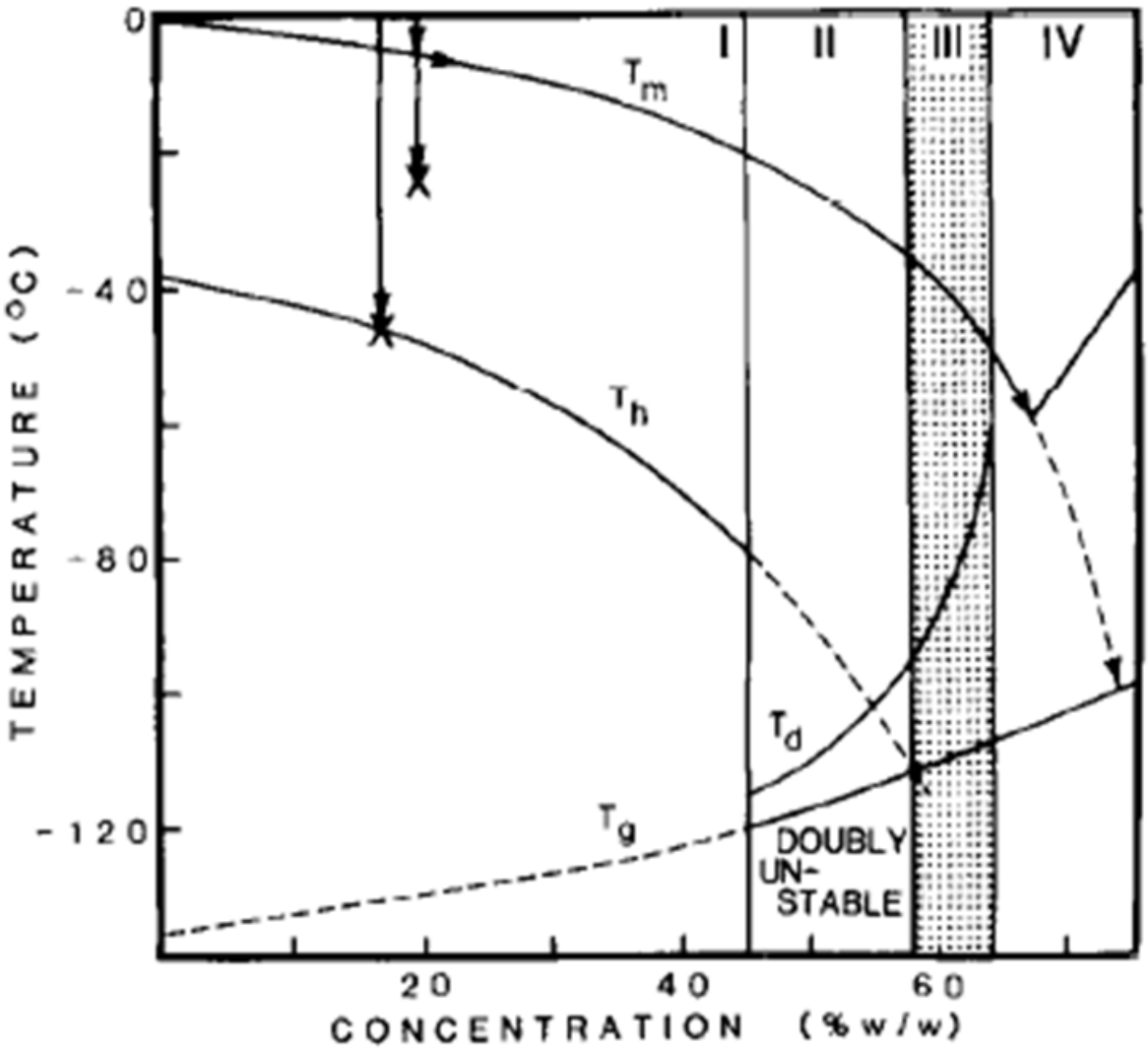

Figure 2.3. Supplemented phase diagram of a hypothetical cryoprotectant. This figure is reused from Fahy et al. [74], Figure 1. Page 408, with permission from Elsevier.

The success of vitrification is relatively easy to measure, it can be observed simply as the absence of opaque ice crystal formation in an otherwise clear solid [75]. However, vitrification can be further divided beyond success/failure into varying stabilities, based on ice formation characteristics. Fahy et al. [75] have described the probability of vitrification with a graph (figure 2.3) that creates four different vitrification regions based on CPA concentration, T_g — the glass transition temperature, T_m — the heterogeneous nucleation

temperature (the freezing point of a non-pure solution, where nucleation occurs from solutes), and T_h — the homogeneous nucleation temperature (the lowest temperature to which a pure substance can be cooled without spontaneously nucleating and freezing). Beginning at the first region, there is a pattern of increasing CPA concentration and T_g, with a decreasing T_m and T_h.

A high T_m and T_h, with a low T_g and CPA concentration characterize the first of these sections. Vitrification in this region is unlikely if not impossible to carry out for cryopreservation purposes, due to the large gap between T_h and T_g (a gap of ~95 ^oC with no CPA and ~40 ^oC at the transition to the second region) and the degree of supercooling that would be required to overcome this. In applications using water, this degree of supercooling is not possible. The homogeneous nucleation temperature of pure water is -40 ^oC, meaning that while it is possible for pure water to form ice crystals above -40 ^oC, it is not possible to supercool below this temperature without ice formation [76]. Vitrification in this region is still possible, but extremely high rates of cooling are required, orders of magnitude higher than other vitrification applications [77].

The second of these regions begins at a threshold of ~45% w/w CPA concentration, and is also considered unsuitable for vitrification. Fahy et al. considers this region "doubly unstable," as a sample must pass through both nucleation regions as it is cooled towards T_g, resulting in ice within the vitrified solid that will promote further ice formation upon rewarming. T_d, the temperature at which a previously vitrified sample will freeze upon warming, is very close to T_g throughout this region, reducing the likelihood that any biological samples will survive the vitrification process here.

The third region constitutes a narrow range of CPA concentration, from ~57-63%, and is where vitrification is generally carried out for cryopreservation. In this region, the T_h has dipped below T_g, marking the lower limit of CPA concentration for vitrification to be certain in the absence of heterogeneous nucleating agents, regardless of cooling rate. T_d has also departed from T_g, reducing the temperature range where ice can form as samples are warmed.

The fourth region is characterized by high CPA concentrations (>63%) which guarantee vitrification, preventing any new ice formation [75]. Unfortunately, the CPA concentrations in this region are far beyond the levels that most cells can tolerate, making it unsuitable for cryopreservation.

Advantages and Disadvantages of Vitrification as a Cryopreservation Process

Vitrification initially appears to be the ideal candidate for cryopreservation applications, as it avoids the standard damage mechanisms that occur during freezing, as well as perfectly preserving the sample for as long as required with no loss of quality [78]. As seen previously, a majority of the damage that is caused during cryopreservation occurs because of ice formation. Obviously, all damage from solution effects, chilling injury, and ice formation is nullified with the absence of ice formation. This then begs the question, why is vitrification not the only cryopreservation method used?

Aside from the successes that it presents, vitrification offers some drawbacks. Comparing freezing and vitrification, freezing is often easier to carry out. Freezing is the

thermodynamically preferred process unless very high concentrations of CPA or very high rates of cooling are used. Vitrification not only requires extreme conditions (during the rapid cool phase to pass T_g, the sample is often plunged into liquid nitrogen at -196^oC) [79,80], but also a longer protocol to load a sufficient CPA concentration to vitrify [54,79]. The requirement of having CPAs at vitrifying concentrations also provides its own difficulties, namely CPA toxicity, although this is not exclusive to vitrification.

One type of damage that is exclusive to vitrification is fracturing. As a sample is cooled during the vitrification process, the volume will continually decrease. As the sample passes the glass transition temperature translational motion is halted and it is considered a solid. If the temperature change beyond this continues too rapidly temperature gradients will form and the uneven contraction can cause the sample to crack or shatter; this is known as fracturing [44,81]. This can be prevented by cooling just beyond T_g, then reheating and holding at T_g to allow temperature gradients to dissipate, followed by further cooling to ensure stability of the vitrified solid [82].

Further, when the sample is being rewarmed the conditions are rarely perfect. As the sample is warmed, it will pass above the T_g, making ice formation once again possible as the solid returns to liquid. In some situations this poses no problems, such as when the concentration of CPA present is high enough to prevent any ice nucleation. However, in most samples the CPA concentration is required to be lower, with initial ice formation during vitrification only prevented through very rapid cooling. In these situations, ice formation is a hazard, so the sample must be rapidly warmed to move it through the ice-forming temperature range quickly enough that only small, non-damaging ice crystals will

be able to form. This transition from amorphous solid, to liquid, to crystalline solid, is referred to as devitrification.

As mentioned, vitrification for cryopreservation requires the use of cryoprotective agents. CPAs are a very important part of vitrification, not only affecting the freezing point and reducing the danger of solution effects but also modulating the freezing temperature, the homogeneous nucleation temperature, and the T_g. Unfortunately, CPAs are also toxic to cells, which has an effect in both vitrification and traditional cryopreservation. However, while cryoprotectants are part of most standard cryopreservation procedures, when used in vitrification a much higher concentration is often needed [83]. This is due to the larger role that CPAs play in vitrification; the use of CPAs in traditional protocols is to reduce the size or frequency of ice crystals and to reduce solution effects, while in vitrification, the CPA concentration must be sufficient to prevent ice formation almost entirely as the solution is cooled to the T_g. While tissue is capable of withstanding exposure to a variety of cryoprotective agents when the concentration is low [84], the concentrations of CPA commonly required for vitrification greatly exceed that which most tissues can withstand [85], especially on the timescales that are required for full perfusion to take place. As such, when designing a vitrification protocol, toxicity must be minimized while still maintaining glass-forming ability and preventing ice formation.

CRYOPROTECTIVE AGENTS

ROLE IN CRYOPRESERVATION

Cryoprotective agents are capable of providing a variety of benefits during cryopreservation, largely because of the variety of different CPAs and the mechanisms through which they work. A general mechanism, characteristic to the majority of CPAs, is a lowering of the freezing (heterogeneous nucleation) and homogeneous nucleation temperatures, the mechanism by which CPAs provide protection against ice formation during traditional cryopreservation. Each cryoprotective agent will attenuate these temperatures uniquely, depending on their particular molecular weight and structure.

Some compounds provide additional benefits beyond temperature threshold reductions. An analysis of dimethyl sulphoxide (DMSO), a common cryoprotective agent, reveals that it is readily toxic to cells; but further examination of this toxicity also reveals a potential benefit. A review of the compound by Brayton [86] identified multiple ways that it is toxic, including "*its own rapid penetration and enhanced penetration of other substances across biologic membranes; free radical scavenging; effects on coagulation; anticholinesterase activity; and DMSO-induced histamine release by mast cells…combinations of DMSO with other toxic agents probably constitute its greatest toxic potential*" (pg. 61). DMSO increases the permeability of biological membranes to both itself and other substances, which provides an advantage when the rate of diffusion is a limiting factor, by reducing exposure times for potentially more-toxic CPAs. DMSO is also reported as a scavenger of free radicals [87], which are known to play a role in inducing apoptosis.

Characteristics of a Cryoprotective Agent

Many compounds can be used as cryoprotectants and, as mentioned above, there are many variables that go into determining whether a compound will be a suitable CPA. There are some general characteristics that every CPA shares. Firstly, they are water-soluble and able to form hydrogen bonds, not readily forming precipitates or eutectics; given that cells are composed mainly of water, the CPA must be able to dissolve in water to be able to provide any benefit. Further, effective CPAs will have a low molecular weight, be relatively non-toxic to cells, and be able to penetrate cellular membranes (unless the compound in question is being used as a non-penetrating CPA) [88,89].

While some of the benefits that a CPA provides are CPA-specific, others are simply a result of the addition of a solute; these are termed colligative properties. Colligative properties are properties of a solution that for dilute solutions depend not on the specific solutes, but on the ratio of solute molecules to solvent molecules. Examples of these include an elevation of boiling point, depression of freezing point, and the modification of osmotic pressure. The previously mentioned hypothesis [33,34] that the protection offered by CPAs was due mainly to their ability to reduce solute concentration and thus osmotic pressure, is an example of a colligative property aiding cryopreservation. A further example is the freezing point depression given with the addition of glycerol to water. While glycerol normally has a freezing point of 17 ^{o}C, when mixed in a proper ratio with water (freezing point 0 ^{o}C), the freezing point of the solution will fall to -37.8 ^{o}C [90].

As mentioned, there are two classes of cryoprotectant, penetrating cryoprotectants (pCPA) and non-penetrating cryoprotectants (npCPA). A comparison of the two indicates that pCPAs are generally more effective at reducing damage during cryopreservation [29,57]. McGann [57] evaluated this difference by exposing cells to a pCPA (DMSO), npCPA (hydroxyethyl starch), or to glycerol with various exposure times, and evaluating the cell damage after cold exposure. It was seen that the pCPA had a stronger protective effect than the npCPA. Importantly, when glycerol was given ample time to diffuse the effect mirrored that of a pCPA, while when exposure was insufficient to allow diffusion it mirrored that of an npCPA. Indeed, DMSO is often seen to be more effective than glycerol as a CPA, and this is believed to be because DMSO is more readily permeable to cells [88]. By penetrating cells, a pCPA forms colligative properties within the cell, both lowering the freezing point and increasing the intracellular solute concentration (particularly beneficial during extracellular freezing, which concentrates solutes outside the cell). This will prevent both intracellular ice formation and excessive cellular dehydration. Non-penetrating CPAs on the other hand, will only show these properties in the extracellular space.

