Tissue Preservation Through Cryogenic Methods

Analyzing different cryogenic compounds and methods

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Abstract

Focus: Identifying and solving problems in the integrity of tissue samples after cryopreservation.

The effective cryopreservation of living tissue would be a significant achievement for areas ranging from medicine to space exploration. The current methods for preserving living cells and tissue are problematic due to toxic cryopreservation agents and the crystallization of water. This study used three agents with vitrification of rat smooth muscle tissue rings. The final results were compared against the commonly used 10% DMSO.

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Chapter 1: Introduction

Cryogenics is the branch of physics that studies the effects of temperatures below -150°C; it first appeared as a scientific field in the late 1940s. Rapid progression did not happen until the 1960s and 70s, when the field bloomed in part due to media representation of space travel. As the field gained relevance, one of the areas of interest was the effect of these cold temperature on different living organisms; this branch of cryogenics is now known as cryobiology (Fuller et al., 2004). Despite the advances in the field, a solution to freezing complex, living systems and reviving them to their original state remains a problem without a clear solution. Progress in technology made it possible for scientists and researchers to start exploring new solutions to issues in cryobiology in the early 2000s. Since then, there have been significant discoveries, such as vitrification. Vitrification is the transformation of a liquid to a glass state, often achieved by rapid cooling; a process that has been proposed as an alternative to traditional freezing of organisms. However, the cryogenic process has remained the same.

Some of the problems that affect current methods for cryopreservation include: the crystallization of ice that result in sharp points that inflict damage upon tissue, cells, and organelles, the adverse effects of cell exposure to high concentrations of commonly used cryopreservation agents, and the thermomechanical effects of water expansion upon changing to different physical states (Fuller et al., 2004). In the case of living samples, the area of interest is freezing a sample and returning it to functionality upon thawing.

This research focused on the hazards of cryopreservation and preventative measures. Specifically, we looked at the thermodynamics and state changes involved in cryopreservation to find a robust solution that addresses multiple hazards such as crystal formation and CPA toxicity. The proposed solution involves multiple mechanisms of action, including careful control of temperature changes and the combination of multiple cryogenic agents, partially based on naturally occurring models. To initially test this methodology, tissue rings grown in-lab comprised of rat smooth muscle cells were used in hopes of scaling upwards to more complex systems like organs. Due to the scope of this project, this upscaling in sample type was impossible. However, the observations made and results found can be used in many future experiments for this field.

The long-term goal of this work would be to apply this process to the safe freezing of organs and tissue for transplantation, freezing of wounds that are difficult to treat, transportation of samples from harsh environments, freezing of people whom science may be unable to treat at its current stage, and even long-term space travel.

Chapter 2: Background/Prior Research

Cryobiology, the study of life under frozen conditions, became a field around the 1940's, when a few scientists in multiple fields started studying why some organisms that encountered freezing conditions could survive, while others could not (Fuller, 2004). Studies surrounding this question and the exact mechanisms of freeze injury followed; however, due to the limitations in technology, most of the studies and theories made at that time were mostly descriptive and based fully on empirical evidence; there was very little understanding of the underlying mechanisms of the freezing process. In the 1950s, scientists started to propose that there might be more than one factor creating freeze damage. The discovery of dimethyl sulfoxide (DMSO) in 1959, and more theories regarding the biochemical, biophysical and thermomechanical interactions of living tissue under cryogenic conditions, helped understand the complexity of the issue. Since then, procedures and compounds for cryopreservation have been researched and developed with a relatively good level of success in cells where general integrity of the sample is not an issue. However, there are still many limitations encountered in the biological use of cryogenics, including the difficulty in scalability to tissue and organs. We challenged the standard method in hopes to find a procedure that will more effectively preserve tissue that undergoes the cryogenic process. To do so, we have summarized the prior research and shortcomings here.

2.1 General Background Information

2.1.1 Problems in cryogenics

Cryopreservation is still an unreliable process with low survival rates; it is not currently recommended for tissue and other complex structures. While the standard methods used for cryopreservation work somewhat efficiently for cells, achieving a 75% survival rate under optimized conditions, when applied to tissue there is a large amount of cell death and loss of integrity and function (Mathew, 2018).