Exposure Criteria

When determining a protocol for cryoprotectant exposure, toxicity is the primary factor to consider. The concentration required to vitrify is known for many CPAs, but determining cellular toxicity is much more involved. Exploratory work has been carried out to examine the effect on toxicity of a variety of different mechanisms, including the interactions of CPAs with each other and with a variety of tissues, over a host of different times, concentrations, and temperatures. In doing so, it has been found that the interplay

between time required for diffusion and the toxicity of the exposure is a delicate balance for each tissue and each CPA. Several different mechanisms have been determined to play a role in CPA toxicity.

TEMPERATURE OF EXPOSURE

Temperature is a relevant factor in CPA toxicity for two main reasons; CPAs are generally more toxic at a higher temperature [84], and CPAs are generally more viscous at a lower temperature resulting in longer diffusion times. It has been seen that, taking both of these into account, the least toxic temperature for exposure is often the lowest that can be used [91]. Lawson, Mukherjee, and Sambanis [91] have shown that while a CPA at a higher temperature is able to diffuse more quickly and thus reduce the length of exposure, the concomitant increase in toxicity negates any benefits. It should be noted as well that the lower toxicity of a cold CPA is measured objectively and is not skewed by referencing the time of exposure in the calculation [91] (e.g. total cell death rather than cell death/exposure time), which would make a shorter exposure look more toxic. The result of this is that the first CPAs are generally added near 0 ^{o}C [79] to prevent as much CPA toxicity as possible without ice formation (because the tissue is immediately exposed to CPA, ice will not form at 0^{o}C). As the intracellular CPA concentration increases with time, the temperature can be lowered to follow the freezing point, for a further reduction in toxicity.

The diffusion rate of the CPAs is the second factor of importance when determining what temperature is necessary for cryopreservation. For the CPAs to have a uniform effect they must be in equal concentration in all parts of the tissue. Given that the flow rate of a

CPA will change based on the temperature, controlling the temperature will affect how long to expose tissue to a given CPA to prevent toxicity or ice formation.

LENGTH OF EXPOSURE

As mentioned, the temperature of exposure is relevant in determining length of exposure. However, the exposure time is ultimately determined by how long it takes the CPA to diffuse throughout the tissue. There has been a variety of work carried out to calculate or experimentally test permeation kinetics of CPAs [91-98], unfortunately, every different type of tissue or cell suspension will behave differently so a new set of equation parameters must be found for each. This was carried out initially based on hypothesis of standard values for a given tissue, and later refined to experimental results. This has been completed in human articular cartilage, porcine articular cartilage, rabbit kidney, and other tissues and species [96,97,99-101].

Through these models and their subsequent experimental trials the diffusion coefficients for several tissues and cell suspensions have been determined [100], allowing minimum exposure times to be calculated [79]. By reducing the exposure times, there is both a reduction in toxicity and in the protocol length, which can be time consuming on account of the diffusion speed of cold CPAs.

The length of exposure is also dependent on whether the sample is composed of a tissue or of individual cells. In a cell suspension, every cell has free and equal access to the cryopreservation media, but when those cells are held within a tissue or an organ the structure and permeability of the surrounding matrix or tissue determines the access to the

media. Additionally, when cryopreserving tissues or organs, the extracellular structures are often important for function, so these must be preserved intact as well, further modifying the length of time that the tissue must be exposed to CPA. Reflections of this complexity are seen in experimental data, where conditions suitable for cell suspensions result in failure when applied to tissues [28,54], with tissues requiring much longer timeframes for successful cryopreservation. In addition, most tissues contain multiple cell types that likely have different sensitivities to CPAs.

Finally, the initial concentration of a CPA can modify the length of the exposure, as a higher initial concentration will drive a greater diffusion gradient, resulting in the intracellular solution reaching a higher CPA concentration more quickly.

CRYOPROTECTANT CONCENTRATION

While a very high cryoprotectant concentration would be ideal, as it removes any chance of ice formation, it is not currently possible in most cryopreservation applications due to the high toxicity that these concentrations elicit. However, it must still be high enough to allow vitrification to take place [26], and at these levels toxicity is still present [102]. Fahy et al. [75] conducted research into the minimum CPA concentrations required for vitrification, with a variety of different CPAs. Of those tested, the concentration to vitrify ranged from 41-65% w/v, although this can be reduced by using multi-CPA cocktails [48,103].

As mentioned, initial concentration affects the time of exposure; with an initial exposure that is greater than the intended final concentration, diffusion will occur more quickly due to an increased concentration gradient.

Beyond this, CPAs are unique, both in their vitrifying characteristics (Table 1 [75]) and in their toxicity threshold. It has been shown that cells and tissues easily tolerate many CPAs at low concentrations, and the point at which tolerance turns to toxicity varies [84,104-108]. Further, the concentration used when initially loading a CPA into a sample can have an effect, as a concentration that is too high can cause osmotic damage.

MULTI-CRYOPROTECTANT INTERACTIONS

It has been seen that multi-CPA solutions can be less toxic to cells during cryopreservation if properly composed [48]. One experiment to evaluate only this toxicity was carried out by Almansoori et al. [109], exploring the effect of interactions between several different cryoprotectants. Five different CPAs were evaluated, in all combinations of one to five CPAs per cocktail, on their toxicity to isolated chondrocytes as measured by a membrane integrity assay (while maintaining total CPA concentration). The results indicated that a four-CPA combination was the least toxic to chondrocytes. The most important finding is not necessarily which combination proved least toxic (as there are many more CPAs that were not tested), but rather that the reduced toxicity is not simply due to the reduction in individual concentrations. The author states that "*multiple-CPA solution toxicities are nonlinear and the final CPA solution's toxicity is not equal to the sum of its individual CPA toxicities*" (page 189) [109]. What this means is that particular combinations of CPAs have synergistic effects in regards to toxicity, allowing for overall higher concentrations or longer exposure times to be used if needed. CPAs are differentially toxic based on the mechanisms by which they act on the sample, which is hypothesized to be

why multi-CPA solutions are less toxic – overall toxicity due to several, reduced intensity mechanisms is lower than the toxicity of a single, concentrated mechanism [109].

ORDER OF EXPOSURE

Finally, in multi-CPA cocktails the order of CPA addition can also play a role in toxicity. It is generally accepted that adding CPAs in a stepwise manner rather than all at once is preferable to avoid osmotic damage [98,110]. The choice of order of exposure will then have an effect on the other factors mentioned above, namely the concentration and time of exposure. For example, by adding a CPA with a low toxicity first, the freezing point can be reduced and the sample can be further cooled, to reduce the toxicity of subsequent CPA additions. Additional benefits can be obtained by putting one CPA before another, with DMSO being an example. As mentioned above, dimethyl sulphoxide is able to increase the permeability for both itself and other CPAs upon exposure; by adding DMSO early in the process it could further reduce the required exposure time by increasing the diffusion rate.

NON-CRYOPROTECTANT VITRIFICATION ADDITIVES

Some researchers that employ vitrification have found benefit through the addition of ice-blocking compounds to their vitrification solution [80,111]. These compounds do nothing to directly promote vitrification, but they do help to prevent ice formation, which serves the same end. Indeed, a concentration of as little as 0.001% of an ice-blocking compound can visibly have an effect on ice formation [111]. Fahy et al. [80] employ an ice-blocker by the trade name X-1000, a synthetic polymer polyvinyl alcohol that acts similarly to antifreeze proteins found in cold-environment fish and insects. The primary means by which it prevents ice formation is by interacting with solutes to prevent heterogeneous ice nucleation [111].

Successes of Vitrification

Vitrification is capable of providing equal, if not better, results in many applications for which classical cryopreservation techniques have been employed. One field that has shown success in using both traditional cryopreservation and vitrification is assisted reproduction. Oktay, Cil, and Bang [63] completed a meta-analysis of oocyte cryopreservation literature and found that the use of vitrification in the field is producing results on par with those from traditional slow-freezing applications. It was further observed that vitrification appeared to be trending as slightly more effective (non-statistical) than traditional methods, with the conclusion that more studies would be needed to fully determine this. Vitrification has also successfully been used in other fields including animal reproduction, human stem cell preservation, and a variety of other applications [112-114].

The true value of vitrification becomes apparent in applications that show little success through traditional cryopreservation. An example that has been discussed previously is articular cartilage. Conventional cryopreservation methods have only shown limited potential, resulting in some live cells in the superficial and deep layers [70]. More recent work however has shown promise by vitrifying AC [79,115,116]. The work reported in the 2012 Jomha et al. publication reports an average cell recovery in cryopreserved samples of 75%, with viable cells spanning the full thickness of the cartilage.

Currently, one of the most difficult puzzles in cryopreservation is that of the intact organ. As previously stated, every cryopreservation protocol must be tailored specifically to the cell type being preserved, as each will react to the process differently. Because of this,

an organ is far more difficult to preserve than even tissues, as there are often multiple cell and tissue types within an organ, all of which must be cryopreserved for function to be maintained. Using vitrification, Fahy et al. [80] demonstrated that this is currently possible. In the experiment, an intact rabbit kidney was successfully vitrified and transplanted. The experimental procedure involved the removal of a kidney from a rabbit, cryopreservation by vitrification, and re-transplantation of that kidney (as well as removal of the second kidney to ensure it was not compensating). The animal maintained health for 48 days postoperatively, at which point it was sacrificed after concluding that the cryopreserved kidney was able to sustain life.

ADDITIVES/SUPPLEMENTS

Cryopreservation is historically a process that is damaging to cells and tissues, so any possibility of protection is worth investigating. One such possibility that has already proven beneficial is the use of additives [68,80,117]. While additives have been discussed already, in the form of CPAs and ice-blocking compounds, it has only been in the role of aiding the cryopreservation process. The additives discussed below have been proven to be useful in various applications, some of which may be applicable to cryopreservation. One strength of these additives is that they can be tailored to an individual cell type to provide benefits on a metabolic level. Further, once CPA damage mechanisms become apparent, it may be possible to locate relevant compounds to mitigate these mechanisms and improve the utility of common cryoprotectants by reducing toxicity.

CHONDROITIN SULPHATE

Chondroitin sulphate (CS) has been previously mentioned in discussing the morphology of articular cartilage; it is one of the predominant building block molecules of the extracellular matrix. No doubt related to this, a prominent use of CS is in the treatment of osteoarthritis (often in conjunction with glucosamine sulphate, another AC cellular matrix component). A majority of the papers in a review article by Jerosch [5] found that CS had a long term benefit in the treatment of OA. Jerosch states that "*in general, CS inhibits cartilage destruction processes and stimulates the anabolic processes involved in new cartilage formation*" (pg. 6). It should be noted however, that these findings are not directly applicable to other applications, such as cryopreservation. These findings come from oral CS exposure, so there are interactions with digestion and metabolism that are not explicitly relatable to the direct exposure of chondrocytes to CS. Further, a pharmacokinetic examination of CS found that plasma concentration of CS did not increase after ingestion, hypothesizing that the effects of CS were due to interactions with the digestive tract or from breakdown products [118].

IN CRYOPRESERVATION

Chondroitin sulphate has also been investigated in other applications and tissues, including cryopreservation. Hagenah & Bohnke [68] conducted an experiment to determine whether CS exposure was beneficial to the cryopreservation of porcine corneas. They found that CS was beneficial, but that this benefit could not necessarily be extrapolated beyond their experiment due to complications in their protocols. The beneficial effect of CS has since been confirmed in human corneas, and an experiment by Fan et al. [119] demonstrated

that it also provided more benefit during cryopreservation than other non-penetrating macromolecules. While the majority of cryopreservation literature using CS is on corneal preservation, CS has also been shown to be effective during the cryopreservation of human vein grafts [117], as well as during the vitrification of human articular cartilage [79].

TETRAMETHYLPYRAZINE (TMP)

Tetramethylpyrazine is another compound that has been investigated for its ability to treat OA, as the major active molecule in a common Chinese herbal remedy. An in vitro experiment by Li et al. [120] was conducted to determine the mechanism through which TMP treats OA, examining the effect of direct exposure of TMP to isolated rat chondrocytes over two generations. The findings of the study indicated that TMP treatment provides an increase in cellular proliferation, which may be how it treats OA. Other work in this area conducted by Ju et al. [121] studied the ability of TMP to act in reducing inflammation and apoptosis in rabbit chondrocytes and articular cartilage explants. This is prefaced with evidence that the production of reactive oxygen species (ROS) is increased with many joint diseases [122], which is important because "*ROS may cause damage to all matrix components, either by a direct attack or indirectly by reducing matrix components synthesis, by inducing apoptosis or by activating latent metalloproteinases*" (pg.747) [123]. Ju et al. found that exposure to TMP can have a beneficial effect in both chondrocytes and intact cartilage in reducing apoptosis, formation of ROS, and formation of inflammatory cytokines (IL-1β). As such, they have suggested that TMP could be used as a treatment to slow the progress of OA.