The multiple studies done surrounding the freezing of living tissue have brought to light most of the mechanisms that affect its low-temperature preservation as pictured in Figure 1. The quantity and morphology of ice, the location of ice crystals in relation to the cells, the toxicity of the cryoprotectant, the temperature dependence of that toxicity, and the osmotically induced changes in volume are some of the most important and problematic issues (Smith, 1961).

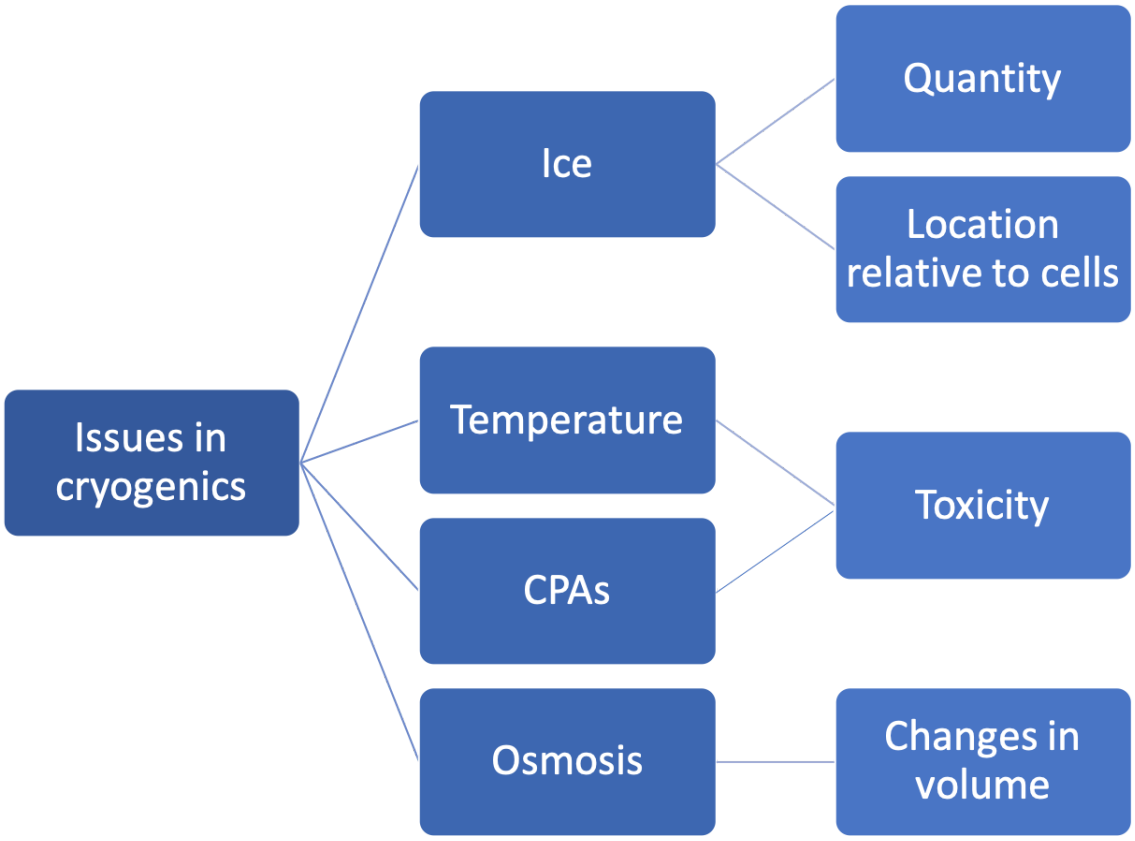


Figure 1. Factors in the cryopreservation process. Factors that affect classical methods for cryopreservation