Beyond its use in treating osteoarthritis, TMP has also been seen to be beneficial in several other applications. TMP is used widely in neurovascular and cardiovascular treatments, and TMP's anti-inflammatory characteristics have also been used as an experimental treatment for ischemic rats - where it has been seen to provide a neuroprotective effect [124,125]. TMP has also been investigated and found beneficial in reversing induced amnesia in a rat model, indicating a potential use in the treatment of dementia [126].

IN CRYOPRESERVATION

Currently, the use of TMP in a cryopreservation application appears to be novel, as indicated by a literature search of the Web of Science and PubMed databases.

ASCORBIC ACID

Ascorbic acid (AA, vitamin C) has also been investigated for use in treating OA, and aside from this has well noted antioxidant properties. AA is a particularly important compound in the healthy functioning of cartilage. This is primarily due to its role in the synthesis of collagen [127] and, to a lesser extent, aggrecan [128], both important components of cartilage extracellular matrix. Experiments have been conducted to evaluate the use of AA in treating OA cartilage defects, with mixed results. Trials completed in humans have shown a successful slowing of the progression of OA symptoms [129], while other trials conducted in guinea pigs found that at high levels of AA ingestion, there was an increase in spontaneous OA symptoms [130]. The negative effects of high concentrations of AA have been shown to reduce cell viability in a tissue engineering application [131].

Aside from AA's benefits surrounding the synthesis of collagen, its antioxidant capacity also indicates its use in the treatment of OA, due to the previously mentioned presence of reactive oxygen species in joint diseases [122,123]. An additional benefit to using AA is that any use in human applications would not have to be further approved due to its known safety and current ubiquitous use.

IN CRYOPRESERVATION

Cryopreservation protocols that incorporate AA have been examined, with minimal beneficial results. A study by Pfeiffer and Arnove [132] found that in an application using glycerol, the inclusion of AA raised the threshold concentration of glycerol required to cause hemoglobinuria in red blood cells by 100% (from 0.75 mL/kg to 1.5 mL/kg). Aside from this there has been limited use of AA in cryopreservation applications, with a study on the preservation of ram semen showing no benefit from the inclusion of AA [133].

GLUCOSAMINE

Glucosamine (GlcN) is another component of cartilage extracellular matrix, and is often paired with chondroitin sulphate in treating OA. Experiments in both rats and guinea pigs have shown that treatment of OA with GlcN reduces destruction of cartilage as well as reducing pain [134,135]. There is also substantial evidence in the literature that the use of GlcN to treat OA in humans is an effective treatment [5]. GlcN is thought to be effective by preventing the breakdown of aggrecan in the cartilage extracellular matrix [136], as well as altering chondrocyte metabolism and having an immunoregulatory role which may reduce inflammation [137]. Additionally, while GlcN is often given in conjunction with CS, there is evidence that these two compounds are not acting in a synergistic manner and are both

effective on their own [118]. GlcN sulphate is a specific form of GlcN that has been indicated to be superior to other forms in treating OA [138], and which has a demonstrated antioxidant capacity [139] that will be beneficial in the ways already mentioned.

IN CRYOPRESERVATION

Varghese et al. [140] have shown that GlcN does contribute to a cryopreservation protocol for human sperm. Trials conducted with GlcN included were significantly better than controls in several measures (% motility, % forward progressive motility, and % normal DNA integrity), with these effects hypothesized to be due to the antioxidant properties of GlcN.

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CHAPTER 3: THE EFFECT OF ADDITIVE COMPOUNDS ON GLYCEROL-INDUCED DAMAGE TO HUMAN CHONDROCYTES

INTRODUCTION

Osteoarthritis (OA) is a disease with a substantial personal and economic burden in modern society. In 2014, the WHO estimated the 2012 disease burden of OA as amounting to a reduction in years of healthy life lost due to disability of over 18 million years worldwide (can be thought of as 18 million individuals being unhealthy for 12 months each year), which has increased since 2000 by over 30% [1]. Currently there is no cure for OA. There only exist treatments that often have a limited period of effectiveness. Further, many of the available surgical treatments are ineffective when treating larger lesions (>2.5 cm^2) due to the minimal healing capability of hyaline (articular) cartilage [2], resulting in few viable options other than joint replacement for advanced cases of the disease. As such, by treating lesions before they develop into OA, complicated issues of global joint arthritis can be prevented. One surgical treatment that shows promise in treating large defects to prevent the development of OA is osteochondral allografting, or the transplantation of bone and cartilage from a cadaveric source. The use of cadaveric tissue is possible largely due to the immune-privileged nature of cartilage [3,4], which limits the immune reaction to foreign tissue on transplantation. One limitation to osteochondral allografting is that there must be donor

cartilage available and that this tissue must be healthy. The structural integrity of cartilage requires healthy cells to continually replace the extracellular matrix, which is responsible for the strength and effectiveness of AC [5]. This limitation is compounded by the fact that cartilage has a limited capacity to tolerate hypothermic storage (the current clinically-used storage technique), noticeably losing viability after 1-2 weeks [6,7] and dropping as low as 68% viable after 28 days [8], which is generally the upper limit of cartilage storage prior to transplantation. With this limitation in place it logically follows that if an improved preservation method could be employed, the utility of osteochondral allografting would be improved.

Much of the early focus in the field of cryopreservation was on the preservation of animal spermatozoa [9], and both classical, controlled-rate freezing and vitrification have since been applied with some success in cryopreservation of various cell suspensions, particularly in the reproductive sciences [10], and in chondrocytes as early as 1965 [11]. While there has been some success in corneas [12] and veins [13] in making the transition from cellular preservation to the more complex tissue preservation, there has been little success in using classical cryopreservation to preserve articular cartilage [14-16]. The limited success of these methods is largely due to the formation of ice and the damage it causes through osmotic and mechanical stresses. This makes vitrification a desirable alternative as the majority of the processes that occur during freezing which damage cells are avoided during vitrification due to the absence of ice formation. The efficacy of vitrification in preserving cartilage has been demonstrated in both porcine [17,18] and, more recently, human applications [19,20]. One of the drawbacks to vitrification, and one of the areas for possible improvement, is the

required use of high concentrations of cryoprotective agents (CPAs) to promote vitrification rather than freezing. At the concentrations required for vitrification these CPAs become cytotoxic, although there are several methods which have been discovered that can begin to alleviate this [21-23], including research into transport kinetics for CPAs into tissue [24-28]. One avenue with the potential to benefit vitrification is the use of additive compounds in the cryoprotectant solutions, specifically those compounds that elicit some benefit that is not directly related to the mechanics of vitrification.

One example of the utility of such an additive regards reactive oxygen species (ROS). It has been previously shown that oxidative stresses are both common and damaging in joint disorders [29], indicating the possible use of an ROS scavenging compound as an additive in vitrification treatments tailored to articular cartilage; particularly those with articular cartilage destined to be transplanted into a joint in which disease is already present. For the present study, additives were chosen based on antioxidant capacity in consideration with other factors such as previous use with cartilage or pre-approval for medical use. Four additive compounds (chondroitin sulphate, tetramethylpyrazine, ascorbic acid, and glucosamine sulphate) were evaluated. Chondroitin sulphate and glucosamine sulphate are both components of cartilage extracellular matrix and have been used clinically to successfully treat OA [30-32]. Chondroitin sulphate has also been used previously in the vitrification of articular cartilage and has been shown to be beneficial to the cell viability in the rewarmed cartilage [19,33]. Some mechanisms by which these compounds are known to act are summarized:

Glucosamine has been shown to alter the metabolism of chondrocytes and play an immunoregulatory function, an action that could reduce inflammation. Chondroitin sulphate in physiological conditions, contributes to the elasticity of cartilage and inhibits its degradation by enzymes such as elastase and hyaluronidase [31].

Ascorbic acid (vitamin C) is a necessary component in collagen synthesis which is in turn a major component of cartilage extracellular matrix. Ascorbic acid treatment in OA settings has also been shown to slow the progression of OA symptoms in humans [34]. Conversely, it has been seen to increase spontaneous OA symptoms if a concentration that is too high is used (albeit in a guinea pig model) [35]. It has been investigated in a cryopreservation application, but was found to have no beneficial effects [36]. Tetramethylpyrazine (TMP/Ligustrazine) was traditionally used in Chinese herbal treatments for back and joint pain, but has since been experimentally shown to have anti-inflammatory [37]and anti-apoptotic/ROS scavenging capacity [38,39], as well as having been used in OA treatments [40].

In the present study, TMP, CS, AA, and GlcN were evaluated for their ability to improve cell viability in intact human articular cartilage after exposure to a toxic glycerol solution. We hypothesized that the use of these additives would have a beneficial effect on cell viability based on membrane integrity measurements. The results from this experiment could then be applied to a vitrification protocol such that post-warming chondrocyte viability could be improved.

MATERIALS AND METHODS

CARTILAGE TISSUE ISOLATION

Articular cartilage was obtained from human knees undergoing total joint replacement in two local operating rooms (Royal Alexandra and Misericordia hospitals, Edmonton, AB). All tissues were immediately placed in sterile phosphate buffered saline solution (PBS, pH 7.1, Invitrogen, Carlsbad, CA) and stored at 4 ^{o}C until use within 1 day of harvest. Although there were no exclusion criteria based on donor characteristics aside from standard research tissue exclusions, information such as height, weight, age, and whether the patient smoked or had undergone cancer or corticosteroid treatment was collected. Ethical approval was obtained from the University of Alberta Ethics Review Board.

Ten millimeter full thickness osteochondral dowels were obtained from the discarded tissue of individuals undergoing knee replacements. The 10 mm diameter samples were cored from the best portion of the articular cartilage after visual inspection and grading on the Outerbridge classification system [41], using only "good" tissue graded as a 0 or 1. Articular cartilage was cut perpendicular to the articular surface into 75 μm thick slices using a vibratome [The Vibratome Company, St. Louis, MO]. The slices were immersed in a petri-dish containing PBS at room temperature (22 ^{o}C) and used within half an hour of slicing.

EXPERIMENTAL PROTOCOL

SOLUTION PREPARATION

Eleven experimental solutions were used, mixed with a base concentration of 1.6 M glycerol (Fisher-Scientific , Ottawa, Ontario) prepared in X-VIVO 10 media (Lonza Inc., Basel,

Switzerland), as previous experiments in our lab had shown glycerol to be second most toxic of the cryoprotectants used in our vitrification protocol [19,22]: (i) 0.1 mg/mL chondroitin sulphate (CS) (Sigma-Aldrich, St. Louis, Missouri), (ii) 200 μM tetramethylpyrazine (TMP; Sigma-Aldrich, St. Louis, Missouri), (iii) 400 μM TMP, (iv) 200 μM TMP + 0.1 mg/mL CS, (v) 400 μM TMP + 0.1 mg/mL CS, (vi) 500 μM ascorbic acid (AA - L-ascorbic acid 2-phospate sesquimagnesium salt hydrate; Sigma-Aldrich, St. Louis, Missouri) , (vii) 1000 μM AA, (viii) 2000 μM AA, (ix) 0.18 mM glucosamine sulfate potassium chloride (GlcN; Medisca Pharmaceutique Inc., St-Laurent, Quebec), (x) 0.36 mM GlcN and (xi) 0.57 mM GlcN.

In addition to the experimental solutions, there were 3 control solutions: A negative control that was composed of 8M glycerol which results in 0% cell recovery of a fresh cartilage sample as determined by membrane integrity stains after immersion; a positive control which consisted of pure X-VIVO 10 (100% viable); and an unmodified 1.6 M glycerol solution (experimental control which gives ~50% viable). Experimental and control solutions were stored in a 4 ^{o}C fridge. 1 ml of each solution was placed into a 48-well cell culture plate and labelled appropriately.

SOLUTION EXPOSURE

For each repetition of the experiment, a sufficient number of slices were obtained from the same donor to perform all treatment conditions as well as the positive and negative controls (duplicate repetitions were carried out in any donors with tissue that supported the increased number of slices). Slices were exposed to the above solutions for 90 minutes at room temperature, and then washed twice in X-VIVO 10 for 5 minutes. In the TMP/CS trials, slices were evaluated: (i) immediately after the completion of the second

wash (immediate analysis trial), and (ii) after a 48 hr incubation at 4 ^{o}C in the wash solution (48 hr analysis trial). A follow-up experiment was performed using ascorbic acid and glucosamine using the 48 hr incubation period time frame only.

CHONDROCYTE VIABILITY STAINING AND QUANTIFICATION

Cell viability within the slices was determined by a dual fluorescent membrane integrity assay using Syto 13 (Invitrogen, Canada) and propidium iodide (PI; Sigma) dyes [6.25 µM Syto 13 and 15.0 µM PI mixed in PBS (v/v)], and viewed under a Nikon Eclipse E600 Microscope (Nikon Canada, Mississauga, Ontario) with a dual filter (Excitation:480 nm, Emission: 530/640 nm) and imaged using a Nikon DXM1200F Digital Camera (Nikon Canada, Mississauga, Ontario). The use of membrane integrity staining correlates well with tissue health, indicated in a study on long-term allotransplant outcomes [42].

The images produced show the stained nuclei of intact cells as green and the stained nuclei of membrane compromised cells as red, allowing for simple quantification. Images were processed using Photoshop (Adobe Photoshop Elements 6, Adobe, Salt Lake City, UT) to stitch photos of each slice and to minimize background noise using the levels function. This served to both increase the homogeneity of image quality as well as make the images more readable by the counting program (identical treatment was applied to every stitched image to reduce background). Images were then entered into a custom program (Viability3, version 3.2, Locksley McGann, Great Canadian Computer Company, Spruce Grove, AB, Canada) that counts and summarizes the number of green and red cells in the image. The program output contains both the number of cells as well as the relative percentages of intact and compromised cells, the latter of which was used for analysis.

The inclusion criterion used in the TMP/CS immediate trials was a minimum 85% cell viability in the positive control, indicating a healthy sample to begin the experiment. Inclusion criteria in the 48 hr analysis trials, both TMP/CS and AA/GlcN, included a minimum 15% cell viability in 1.6 M glycerol controls, due to a hypothesized inability to accurately measure the effect of the additives below this point and indicating that the control tissue was unusually sensitive to glycerol. The upper limit in the 48 hr trials of 1.6 M glycerol control cell viability was limited to 65% cell viability because viability above this level suggests that these cells are resistant to glycerol toxicity and would compromise the ability to determine if an additive could limit glycerol toxicity.

STATISTICAL ANALYSIS

Statistical differences in cell viability between the 1.6M glycerol control group and each of the 11 experimental groups over the two experiments were assessed using a one-tailed t-test analysis to determine if any individual experimental condition was significantly better than the 1.6 M glycerol control. This 1.6 M glycerol solution was used as the control as it was determined to provide ~ 50% cell viability after the 90 min exposure period, allowing simple measurements of relative improvement. The immediate TMP/CS trials underwent a total of 16 replications, while each of the TMP/CS, AA and GlcN 48 hr analysis trials underwent 10 replications. All statistical differences were considered significant when $p<0.05$.

RESULTS

TMP/CS IMMEDIATE ANALYSIS TRIALS

Figure 3.1 provides the average percent chondrocyte viability data for the immediate TMP trials, normalized to the X-VIVO control. The tissues included in these trials were taken from adults ranging from 56-72 years of age (average 65.2, N=16). The average percent cell viability of the 1.6 M glycerol control group, to which all other trials were compared, was 56.45 ± 4.87% (mean ± SEM, range: 17.5-89.1%) as obtained from the Syto 13/PI membrane integrity staining as detailed above, after two samples were removed from the data set for not meeting the minimum 85% cell viability in the X-VIVO control. Statistical data analysis showed no significant difference when comparing the mean control to each of the mean experimental trials (N=16). Recoveries in the experimental trials ranged from 55.5 ± 5.58% to 60.7 ± 4.77%, showing substantial overlap with the values in the control group. After completion, this trial was used as baseline information and provided a reference point for the results when the same methodology was applied to a longer time frame in the 48 hr analysis trials.

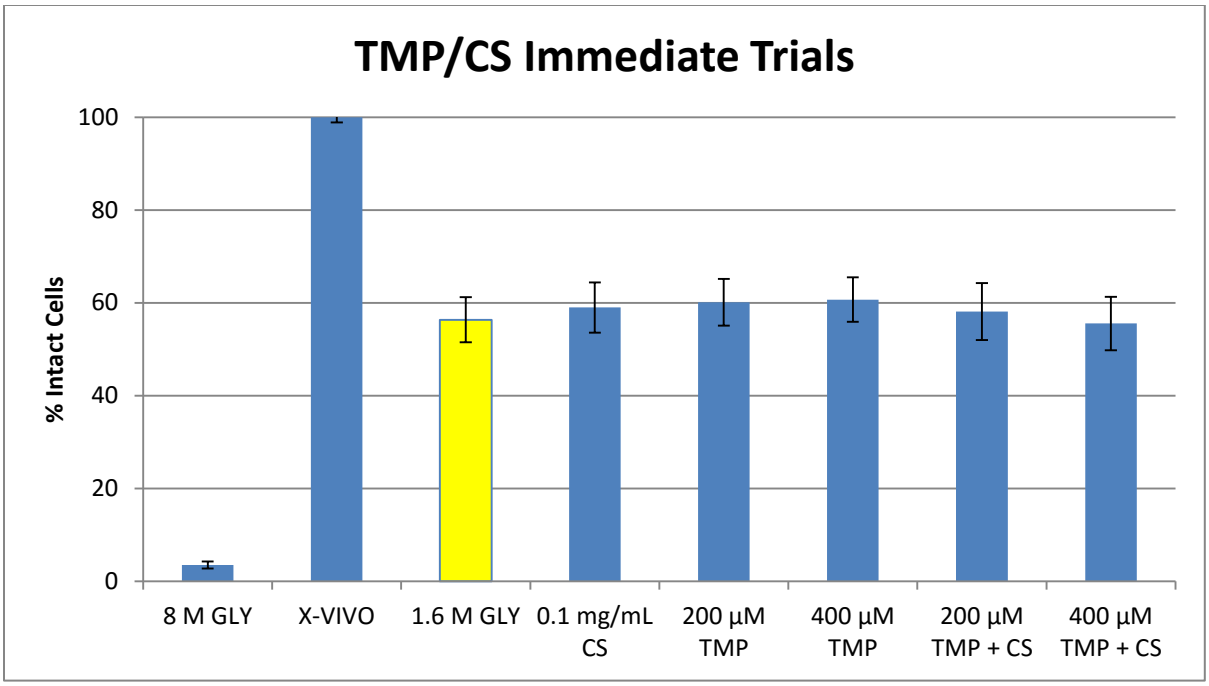

Figure 3.1. Average chondrocyte percent viability (mean ± SEM) in immediate analysis TMP/CS trials based on Syto 13/PI membrane integrity staining normalized to X-VIVO control. Each treatment was normalized to its own control from the same sample, then averaged individually (N=16). Cartilage slices incubated for 90 min in any of the five experimental groups did not show significant differences in cell viability when compared to the 1.6 M glycerol control group.

TMP/CS 48 HR ANALYSIS TRIALS

For the TMP/CS 48 hr analysis trials, the average percent cell viability for each additive normalized to the X-VIVO control is shown in figure 3.2. The average cell viability of the 1.6M glycerol control, to which all other trials were compared, was 42.32 ± 4.71% (range: 20.5-60.9%) with donors ranging from 52-80 years of age (Average 64.3, N=10), after exclusion of one replicate for exceeding the 65% maximum cell viability in the 1.6 M glycerol control. When compared to this control there were three additive combinations that were found to be significantly different: 200 µM TMP with CS (57.75 ± 4.40% p=0.004), 400 µM TMP with CS (55.45 ± 4.21% p=0.012), and 400 µM TMP alone (50.87 ± 2.89%

p=0.026). Based on these results, a subsequent experiment was performed to include new additives, and the trials were repeated with ascorbic acid and glucosamine sulphate.

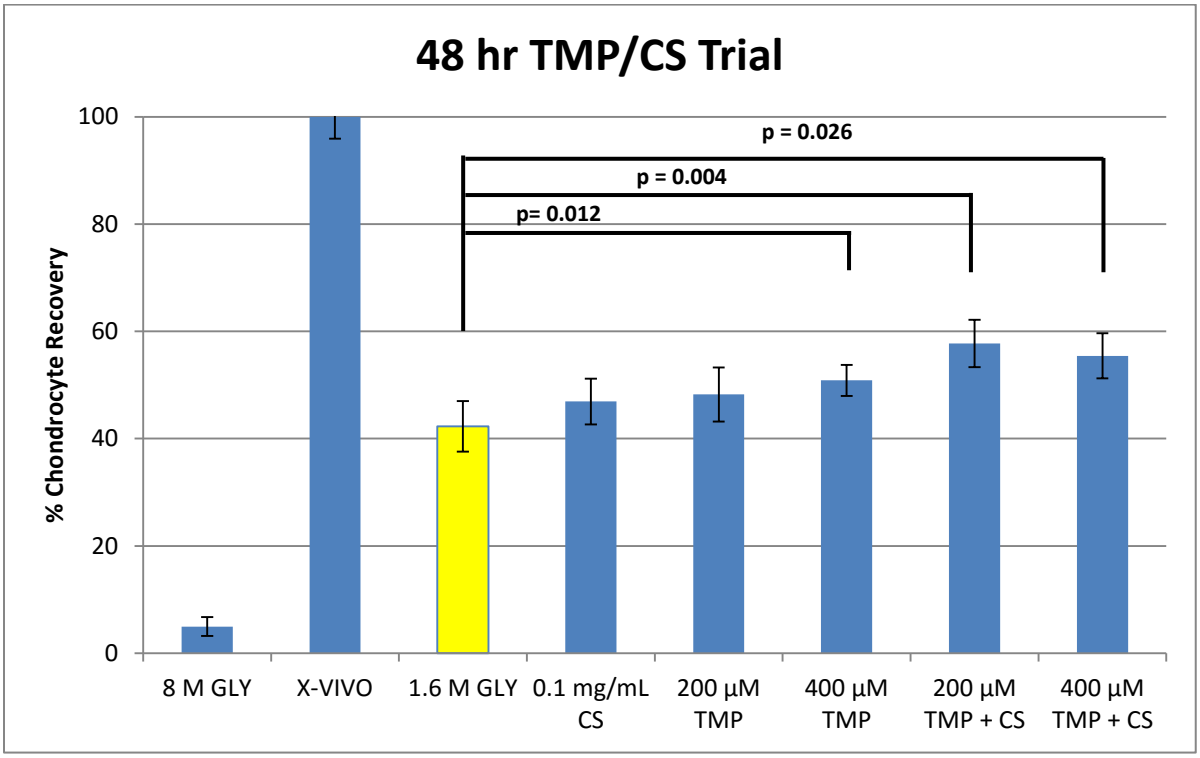

Figure 3.2. Average percent chondrocyte viability (mean ± SEM) results in 48 hr analysis TMP/CS trials based on Syto 13/PI membrane integrity staining (N=10) normalized to X-VIVO control (each sample normalized to its own control, then averaged individually). Cartilage slices incubated for 90 min in presence of TMP, CS or TMP+CS in a 1.6 M glycerol solution followed by 48 hr incubation at 4 °C showed significant differences in cell viability in three trials when compared to the 1.6 M glycerol control group (400 µM TMP, 200 µM TMP+CS, 400 µM TMP+CS - p-values in figure with $\alpha = 0.05$).

AA/GlcN 48 Hr Analysis Trials

The results of the 48 hr analysis trials evaluating ascorbic acid and glucosamine are summarized in figure 3.3. The average percent cell viability of the 1.6M glycerol control samples, to which all other trials are compared, was 45.91 ± 6.89% (range: 15.4-57.9%) with the donor ages ranging from 52-88 years of age (average 64.2, N=10), after exclusion of two

samples which exceeded the 65% maximum cell viability in the 1.6 M glycerol control. There were three additive trials that showed significance in percent cell viability after ten repetitions when compared to the 1.6 M glycerol control. Those experimental groups that reached significance included the 2000 μM ascorbic acid (67.61 ± 5.63%, p=0.0109), the 0.18 mM GlcN trial (66.06 ± 4.30%, p=0.0141), and the 0.36 mM GlcN trial (70.17 ± 6.01%, p=0.0125). Furthermore, all other experimental groups aside from 0.56 mM GlcN (CS, 400 μM TMP, and 500 μM & 1000 μM AA) were approaching significance in these trials (average viability of 59-62%, p=0.058-0.089).