One of the biggest issues discovered is the presence of extracellular ice. Analysis of samples showed that intracellular ice formation (IIF), which is a major cause of cell death, was usually dependent on the formation of extracellular ice (Luyet, 1937; Luyet and Gehenio, 1940; Hunt et al., 1982; Karlson and Toner, 1996). Multiple solutions were designed to try to chemically and physically limit the formation of ice crystals, with some degree of success (Fuller et al, 2004). However, a solution that limits the formation of ice and preserves functional integrity remains elusive. Many studies using a variety of CPAs under highly controlled conditions conducted by Taylor and Pegg during the 1980s showed that the formation of extracellular ice during slow freezing is inevitable. These experiments were conducted with the standard freezing method, which is reducing the temperature of the sample by -1℃/minute. This proves that the current process of cryopreservation is flawed from the start (Fuller et al., 2004).

The addition of CPAs that cause thermal hysteresis, a temperature difference between the freezing and melting points of a material, to the samples can result in lethal toxicity (Huntington, 2014). DMSO, the most commonly used CPA, causes multiple problems during the cryogenic process. Exposure to DMSO during the standard freezing process can result in toxic damage to the cells and it is not effective enough for satisfactory freezing of large tissue samples (Best, 2015). It is common for the viability of samples to be as low as

50% after initial thawing when using DMSO (J. Kearns, personal communication, December 12, 2018). There is also an increase in concentration as more water is removed from the system via crystallization (Fuller et al, 2004). This results in the possibility of the samples absorbing dangerous concentrations of DMSO, even if the initial amount of DMSO is low. Thawing fast has not been shown to damage the system further, and therefore is the method used to remove the DMSO from the solution as quickly as possible to prevent toxicity (Fuller et al, 2004).

Finally, the phase changes that occur during the cryopreservation process and the changes in chemical equilibrium between the tissue and its surroundings result in osmotic disequilibrium (Muldrew and McGann, 1990). This will inevitably lead to changes in volume, which in turn may be too much stress for the cell or contribute to the absorption of high concentrations of CPAs (Wolf, 1999). As water solidifies outside of the cell, there is a decrease in equilibrium of liquid water inside and outside of the cell and the channels will lose water so that a balance is restored, resulting in the desiccation of cells (Wolf, 1999).

The unavoidable damage makes the integrity of samples, and thus the results obtained from the samples, questionable (Kearns, personal communication, 2018). By finding a more effective way to preserve these samples that is non-toxic, researchers will be able to have a longer lifespan of samples, with less work on those which would normally have to be regrown and maintained daily.

2.1.2 Applicable fields

Applications for tissue preservation can be found in multiple fields, the largest of which is tissue and organ preservation. Vital organs such as kidneys, hearts, and livers that are used in donations cannot be frozen (Kline et al, 2015). This gives a short life-span for donated organs, which is unideal for sudden deaths such as car accidents where a victim is a donor. If these organs could be frozen in-transit and thawed in a reliable state for transplantation, many more patients could receive organs necessary for their survival. This would not only be applicable to extreme cases such as car accidents, but also in stable conditions such as the removal of a kidney from a healthy donor to be given to a patient that is hundreds of miles away. This would reduce the waste of organs and make for shorter waiting-lists. Furthermore, certain tissues, like connective tissue, vascular tissue, and corneas, are more prone to problems due to cryopreservation. This results in low percentages of viability after freezing. For example, cartilage has a 0% to 20% viability after standard cryopreservation procedures (Fuller, 2004). Increasing the viability of frozen samples would lead to more resources and solutions for common medical treatments and research.

Another application of this technology can be found within the International Space Station (ISS). The researchers on the ISS perform experiments with living tissue and run tests that range from microgravity to exposure to elements. The samples from the ISS are then sent to Earth for further analysis. While freezing the tissue will preserve it better than transporting it unfrozen, there are still differences in the initial and end states due to crystal formation or crystal grain damage (Fuller et al, 2004).

Another applications in space aside from transportation of tissues is deep-space travel that is unideal for human life-spans. Cryogenics would slow down or even stop the natural decaying process that the body undertakes over time, allowing travelers to awaken in a similar state as when they departed from the origin location. While this would then raise other problems such as who would awaken the travelers, this research would provide a starting point for human preservation.