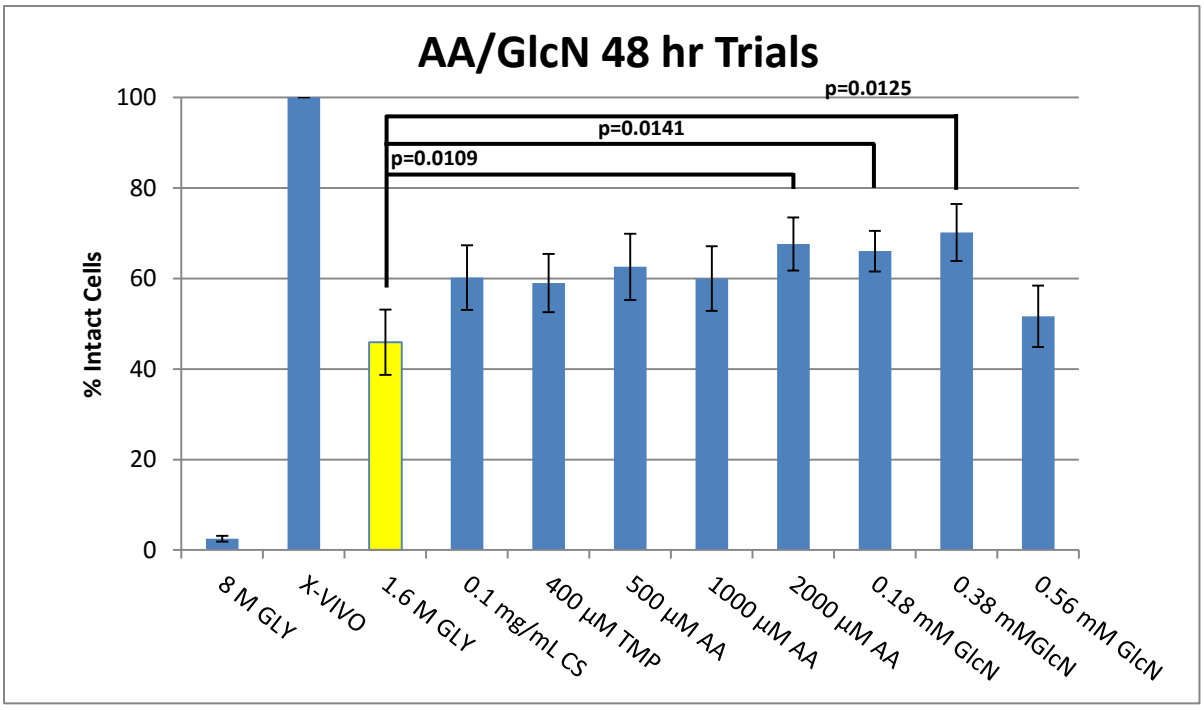

Figure 3.3. Average percent chondrocyte viability (mean ± SEM) results in 48 hr analysis in AA and GlcN trials based on Syto 13/PI membrane integrity staining (N=10) normalized to X-VIVO control (each sample normalized to its own control, then averaged individually). Cartilage slices incubated for 90 min in presence of TMP, CS, AA, or GlcN in a 1.6 M glycerol solution followed by 48 hr incubation at 4 °C showed significant differences in cell viability in three trials when compared to the 1.6 M glycerol control group (2000 μM AA, 0.18 mM GlcN, 0.38 mM GlcN - p-values in figure with $\alpha = 0.05$).

DISCUSSION

The aim of these experiments was to evaluate the effectiveness of the selected compounds in two capacities. In the first study of the immediate trials using TMP & CS, percent cell viability was evaluated to assess any effect in reducing the toxicity of glycerol exposure. Due to the relatively short time frame of the experiment coupled with the analysis immediately following the washing step, there was not enough time for the effects of apoptosis to be observed in these samples as observable effects of apoptosis in tissues have been estimated to generally occur on the order of 11-14 days as opposed to a matter of hours in isolated cells [43]. It is likely that apoptosis in the thinly sliced tissues samples used here would occur sometime between these two time periods and outside the scope of the immediate trials, leaving only cell death that has occurred as a direct result of glycerol toxicity. The second study in which the compounds were assessed was for their general ability to either exert a positive effect on the regenerative capacity of damaged chondrocytes, or to reduce continued damage over the incubation period set in motion by the glycerol exposure, possibly due to oxidative stresses. Successful mitigation of damage in either of these contexts would then qualify an additive for potential use in a vitrification protocol.

The results presented for the immediate trials indicated that there was no capacity of CS or TMP to reduce the toxicity of glycerol in cartilage after incubation for 90 minutes. This is not surprising, as one of the few identified mechanisms of glycerol toxicity involves manipulation of the glycerol phosphate cycle to promote protein glycosylation and mitochondrial dysfunction [44], on which none of TMP, CS, AA, or GlcN appear to have a

known effect. Glycerol is reported as having a low toxicity when tested in almost all organisms [45], which is very different from its well-known toxicity when being used on isolated cells or tissues [22,46,47]. It is possible that glycerol is more toxic in cell and tissue applications due to its high viscosity and more direct interactions with the cells as opposed to whole organism applications. Glycerol can act by osmotically damaging cells due to low glycerol permeation rates resulting in rapid efflux of water soon after exposure. This osmotic damage could initiate an apoptotic response that could be blocked by additive compounds and noted in our delayed trials. This will need to be investigated further.

The 48 hr analysis TMP/CS trials showed statistically significant results with three of the additive combinations (400 μM TMP, 200 μM TMP+CS, 400 μM TMP+CS) having higher cell viability than controls. An important point to be made regarding the data in these trials is that these groups in this set of experiments displayed a viability on par with the 1.6 M glycerol controls in the immediate trials. This would not normally be expected due to natural cell degradation during the 48 hr incubation period. This suggests that over the 48 hr time course, there may have been some cell membrane recovery from sub-lethal damage or halting of further cell death from other processes that resulted in cells maintaining or regaining cell membrane integrity. The use of TMP in attempting to mitigate CPA toxicity in either of the capacities tested in the current study is novel, with current uses centering on clinical treatments of reducing inflammation and apoptosis in a variety of settings, including the treatment of osteoarthritis [37-40,48-51]. However, as TMP is historically a compound derived from a traditional Chinese herbal treatment for back and joint pain, much of the literature is available only in Chinese. This makes determining the full spectrum of TMP

investigation difficult, so the claim that this investigation is novel does come with this caveat.

The results of the TMP/CS 48 hr analysis trials are more appropriately put into a useful context when compared with the results of the AA and GlcN trials. While there were statistically significant results present using TMP & CS, the maximum improvement of the experimental groups was ~13% compared to the glycerol control group. Although important, it is not as significant as in the AA/GlcN trials where there was a maximum increase in cell viability of ~23%. The fact that the 23% increased viability trial is not more statistically significant than the 13% increased viability trial can be accounted for by the higher SEM of both the experimental trials and the 1.6 M glycerol control in the AA/GlcN set of experiments. Thus, it is more likely that the AA/GlcN compounds will have a clinically significant effect. An important note in making this comparison between different groups of trials is that while the trials were completed on different sets of donors making a direct comparison perhaps inappropriate, both sets of trials had nearly identical results for the control group (~3% difference, with ~2% difference in SEM) supporting that comparison of these two experiments is valid.

There has been little investigation of AA's effect on glycerol toxicity in RBCs [52], with the majority of uses in the literature centering on growth of cartilage extracellular matrix via collagen synthesis [53,54]. Ascorbic acid has also been shown to mitigate OA progression in guinea pig articular cartilage in vitro at concentrations of 189 μM – 769 μM [55], and in humans [34,55], although a high concentration of ascorbic acid in guinea pigs in vivo (150 mg/day) appears to have a positive correlation with OA severity [35]. The use of glucosamine

to prevent glycerol damage also appears to be a novel investigation, with the majority of the literature regarding glucosamine's effect on OA severity and progression [30,31,56-58].

The results of the AA and GlcN trials provide some indication that an examination of these compounds using immediate analysis trials could be beneficial. Based on the assumption that there was no reduction in direct glycerol toxicity, as was found in the TMP/CS immediate trials, the maximum cell viability that could be attained after the 48 hr incubation period would be the cell viability at the time that the glycerol exposure ended, when all further cell death was prevented. As there are no controls that represent what this cell viability value might be in the AA/GlcN trials, a potential proxy would be to use the controls from the TMP/CS immediate trials. This comparison is not entirely unsuitable given the relation between the TMP/CS immediate trials and the TMP/CS 48 hr trials and the subsequent relation between the TMP/CS 48 hr trials and the AA/GlcN trials. In making this comparison it is seen that the highest attained viability of 70.2 ± 6.3% in the 0.38 mM GlcN trials is much higher than the benchmark viability of 56.3 ± 4.9% 1.6 M glycerol controls in the immediate trials and is nearing significance ($p=0.058$). This calls into question the assumption that there was no influence by GlcN on the direct glycerol toxicity, given that it has improved cell viability results beyond the benchmark set in the immediate TMP/CS trials. However, because these are different sets of experiments, this is largely speculative. A more suitable option than running a set of immediate analysis trials using AA and GlcN may be the introduction of an immediate control in future additive investigations.

A strength of the experimental design is that it is easily adapted to testing additional compounds and combinations of compounds. An example of this would be adding ascorbic

acid and glucosamine together to see if any additional benefit could be produced, similar to that seen when combining TMP with CS. There is evidence that the addition of CS to GlcN may be beneficial, as this particular combination has been utilized successfully in clinical applications [31,32]. Further, any number of new compounds hypothesized to have an effect could be tested efficiently. Finally, the data presented here could be used to confirm or formulate hypotheses about the specific mechanisms of cell death occurring over the 48 hr analysis incubation period. These inferences could be made by comparing known mechanisms of action of TMP, CS, AA, and GlcN (how they might reduce cell death) to the results of these studies, where reductions in cell death have been measured.

The limitations of this study are primarily situated around the choice of tissue and the protocol used. The use of OA tissue for experimentation was necessary in this case due to the difficulty in obtaining normal human articular cartilage, but nonetheless introduces a bias towards potentially exaggerating the results. This is due to the fact that OA chondrocytes are more likely to die by apoptosis than their healthy counterparts [59], even if the tissue is taken from a healthy-looking region of a joint where OA is present [60], so any portion of the results that are due to the anti-apoptotic properties of our compounds would not be likely to have as much of an effect in healthy cartilage. The limitations arising from the experimental protocol are specifically due to the fact that the intended final application is to a vitrification protocol, while the protocol to evaluate the compounds is not vitrification, or even cryopreservation, based. By simplifying the vitrification process to exposure to a single CPA, the effects of both warming and cooling as well as the toxicities and CPA-CPA interactions [22] of a multi-CPA solution are removed from consideration;

however, this simplification is necessary due to the long time frame and increased stress on OA cells that a full vitrification protocol necessitates. Finally, since the compounds had the greatest effect when given a longer period of time, a potential weakness of this design was the inclusion of the compounds only during the glycerol exposure period. There is evidence that pre-exposing cartilage to TMP will increase the effect that it can have after introducing an event [49], and our own results indicate that continued exposure to TMP, TMP/CS, AA, or GlcN after the glycerol exposure may be beneficial given that this is the time frame in which they appeared to act. This portion of the experimental design was intended to ensure that the results of the immediate and the 48 hr analysis trials were comparable, by keeping the exposures the same.

CONCLUSIONS

Tetramethylpyrazine, ascorbic acid, glucosamine, and chondroitin sulphate combined with tetramethylpyrazine had a significant beneficial effect on chondrocyte viability after exposure to 1.6M glycerol and a 48 hr incubation period. TMP and CS alone were ineffective at reducing direct glycerol toxicity, but all of TMP, TMP/CS, AA and GlcN were able to reduce further cell death over the 48 hr incubation period. The most effective treatment in this capacity was glucosamine at an intermediate concentration of 0.38 mM. Further investigation into combinations and additional concentrations of the above compounds are warranted, and the data justifies the inclusion of additive compounds into vitrification solutions.

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CHAPTER 4: TESTING THE EFFECT OF CRYOPROTECTANT PERFUSION PERIOD IN HUMAN ARTICULAR CARTILAGE VITRIFICATION

INTRODUCTION

Osteoarthritis (OA) is a degenerative disorder resulting in the breakdown of cartilage and bone within joints, leading to disability. Treatment of OA and losses of productivity attributed to OA account for expenses of up to 1-2.5% of national GDP in many first world nations [1], with a recent estimated lifetime risk of developing knee OA being approximately 40% in men and 47% in women [2]. OA is a substantial global issue that requires some form of intervention. One of the reasons that OA continues to have such a profound effect even in the established medical systems around the world is that it is among the list of diseases that are incurable, so while a variety of successful treatments exist that can mitigate the symptoms of OA, the afflicted individuals will always return to the health care system. One surgical treatment that aims to delay or prevent OA and shows promise particularly in young individuals is osteochondral allografting. Osteochondral allografting involves the transplant of bone and cartilage from a tissue donor to fill in cartilage lesions in an affected joint before they can deteriorate into clinical OA. This is in contrast to total knee arthroplasty (effectively the synthetic implant equivalent of allografting), which is generally used as a last resort and is considered less suitable for younger patients due to the increased demands on the joint and longer post-transplant lifespan [3,4].

One of the major shortfalls in the use of allografting is the need for tissue to be fresh and healthy for a successful transplant [5], as the extracellular matrix must be continuously replenished by the chondrocytes. Chondrocytes recovered from a tissue donor are able to be stored at 4 ^{o}C for up to a week in media with no substantial cell death occurring [6], but with longer storage periods over two weeks the viability of the tissue begins to degrade [6,7]. This places an upper limit on the timeframe after death that transplantation of recovered tissues can still occur, generally set at approximately 28 days after death [6,7]. This short timeframe, coupled with the poor supply of donor tissue results in a limitation to the application of allografting, which could be alleviated with the introduction of an improved preservation method. Simple freezing, controlled-rate (two-step) freezing, and vitrification are examples of cryopreservation methods that have been used in an attempt to improve the outcome over refrigeration at 4 ^{o}C. Simple freezing, or the placement of the tissue at sub-zero temperatures, has been shown to be ineffective for cartilage as this method of cryopreservation destroys cells [8,9]. The use of two-step freezing and cryoprotectants (CPAs) has been more successful in sheep and cows [9,10], but when applied to human articular cartilage it failed to perform adequately [11,12]. Vitrification is a technique that has been applied more recently, and which has been more successful in improving chondrocyte viability in rewarmed cartilage [13].

Vitrification is a cryopreservation method that completely avoids the formation of ice [14,15], eliminating the majority of the dangers to cells that are present in freezing procedures [16,17]. Further, vitrification occurs typically around -130 ^{o}C, which is a temperature at which all biological activity has effectively ceased [18], allowing for indefinite

preservation if applied successfully. This is accomplished with the use of high concentrations of CPAs that prevent ice formation during high cooling rates. The difficulty with this technique stems from the requirement for relatively high concentrations of CPA when vitrifying, because CPAs become toxic to cells at the concentrations required for vitrification [19]. This toxicity can be reduced through multiple techniques, such as the use of multi-CPA cocktails [20], lower temperature exposure [21,22], and choice of CPA to account for CPA-CPA interactions [23]. One aspect of the previously established vitrification protocol [13] that can be further optimized is the length of the diffusion periods for each of the CPAs.

The diffusion periods (CPA loading periods) in the established vitrification protocol were designed by measuring diffusion characteristics of various CPAs into porcine articular cartilage [24] and applying these values with diffusion models to approximate concentrations throughout human articular cartilage [24,25]. These calculations determined the time required in each step for CPA concentration to reach the final intended concentration. In a further attempt to optimize the CPA loading steps around the diffusion characteristics, Nadia Shardt calculated the minimum time required for each CPA to perfuse the tissue over the span of all of the loading steps [26]. Essentially, the initial loading steps could be reduced because the later loading steps will continue to allow diffusion of initially added CPAs due to the holding concentrations present in later loading steps (see table 4.1). Based on Nadia Shardt's proposed loading steps, experiments were carried out to examine the effect that optimized CPA loading periods would have on cell viability in vitrified and rewarmed human articular cartilage.

Vitrification is a potential method of preserving articular cartilage for transplant, and the optimization of a protocol for this purpose is important. The reduction in both protocol time and exposure concentration of some CPAs will be beneficial to the cryostorage of articular cartilage. It was hypothesized that using optimized exposure periods in the standard vitrification protocol will improve the post-warming viability outcome of the tissue, as well as increasing the utility of the process for practical application by reducing the protocol period to a more manageable timeframe.

MATERIALS AND METHODS

CARTILAGE TISSUE ISOLATION

Articular cartilage was obtained from human cadaveric donors within 24 hours of death. Exclusion criteria based on donor characteristics include standard tissue donation exclusions, as well as a minimum age limit of 16 years old and a maximum limit of 80 years old and the exclusion of cartilage which did not appear overtly healthy. Basic information such as height, weight, age, and whether the patient smoked or had undergone cancer treatment was collected. Tissue was determined to be unsuitable for inclusion into the study based on a visual inspection and a grading on the Outerbridge classification system [27], with tissue that was graded higher than either a 0 or 1 deemed to be of unacceptable quality for inclusion.

Ten millimetre diameter full thickness osteochondral dowels were cored out of the healthy donor tissue using a custom coring device and the dowel was immediately placed in X-VIVO 10 (Lonza Inc., Basel, Switzerland) at 4 ^{o}C for no more than 30 minutes prior to the start of the experiment. Healthy control slices to determine baseline cell viability were

taken from one of two sources, either as slices from each of the experimental dowels prior to the beginning of the experiment or taken from a separate non-experimental dowel cored from the same condyle to be used only as a control.

EXPERIMENTAL PROTOCOL

SOLUTION PREPARATION

Cryoprotectant solutions were prepared using weight/weight mixtures of CPA and X-VIVO 10 supplemented with 0.1 mg/mL chondroitin sulphate (CS, Sigma-Aldrich, St. Louis, Missouri) as calculated in figure 4.1. X-VIVO and CS were mixed until the CS was fully dissolved, next the CPA and X-VIVO/CS were weighed and mixed by stirring at room temperature for 15-30 minutes before being cooled to the required temperature (see figure 4.2). In accordance with the previously published vitrification protocol [13], four solutions were prepared to facilitate a stepwise increase in cryoprotectant concentration as shown in table 4.1. For each dowel that was to be included in the trial (up to 4), 50 mL of each solution was prepared at room temperature and cooled as above.

Figure 4.1 Equations to convert intended molar concentration to weight/weight for solution mixing. Beginning with known molarity and volume, (1) find moles of solute (M=molarity, V=volume), (2) calculate the mass of the solute (m=mass), (3) calculate the volume of the solute (ρ = density), (4) calculate volume of the solvent, (5) calculate mass of solvent, (6) final.

1. $moles_{solute} = M_{solution} * V_{solution}$ 2. $m_{solute} = moles_{solute} * molar\ mass_{solute}$

3. $V_{solute} = \frac{m_{solute}}{\rho_{solute}}$ 4. $V_{solvent} = V_{solution} - V_{solute}$

5. $m_{solvent} = V_{solvent} * \rho_{solvent}$ 6. $\frac{w}{w} = m_{solute}/m_{solvent}$

Table 4.1. Chemical composition and total concentrations of the four CPA-loading solutions used in the standard vitrification protocol

Solution 1	6 M DMSO				6 M Total
Solution 2	6 M Glycerol	2.438 M DMSO			8.4 M Total
Solution 3	6 M (PG) Propylene Glycol	1.625 M Glycerol	2.438 M DMSO		10.1 M Total
Solution 4	6 M (EG) Ethylene Glycol	0.8125M PG	1.625 M Glycerol	2.438 M DMSO	10.9 M Total

STANDARD VITRIFICATION PROTOCOL

The vitrification protocol pioneered previously [13] has been established for use in these experiments as a standard protocol, against which the experimental vitrification protocol below has been tested.

This established standard protocol carefully controls the temperature of the dowel and CPA solution relative to the freezing point during stepwise increases in total CPA concentration, to continually reduce the freezing point within the tissue sample, prior to immersion in liquid nitrogen and formation of a glassy solid. The protocol requires a minimum time of 9:30 hr to complete, consisting of four CPA perfusion steps 90 minutes, 220 minutes, 180 minutes, and 80 minutes in length. The general layout of the protocol can be seen in figure 4.2 (see [13] for further information). The temperature was controlled by carrying out the perfusion steps in an ice water bath (0 ^{o}C) for steps 1 and 2 or an alcohol bath (-10 ^{o}C and -15 ^{o}C) for steps 3 and 4 respectively for the specified times (see figure 4.2), and by pre-cooling solutions prior to dowel immersion. Transfer from one solution to the next was performed by moving both the sample container and the pre-cooled solution for the next

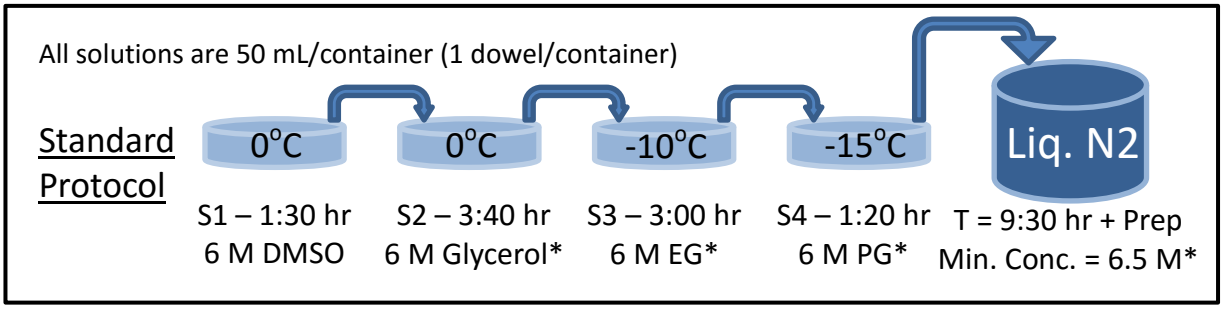

Figure 4.2. Standard vitrification protocol. Dowel is placed in a conical tube containing 50 mL of pre-cooled solution for each stage (S1-4). Solutions are mixed as in table 4.1, with holding concentrations of previous CPAs in every solution after S1 (e.g. S2 contains 6 M glycerol & 2.438 M DMSO). The lower temperatures in later stages reflect a lowered freezing point due to CPA diffusion within the tissue. The time that the tissue is held in the solution for each step is given. All stages are agitated throughout (orbital shaker in S1&2 and alcohol bath stirring in S3&4) and inverted every 15 minutes to prevent concentration gradient formation.

perfusion step to an ice water bath to prevent excessive heat gain, quickly blotting the cartilage with a Kimwipe after removing the sample from the container, placing the dowel in the pre-cooled solution and moving it to the bath to maintain exposure temperature as specified above. The final concentration of CPA in the tissue will reach a minimum value that will allow vitrification to occur with immersion in liquid nitrogen. The concentration present at the bone-cartilage junction in the tissue by the end of the exposure protocol is expected to be 2.4375 M DMSO, 1.625 M glycerol, 0.8125 M PG, and 1.625 M EG (6.5 M total) for a dowel with articular cartilage that is 2 mm thick [13].

EXPERIMENTAL VITRIFICATION PROTOCOL

The experimental vitrification protocol focused on limiting CPA-induced toxicity that arises due to the extended CPA loading periods. Nadia Shardt proposed the loading steps through the use of a 1-D Fick's law based approximation of CPA loading [26]. In her work the

flow of water was not included in the calculation and the diffusion constants used were assumed to be identical to those of porcine articular cartilage [24,25].

Based on a 2.12 mm cartilage thickness model, Nadia Shardt calculated CPA concentration profiles for each step of the standard protocol and together with Drs. Jomha and Elliott used them to determine which steps were eligible for exposure period or concentration reductions. Three modifications were included in the experimental protocol. In the first loading step there was a reduction in the concentration of DMSO from 6 M to 3 M, as well as a reduction in exposure time from 90 to 70 minutes. In the second loading step, there was a reduction in exposure time from 220 to 150 minutes. These modifications combined result in a 90 minute reduction in total exposure time while still maintaining the minimum concentration at the bone-cartilage junction required for vitrification [26,28]. Aside from these three changes, the experimental protocol was identical to the standard protocol.

Sample Warming and Processing

Sample Warming

Vitrified samples were rewarmed following the previously published protocol after a minimum of 12 hours in liquid nitrogen. Sample tubes were removed individually from liquid nitrogen and placed in a 37 ^{o}C water bath for 30 seconds. The tube was constantly moved to ensure even warming and, after removal from the bath, the dowel and molten CPA solution were removed with forceps. The dowel was then removed from the CPA and wiped with Kimwipe to remove any excess CPA. The dowel was washed in 25 mL of X-VIVO 10 supplemented with 0.1 mg/mL CS on an orbital shaker at 4 ^{o}C for 30 min. The dowel was moved to a fresh wash solution every 30 minutes for a total of 3 washes.

SLICING AND ANALYSIS

Following washing, the dowels were stored at 4 ^{o}C for up to 30 minutes during slicing. Articular cartilage slices were cut perpendicular to the articular surface in 75 μm thick slices using a vibratome [The Vibratome Company, St. Louis, MO]. Slices were placed in a petri dish containing X-VIVO 10 + CS on ice to maintain temperature before being stained. Cell viability within the slices was determined by a dual fluorescent membrane integrity assay using 6.25 μM Syto 13 (Invitrogen, Canada) and 15.0 μM propidium iodide (PI; Sigma) mixed in PBS, and viewed under either a Nikon Eclipse E600 Microscope (Nikon Canada, Mississauga, Ontario) with a dual filter (Ex:480 nm, Em:530/640 nm) and imaged using a Nikon DXM1200F Digital Camera (Nikon Canada, Mississauga, Ontario), or under a Nikon Eclipse Ti (Nikon Canada, Mississauga, Ontario) with a dual FITC/TRITC filter (Excitation: 480/555 nm Emission: 520/600 nm) and imaged using a Nikon DS-Fi2 Digital Camera (Nikon Canada, Mississauga, Ontario). The use of membrane integrity staining correlates well with tissue health, as indicated by a study on long-term allotransplant outcomes [5].

The images produced show the stained nuclei of intact cells as green and the stained nuclei of membrane compromised cells as red, allowing for sample quantification. Images were processed using Photoshop (Adobe Photoshop Elements 6, Adobe, Salt Lake City, UT) to stitch photos of each slice and to minimize background noise using the levels function. This served to both increase the homogeneity of image quality as well as make the images more readable by the counting program (identical treatment was applied to every stitched image to reduce background). Images were then entered into a custom program (Viability3,

version 3.2, Locksley McGann, Great Canadian Computer Company, Spruce Grove, AB, Canada) that counted and summarized the numbers of green and red cells in the image. The program output contained the number of intact and membrane-compromised cells as well as the relative percentages of each from the total cell count, the latter of which was used in analysis.

STATISTICAL ANALYSIS

Statistical differences between the standard and experimental vitrification protocol were assessed with a 2-tailed t-test between the average viability of each group. Each of the groups underwent a total of 14 trials (8 after the maximum number of exclusions), and differences were considered significant at $p<0.05$.

RESULTS

Given that there were no exclusion criteria determined at the outset of the experiment, there is no one criterion that can be chosen in the analysis phase. The results are therefore presented below according to four different exclusion criteria to gain a better insight into the existing data. There were no instances where the results demonstrated a significant difference between the experimental and standard protocols, although in the final exclusion criterion listed the difference is approaching significance ($p = 0.072$ with a difference in percent cell viability of 6.03%).