2.2 Cryogenic Process

2.2.1 Current cryogenic process

The current cryogenic process implements DMSO as the main cryoprotectant and uses a freeze slow, thaw fast methodology. The sample that needs to be preserved is put into a solution of 10% DMSO with standard cell culture media before being placed in styrofoam or other insulating material to slowly freeze at about -1°C/minute. The final temperature is -80°C. When the sample needs to be thawed, it is placed in a water bath at 37°C and the sample is resuspended and removed from the DMSO solution as quickly as possible. The necessity for speed here is due to the toxic properties of DMSO. While this is not the only procedure for cryopreservation, it is the most common among many cell and tissue lines.

Aside from the toxicity of DMSO, another problem with this method is the slow-freezing of samples. A number of studies by Pegg and Taylor have evidence that standard freezing will always produce crystal nucleation, and as the ice forms from these nucleation points it becomes a hazard to the cells. The only solution to prevent intra- and extracellular ice formation is vitrification (Fuller et al, 2004). Vitrification is the process of supercooling a liquid into the glass state without forming ice crystals. The glass state is amorphous and highly viscous and the method of supercooling is what prevents crystallization, as it does not allow time for the crystals to form by before the liquid passes its freezing point.

2.2.2 Nucleation and Ice Formation

Nucleation is the process in which liquid becomes a solid through an interface (G. Vali, 2014). Liquid water forms ice crystals by the formation of a seed crystal, which is usually in the form of a particle within the water, however sometimes spontaneous homonucleation occurs. Homonucleation occurs when water molecules are clustered and aligned similarly to ice. In heterogeneous nucleation, particles within the water as well as electric fields and pressure waves have an impact on nucleation (Echlin, 1992). The geometry of the particle also influences the nucleation, as molecules that are geometrically similar to the organization of the water molecules are more likely to result in the formation of ice. This is dependent upon the interfacial free energy of the particle.

Nucleation is a time dependent process. The speed at which a sample of water is cooled determines the type of ice that is formed, if any. The phenomenon of vitrification by

fast freezing is discussed in the next section. The alignment of water molecules also plays an important role in nucleation, which is also related to time. If there is a very short time for the temperature drop, water molecules are unable to line up to grow on the crystal seed (Knight, 1967). Additionally, the temperature at which nucleation occurs determines the structure of the ice. If just a section of a sample is undercooled, which occurs in large samples, nucleation occurs at the edges and the crystals penetrate inward (G. Vali, 2014).

So far, laboratory testing and analysis of how ice forms under different conditions has shown a very complex phase diagram for water. On Earth, solid water consists mostly of ice I_h, which means that the common ice crystal is made out of a hexagonal unit cell, followed in much smaller quantities by ice I_c, which has a cubic unit cell. Experiments of water crystallization under different cooling rates and pressure conditions, however, has shown at least 17 different types of ice crystals (Pang, 2014). Due to the nature of the procedures, the main forms of ice that are expected to be found during the cryogenics process are the common ice I_h and cubic ice I_c (Fuller et al, 2004).

Since the current study is focused on preventing any type of crystallization, it is expected we will find mostly vitreous or amorphous ice. Amorphous ice is a particular kind of solid water that is found after it undergoes vitrification. Fast cooling and/or viscosity increments in the solution and the lack of nucleation points due to the transformation into glass result in the arrested state of the water molecules without a particularly shaped unit cell.

The formation of ice outside of the cell can have detrimental effects. When there is ice outside of the cell, the water equilibrium outside and inside the cell becomes unbalanced. This results in swelling of the cell due to increased water concentrations. Following this, there is an imbalance in the ions which results in the pumps becoming passive. Since there is more water that is pumped into the cell, the likelihood of the formation of intracellular ice increases, which results in expansion and eventual rupture of the cell membrane.