No Exclusion Criteria

The average percent cell viability in the rewarmed samples, normalized to pre-experimental controls, with no exclusionary criteria applied was 13.44 ± 2.72% in the experimental protocol and 9.46 ± 2.01% in the standard protocol samples (p= 0.249). Trends present in this data set include two trials where the cell viability in the rewarmed cartilage was 0% and, of the remaining 12 trials, there were 7 trials in which the experimental protocol viability exceeded the standard protocol by more than 2%, 1 trial in which the standard protocol exceeded the experimental protocol by more than 2%, and 4 trials in which the difference between the protocols was less than 2% (excluding 0% viable trials, see figure 4.3).

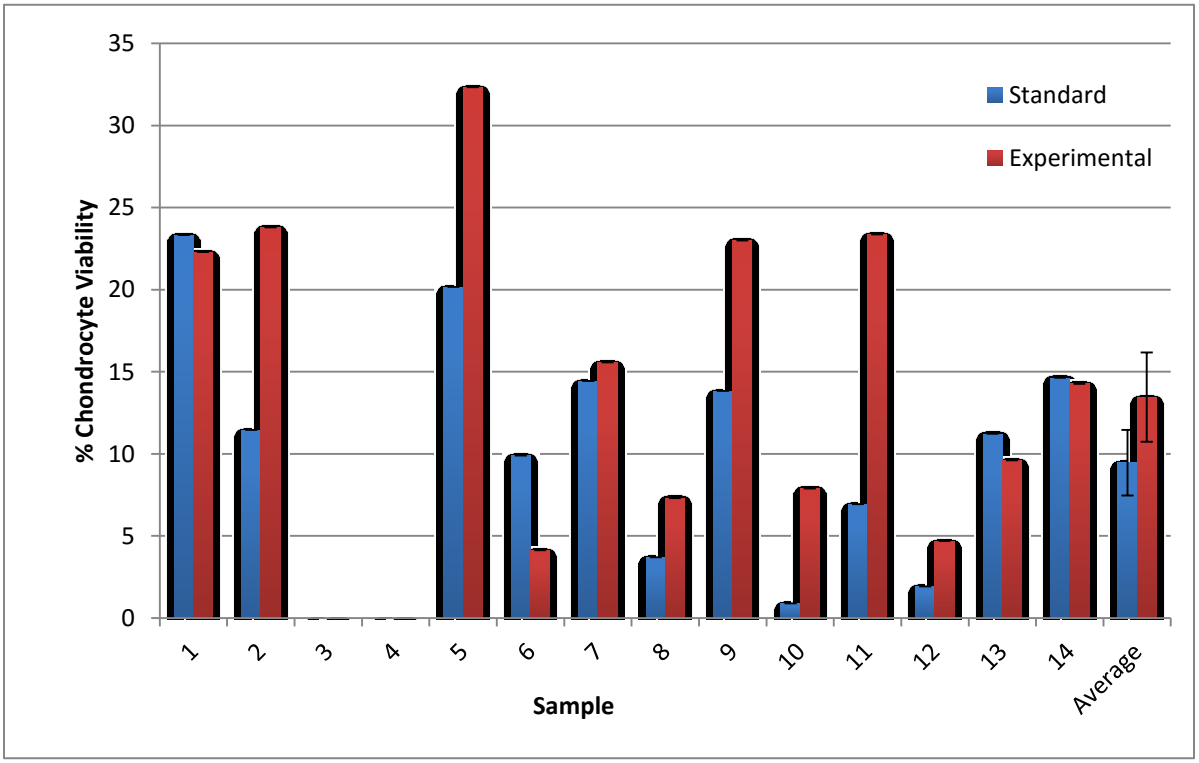

Figure 4.3. Percent cell viability data from all vitrified samples after rewarming (N=14). There was no statistical difference between the standard and experimental protocols (p=0.249, $\alpha = 0.05$) when no exclusionary criteria are used. Values are normalized to pre-experimental controls.

0% Viable Samples Excluded

The average percent cell viability, normalized to pre-experimental controls in the rewarmed samples with the 0% viable samples removed (2 samples removed, N=12), was 15.68 ± 2.72% in the experimental protocol and 11.03 ± 2.01% in the standard protocol samples (p= 0.174). The previously observed trends, aside from those regarding the 0% viable trials, are unchanged using this exclusion criterion (see figure 4.4).

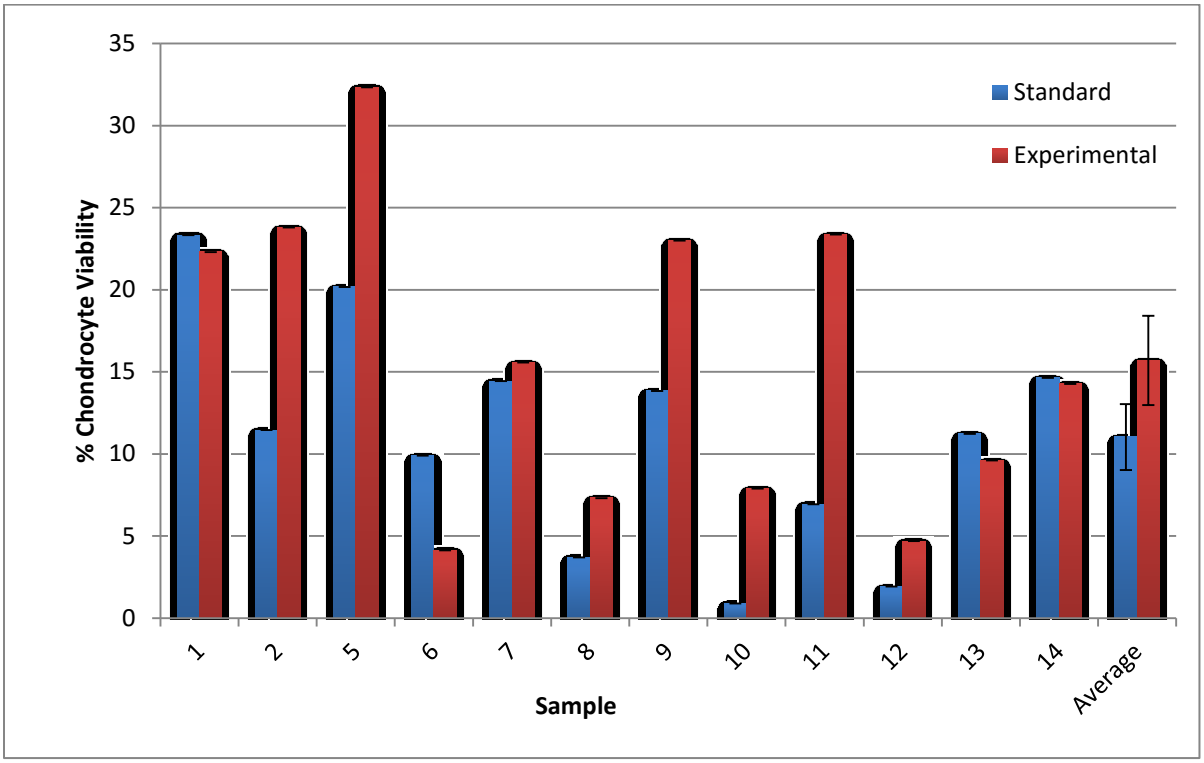

Figure 4.4. Percent cell viability data from 12 vitrified samples after rewarming and removing samples that had 0% cell viability. There was no statistical difference between the standard and experimental protocols (p=0.174, α = 0.05) using this exclusion criterion. Values are normalized to pre-experimental controls.

LESS THAN 5% VIABILITY IN BOTH PROTOCOLS EXCLUDED

The average percent cell viability normalized to pre-experimental controls in the rewarmed samples, with all trials not meeting at least 5% viable in one protocol excluded (3 samples removed, N=11), was 16.69 ± 2.72% in the experimental protocol and 11.86 ± 2.01% in the standard protocol samples (p= 0.164). Using this exclusion criterion, there were 6 trials in which the experimental protocol viability exceeded the standard protocol by more than 2%, 1 trial in which the standard protocol exceeded the experimental protocol by more than 2%, and 4 trials in which the difference between the protocols was less than 2% (see figure 4.5).

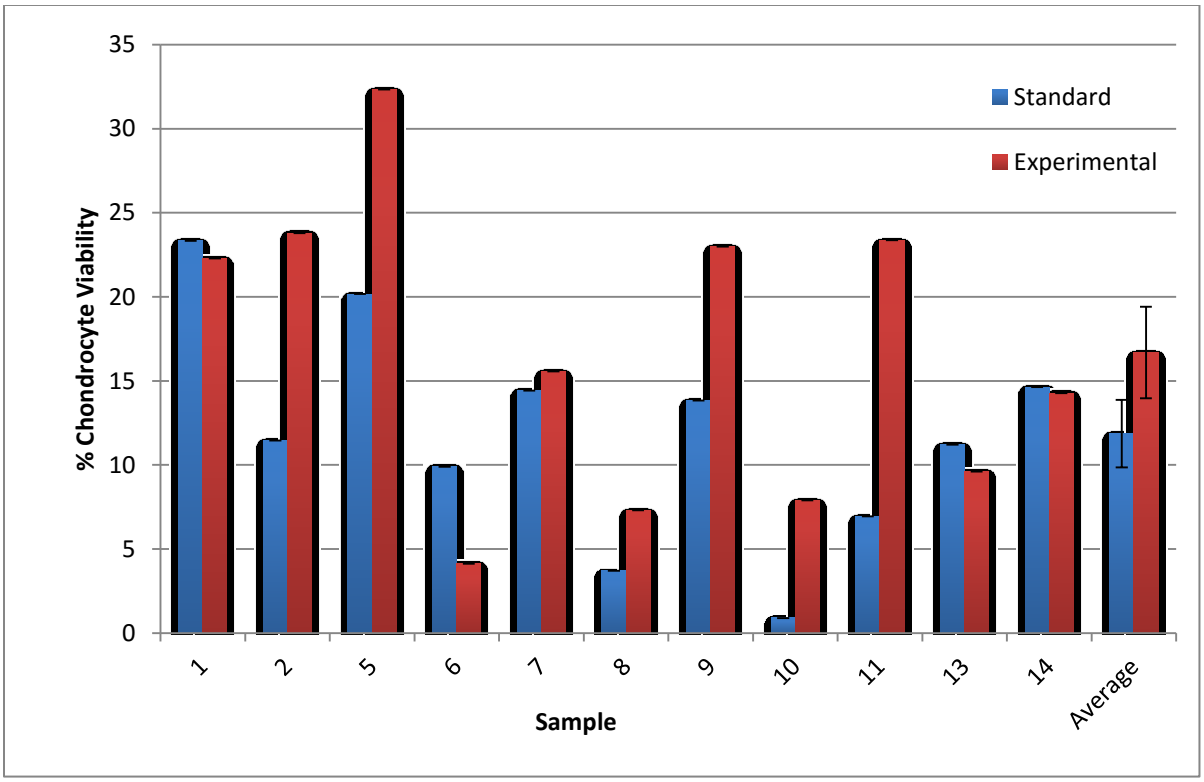

Figure 4.5. Percent cell viability data from 11 vitrified samples after rewarming and removing samples not meeting at least 5% viability in one protocol. There was no statistical difference between the standard and experimental protocols (p=0.164, $\alpha = 0.05$) using this exclusion criterion. Values are normalized to pre-experimental controls.

LESS THAN 10% VIABILITY IN BOTH PROTOCOLS EXCLUDED

The average percent cell viability in the rewarmed samples, normalized to pre-experimental controls with all trials that were not at least 10% viable in one protocol excluded (6 samples removed, N=8), was 20.53 ± 2.72% in the experimental protocol and 14.49 ± 2.01% in the standard protocol samples (p= 0.072). Using this exclusion criterion, there are 4 trials in which the experimental protocol viability exceeded the standard protocol by more than 2%, 0 trials in which the standard protocol exceeded the experimental protocol by more than 2%, and 4 trials in which the difference between the protocols was less than 2% (see figure 4.6).