2.2.3 Vitrification

Vitrification is the formation of an amorphous, highly viscous, arrested liquid state, also known as glass state (Fuller et al., 2004). In this case, the molecules are not organized enough to form a solid crystal, but the molecules are bound in a way that does not allow for the same freedom of movement as the liquid state (Fuller et al, 2004). The large kinetic restraints that ensue result in the inability for the thermodynamically favored crystallization to occur (Fuller et al, 2004). Since a good vitrification procedure would eliminate all the complications that come from the formation of crystals, it has been suggested as a potential solution to the issues that come up when moving from cells to more complex multicellular systems (Fahy & Wowk, 2015).

While measurements of freezing points and phase transition diagrams have been extensively made, it is unusual for matter to change states at the exact expected temperature. When a pure liquid undergoes its expected freezing point without changing into a solid state due to the lack of nucleation, it is said that it is supercooled (Knight, 1967). This

event can be influenced by many factors, such as purity of the liquid, the rate of freezing and the effect of pressure. Eventually, it is likely that nucleation will happen in the supercooled liquid and lead to crystallization unless it reaches its glass transition temperature before then (Knight, 1967). This is the temperature where the material transitions from the hard and brittle glassy state into a viscous, amorphous state.

Freezing point depression is a separate phenomenon, where a solution freezes below its expected freezing point due to the presence of solutes. This causes the point where the liquid and solid pressures are at equilibrium to be lower than that of a pure liquid, even while in the presence of nucleation (Knight, 1967). The resulting temperature difference between the actual freezing point and the melting point is called thermal hysteresis.

Two approaches were initially proposed in order to properly employ vitrification for the preservation of tissue samples: the equilibrium approach and the non-equilibrium approach (Fuller et al, 2004). The equilibrium approach, suggested by Farrant in 1965, proposed the methodical step-by-step replacement of about 60% of water by a CPA solution like DMSO. This would prevent any freezing up to -70˚C, preventing freeze injury and concentration toxicity. While it was initially proposed to solve the electrolyte problems that occurred during cryopreservation, further experimentation showed that it had a positive effect on survival rates (Elford & Walter, 1972; Taylor, 1982; Taylor et al., 1978). However, further mechanical testing showed that smooth muscle preserved this way failed to provide adequate contractile function (Fuller et al, 2004). It was then theorized that the long exposure to toxic solutes and the lack of enough time for permeation of CPAs before temperature changes were the most probable reasons for the failure of this solution (Fuller et al, 2004).

The non-equilibrium approach, which is currently the most used vitrification process, involves using high cooling rates to achieve the glass state (Fuller et al, 2004). This method has proven to be effective for the preservation of monocytes, ova, early embryos, and pancreatic islets. Ideally, this would result in no biological damaging effects, the possibility of storage without degradation over time, exposure to lower concentrations of CPAs, and lesser contact time with potentially toxic solutes (Fuller et al., 2004). The combination of all these factors would thus make it applicable to all biological systems. However, there are still two issues with this approach. Firstly, the amorphous state of glass exists in a metastable state and differences in cooling rates, storage rates, and CPAs used result in more or less stable conformations (Buittink et al, 1998; Crowe et al, 1998). The less stable the glassy state is, the more likely it is to undergo devitrification upon warming. Devitrification is the process of recrystallization that can happen when a vitreous sample is warmed. Remaining at or fast warming past certain temperatures may result in the spontaneous nucleation of ice crystals or the continous growth of crystals from any ice nuclei that may have formed from non-uniform freezing. The other issue is thermomechanical stresses of the tissue being cryopreserved. Stress created by the constrained contraction of the vitrified tissue can result in the formation of microcracks and fracturing. The former could accumulate and cause integrity failures, while the later would result in immediate loss of integrity of the sample. It should be noted that fracturing of samples usually happens upon thawing (Fuller et al.). The fracturing of samples is a when the tissue continues to contract under cold environments but

is not longer able to due to the surrounding crystals or glassy state, and therefore forms cracks separating the tissue from itself (Alcor n.d.).