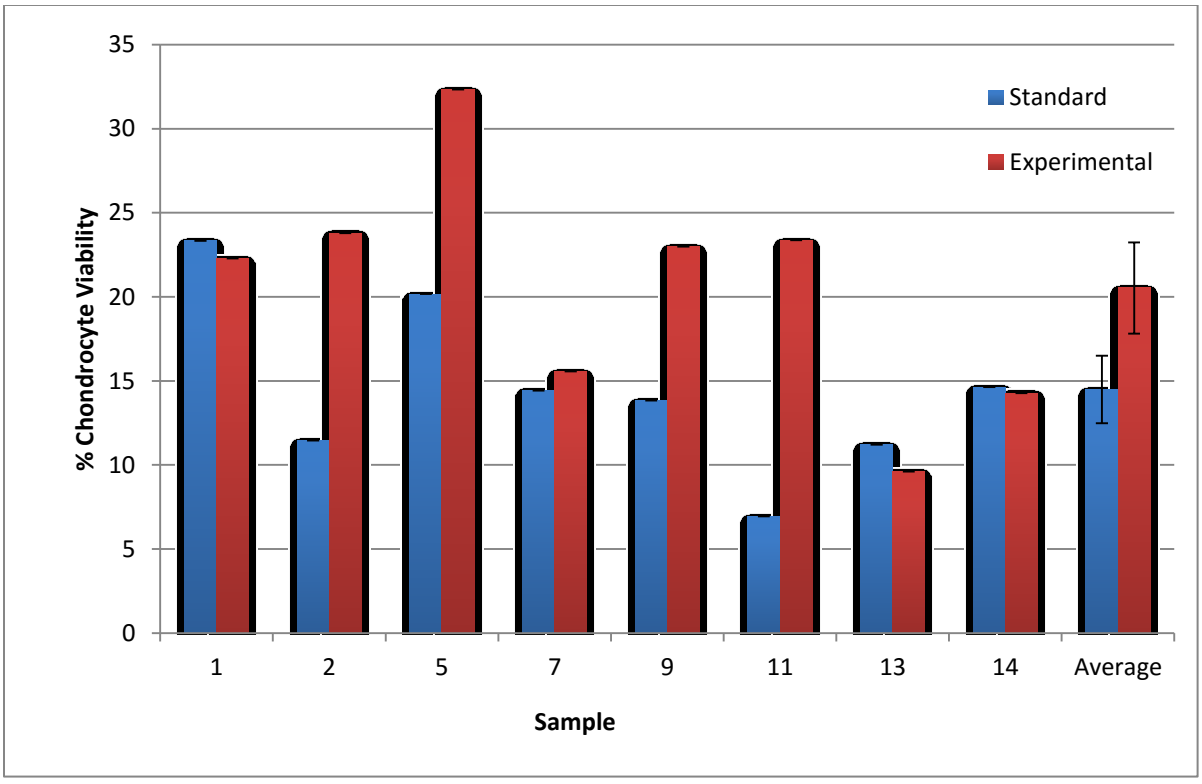

Figure 4.6. Percent cell viability data from 8 vitrified samples after rewarming and removing samples not meeting at least 10% viability in one protocol. The difference between the standard and experimental protocols using this

exclusion criterion was approaching significance ($p=0.072$, $\alpha = 0.05$). Values are normalized to pre-experimental controls.

DISCUSSION

The aim of this experiment was to assess the capability of the shorter experimental protocol to improve chondrocyte viability in post-vitrification warmed articular cartilage over the previously established vitrification protocol [13]. The current data indicates that by reducing the overall time of the vitrification protocol, there is possibility for either an improvement or a reduction in cell viability. The viability was expected to increase due to a reduction in CPA exposure and therefore a reduction in the associated toxicity. However, the viability could also decrease due to incomplete vitrification (the formation of ice crystals occurring during the process) as a result of excessive lowering of the CPA exposure to the point where vitrification no longer preferentially occurs over freezing [28]. An increase in the post-warming viability of one of these protocols over the other would indicate it for use as the primary vitrification protocol for intact human articular cartilage.

One of the most important facets of the obtained data is that the recovered cell viability is much lower than the previously published recoveries [13]. With a previously reported average recovery of ~75% after re-warming, there is a clear difference between the previously published data and the new data sets in this thesis. The data here raises concerns about some unknown problem in the protocol resulting in these low recoveries. This disparity is currently under investigation. Aside from this, the current data was analyzed below to determine if any trends were apparent.

Based on the results in this study it is unclear whether the experimental protocol is outperforming the standard protocol. While the data seems to favour the experimental protocol by a few percent (4-6% improvement depending on exclusion criteria), this difference is not significant enough to make any conclusions but it does suggest that the experimental protocol is not detrimental to cell recovery. That said, the experimental protocol does offer one definitive benefit over the standard protocol, a reduction in protocol time. The standard vitrification protocol takes nine and a half hours to complete without including preparation time. This is a noticeable contrast to the experimental protocol, which only takes eight hours to complete aside from preparation. This improvement is important because it reaches the important "eight-hour work day" threshold such that the same technician can perform the whole protocol, which tends to be technically precise. The intention behind vitrifying human articular cartilage in this case is for tissue banking prior to transplantation. The fact that the protocol is now effectively one working day will be a significant improvement in applicability for any health agencies that would be adopting this protocol for use.

As there was no established exclusion criteria for these trials it is difficult to definitively make conclusions in the data. However, by assessing several possible exclusion thresholds, each justifiable in one way or another, the data can be more adequately described. The first criterion, using no exclusion criteria, could be argued for simply on the basis that any exclusion criteria that are added after data collection has finished are subject to preconceived hypotheses, and are likely biased. The second threshold, that the viability must be greater than 0%, is justifiable with the explanation that a 0% viable sample

indicates either a vital problem with the tissue sample or an issue in the vitrification protocol, neither of which would allow for meaningful conclusions to be drawn about the efficacy of one protocol over the other, regardless of any bias. Both of the final two criteria are extensions of this, as a minimum percent cell viability of 5% or 10% in at least one protocol would also be excluding on the basis that these miniscule viabilities are incapable of being used to model a comparison. While perhaps not as obvious as when compared to the 0% viable samples, at these low viabilities the variability of human tissue becomes much more likely to drive the results.

Once the problem of lower than expected viability has been solved, the next step in this research would be to repeat the experiment to determine if the trend towards improved viability seen in the experimental protocol would become significant when the overall viability is improved, with the addition of appropriate exclusion criteria. Once this has been completed, future work would likely focus on continuing to work toward making the protocol ready to be used for tissue banking. Specifically, this would be to adapt the protocol to the vitrification of femoral hemicondyles rather than dowels as the ability to use larger osteochondral pieces is one of the main advantages to the previously mentioned osteochondral allografting transplantation procedure.

A limitation is that this set of experiments was conducted on osteochondral dowels, and with the intended future application of vitrification as a method to cryopreserve cartilage that is to be transplanted, the use of dowels is limiting. A more useful application would be the vitrification of larger osteochondral pieces, with research currently being conducted in this avenue within the lab.

CONCLUSIONS

In exploring the use of vitrification to cryopreserve articular cartilage, neither the standard nor experimental vitrification protocols provide significantly better viability after warming with the current number of replicates. While the experimental protocol does offer a reduction in protocol time and a slight trend of improvement in cell viability, the generally poor cell viability in both trials does not allow for a definitive choice of protocol to be made from these data. Further trials exploring this relationship are warranted, but should not be conducted until it is discovered why these results are worse when compared to the published viability results using the standard protocol.

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CHAPTER 5: CONCLUSION

An examination of the literature regarding cryopreservation of intact human articular cartilage quickly identifies the ineffectiveness of most current protocols. Traditional cryopreservation methods have proven unsuitable in some tissues, with articular cartilage being one example [1,2]. The only real successes observed in the traditional cryopreservation of articular cartilage in the presence of ice have been in animal models [3,4] and in cell suspension models [5,6]. The use of vitrification has been successfully applied to cryopreserving intact human articular cartilage [7], with research aimed at improving this protocol contained in this thesis.

Focussing specifically on the cell viability in rewarmed cartilage it was observed that there was capacity for improvement in the current vitrification protocol. The first set of experiments relating to the improvement of cell viability, which tested the benefits of various additive compounds in reducing cryoprotectant toxicity, produced several important findings. One finding was that cell death in cartilage continues for at least 48 hours after exposure to toxic concentrations of glycerol, indicating that previous measurements of post-vitrification viability may have been over-estimated due to the measurement being taken shortly after rewarming. The results have also indicated, however, that this cell death can be mitigated by the addition of certain compounds, even though the compounds tested appear incapable of reducing the direct toxicity of glycerol. Specifically, the use of ascorbic acid and glucosamine sulphate have shown to be the most

potent in reducing continued cell death over a 48 hour period following cryoprotectant exposure.

The second avenue of experimentation, focusing on testing a modification of the vitrification protocol itself in an effort to improve post-warmed cell viability, has experienced more complications. The lack of predetermined exclusion criteria in this set of experiments contributed to the results being inconclusive, but beyond this, these experiments have shown a reduction in overall post-warmed cell viability when compared to the previously published vitrification protocol by as much as 55%, potentially invalidating the data. Aside from these clear issues, the data showed that the use of an experimental protocol with reduced CPA exposure period length and concentration was able to produce a marginal increase in post-warmed cartilage cell viability that was at best approaching significance ($p=0.072$) and at worst insignificant ($p=0.249$), depending on the exclusion criteria chosen for analysis. The only conclusive result that these experiments produced is that the experimental protocol that is significantly shorter, showing a 90 minute reduction in protocol length over the standard protocol, is at least not worse than the standard protocol.

The central conclusion from the experiments using the additive compounds is that they have been shown to be beneficial in reducing further cell death after CPA exposure for up to 48 hours. The use of these compounds may be beneficial in a vitrification application, due to the high CPA exposures inherent in these protocols. Due to the previously mentioned problems in the vitrification protocol modification experiments, there were no definitive conclusions that could be made regarding the use of either protocol. The main

conclusion was that by reducing the protocol length by 90 minutes to an even eight hours, the ease with which vitrification can be carried out, both in further research and later in tissue banking applications, is greatly increased. Additionally, if it is assumed that whatever was the source of the lower viability in this set of experiments affected both protocols proportionally, then the use of the experimental protocol would be suggested for use due to a minor trend of viability improvement and the reduction in protocol time.

There were a variety of limitations associated with both of the experiments carried out in this work. In the examination of the effect of additive compounds on CPA toxicity, the majority of the limitations stem from the use of a simple experimental design. The use of only a single CPA in these experiments is limiting in part because there is little information on the toxicity mechanisms of most common CPAs. As such, these results may be focusing on a single facet of toxicity that is unique to glycerol, requiring that future trials with additional CPAs are conducted; particularly if the use of additive compounds is intended to be applied to multi-CPA vitrification protocols. Another limiting factor is that although the experiments were designed to explore the potential use of additives in a vitrification application, the experimental design involved no vitrification or cooling. This limitation is not expected to play a large role however, as cooling itself does not generally pose a significant risk to cells, particularly in the presence of CPAs. Finally, the use of tissue that is sourced from individuals undergoing a total knee replacement, generally for severe osteoarthritis, requires questioning whether the results obtained from this tissue can be extrapolated to healthy tissue. While the tissue used appeared to be healthy, no tests were carried out to directly assess the health of the tissue aside from membrane integrity testing,

and it is known that tissue within a joint that has OA is not entirely healthy [8]. However, in past membrane integrity results have been correlated with tissue health in articular cartilage [9] and since only tissue that appeared normal was used, these results are expected to be applicable to healthy cartilage. The predicted outcome of the use of non-normal cartilage is that the effect of the additive compounds that was seen may be slightly exaggerated when compared to the effect that they would have in normal cartilage. Worth noting as well is the use of a t-test to determine any significant difference. By employing a t-test we have limited the conclusions that can be made of our experimental groups to whether they are improved over the control, there can be no comparison between groups.

Many of the limitations in the experiments examining a proposed optimization of the vitrification protocol have already been mentioned. The generally poor results compared to the previously published protocol constitute the principal limitation to this study. Because the results were appreciably different even in the standard protocol, a definitive evaluation of whether the experimental protocol is an improvement is impossible. As mentioned, to overcome this limitation the source of the low cell viability would have to be located and several trials would have to be conducted to see if the same trend in the data is continued. Aside from this, the lack of a suitable exclusion criteria being established prior to data collection is a substantial limitation as well. The difficulty in establishing exclusion criteria was directly linked to the results in these trials falling far short of the expected values. When the previous limitation has been addressed, appropriate exclusion criteria will be relatively straightforward to decide upon.

With the above limitations in mind, there is potential benefit to further work in these areas. The additive compound experiments are well designed for further expansion to new cryoprotectants and additive compounds. The expectation is that new concentrations, compounds, and combinations of compounds can be tested to find an optimum application for articular cartilage with exposure to a cryoprotectant. Additionally, while the compounds already examined did not show evidence of a reduction in direct cryoprotectant toxicity, future compounds may be able to reduce this damage. The vitrification protocol modification experiments are also appropriate for continued research, as can be seen from the previously detailed poor results. The first and most important avenue for continued research into vitrification protocol modification is the determination and correction of the source of the reduced overall cell viability. As the previous trials produced an average cell viability of 75%, it seems reasonable that at least this level of viability must be reached before meaningful research can continue. Once this is accomplished, there are still multiple questions that can be investigated. After these experimental trials had already begun, an additional protocol was proposed by Nadia Shardt working with Drs. Elliott and Jomha but not investigated due to time constraints. Another future direction to this research is the expansion of the protocol from osteochondral dowels to femoral hemicondyles, which could then be used in a tissue banking application.

The work carried out here is admittedly useful only to a narrow scope of clinical practice, aiming to primarily increase the available supply of donor tissue for osteochondral allografting procedures of the knee. The use of additive compounds in an effort to reduce cryoprotectant damage to chondrocytes is a novel examination and the improvement of the

vitrification protocol that has been examined is unique to this lab. While the applications of the results from this work are currently limited, the fact that the use of vitrification to preserve articular cartilage is still a relatively new and little-researched field means that the possibilities for the future application of this research are many and varied. If the cryopreservation of a complex tissue such as articular cartilage can be mastered, it sets a precedent for the creation of protocols to cryopreserve many other tissues and even organs. It is not difficult to imagine the benefits to the medical field that these advancements would have, and it is hoped that the work carried out here can be expanded beyond the lab and contribute to those eventual benefits.

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