Further studies regarding the mechanisms that surround both issues have been done in the hopes of coming up with a solution to these two major problems. A proposed solution to the problem of devitrification is to use an ice inhibitor to reduce the possibility of nucleation. The ideal compound would be able to form hydrogen bonds with the water and prevent it from binding to itself. Several compounds have already been successfully tested for this, such as 1,3 and 1,4 cyclohexanediol, antifreeze glycoproteins (AFGPs), and antifreeze glycolipids (AFGLs). The problem of fracturing and microcracking has so far been avoided by cooling and warming slowly below the T_g and allowing the sample to remain for longer times at certain temperatures (0˚C).

Finally, regular cryopreservation methods are not free of vitrification. Methods that count on lowering the freezing point of water surrounding the samples and supercooling the cells while preventing ice formation end up with the cells in a vitreous state. The increasing concentrations of solutes result in the vitrification of cells in the presence of ice. The mechanisms behind this are a combination of dehydration, cooling and promotion of vitrification by some intramolecular macromolecules (Wolf, 1999). However, it should be mentioned that vitrification and crystallization are not necessarily separate events. The current techniques of standard freezing and fast thawing also result in the insides of the cells supercooling and vitrifying once they reach a certain temperature (Fuller et al., 2004). Furthermore, studies on vitrification techniques have shown that the formation of small, nucleating areas while vitrifying can happen when the water molecules are not properly bonded.

2.2.4 Thawing

As controlled as the preparation and freezing methods must be for the cryopreservation of tissue, the thawing method is equally intensive. When being thawed, cells are susceptible to thermal shock. Thermal shock occurs during any quick temperature change. Even without the presence of ice crystals, this temperature fluctuation can cause cellular lesions that can cause cell death. Thermal shock typically occurs around 15°C to 37°C, but can also occur from 0°C to -80°C (Huntington, 2014). In order to prevent this, current thawing techniques are performed in less than a minute, to reduce the time the frozen cells are in this dangerous range. Lesions caused by thermal shock have shown to be reduced by certain phospholipids, such as phosphatidylserine.

There are two approaches that have been applied to thawing cryopreserved tissue; the standard (rapid) thaw and the checkpoint thaw. The standard (rapid) thaw involves placing cryopreserved cells into a warm culture medium at 37°C to completely thaw the sample in less than one minute. This method is effective and simple, but not immune to thermal shock. To have more control over the behavior of ice crystal nucleation, Fuller et al. have proposed and attempted a checkpoint thaw (Fuller et al., 2004). The general idea behind a checkpoint thaw is that the sample is thawed at a controlled rate and allowed to sit for some time at temperatures related to phase changes. Changing the rates of thawing

between those key points also helps prevent structural damage that can be caused by fracturing and cracking (Fuller et al., 2004).

While a checkpoint thaw grants slightly more control over crystal formation, there is not yet a standard procedure for this kind of thawing and, therefore, there is room for improvement. With standard and checkpoint thawing methods an even distribution of heat is required to ensure uniform thawing, since even slight variations of temperature can lead to unwanted crystal nucleation. One new advancement in the field of thawing is the introduction of magnetic nanoparticles into the frozen sample (Finger & Bishop, 2018). The sample is placed inside a magnetic field when being thawed. The magnetic nanoparticles in the sample all begin vibrating at the specified frequency all throughout the sample (Finger & Bishop, 2018). This creates a very uniform heat distribution while thawing, and has proven to be very successful for samples up to 50 mL (Finger & Bishop, 2018). This technology is not commercially available, as the high cost and large equipment is not attainable in all lab spaces.

2.3 Cryoprotectants and Antifreeze Agents

There are multiple compounds that affect the cryopreservation process commonly known as CPAs. This group of compounds include cryoprotectants, substances that aid the prevention of freeze damage, and antifreeze agents, compounds and proteins that prevent the formation of ice crystals. Cryoprotectants can be further separated by whether or not they are able to penetrate the cell membrane; this distinction helps to differentiate in their mechanisms of action.

Some naturally occuring CPAs include urea, glucose, and xylomannan; however, there are a number of other commonly used and useful CPAs. Penetrating CPAs include, but are not limited to, DMSO, propylene glycol (PG), and glycerol. While PG is not inherently toxic, as it is used in many food products, when implemented as a cryoprotectant it is due to the concentration used. In high concentrations, more than 2.5 M, it decreases intracellular pH (Best, 2015). While this is not the only adverse effect PG creates on cells, it also does not mean that PG is always toxic. Often times with CPAs, they are more toxic to certain types of tissues and cells. One way that the toxicity can be decreased for most CPAs is by combining 20% methanol with 5% of the CPA (Best, 2015). PG can be beneficial as a CPA if sustaining cells is not necessary, such as only needing a low survival rate due to quantity frozen, or it is used in a tissue or cell culture where it is not as toxic. It has one of the highest permeabilities for human oocytes and sperm, and acceptable permeability compared to DMSO for red blood cells (Best, 2015).

The rate of diffusion varies depending on the tissue as well as the CPA. For example, PG will diffuse through pig articular cartilage at a rate that is 50% less than it will for oocytes. Finally, PG commits critical damage to chromosomes, increases calcium concentrations inside the cell, and increases formaldehyde formation more than any of the other CPAs mentioned, making it the CPA to alter samples the most. However, it is one of the more effective CPAs for reducing freeze damage when used in tissue where it is less toxic, like vascular endothelial cells.

Comparatively, glycerol is much less effective at combating ice formation, but substantially less toxic than PG. Glycerol is also less reliable when testing on different samples. For example, it is very permeable to cell membranes in human red blood cells, but not in bovine red blood cells (Best, 2015). It is most effectively used in human sperm cells. It also contains some toxic properties, as it can lead to oxidative stress and renal failure in some specimens, like rats, if the concentration is too high (Best 2015). This compound is best for sperm preservation since it is most effective and least toxic to those cells.

Glucose is primarily used alongside other cryoprotectants, like glycerol, for freezing bovine sperm (Reyes et al, 2002). Its primary function is to prevent dehydration or facilitate non-lethal dehydration and it may also stabilize the plasma membrane (Larson et al, 2014; Reyes et al, 2002). It can be used at low concentrations for systems that are unable to process large amounts of glucose, but is more effective as a CPA when used at higher concentrations (Reyes et al, 2002). The main toxicity of glucose is for systems that cannot process large amounts of sugar at once, which makes this unreliable as a cryoprotectant in large systems, but still usable for small cryopreservation, like with sperm. However, this data is not unique solely to glucose, as many sugars can have these types of effects during cryopreservation, as shown next with trehalose.

Trehalose is a sugar-based CPA that makes cells able to survive the drying process. This is a large issue in cryopreservation as some cells lose water as the outside freezes while others may gain water intracellularly but dehydrate extracellularly (Wolf, 1999). It is most commonly studied in baker's yeast where it not only helps with dehydration, but also prevents damage from compounds such as ethanol, which is produced during fermentation (Fuller et al, 2004). Trehalose is also found naturally in higher plants, not just in yeast, making it more commonly used than CPAs like xylomannan (Fuller et al, 2004). There have also been more studies done with trehalose, including ones using mammalian cell lines and organs (Beattie et al. in 1997; Bando et al. in 1994; Fuller et al, 2004). Fuller et al. have also theorized that trehalose is the ideal candidate for preventing negative dehydration effects in cells. This is primarily because compared to sugars like glucose, trehalose has a much higher hydrated radius causing its usefulness in stabilization of biomaterials (Fuller et al, 2004). Some other beneficial functions of trehalose are having cells maintain the ability to transport calcium, prevent enzymes like phosphofructokinase from being denatured, and keeping liposomes intact (Fuller et al, 2004). While trehalose is not the only sugar than can perform these functions, it has the highest rates of success and is one of the few that can do all of this and more.

Trehalose is safe for human consumption based on a study done with commercial products in Japan (Richards et al, 2002). No adverse effects were found at high concentrations, 10% of the dietary intake, in mice, rats, and rabbits, including tests done on chromosomal changes like what occur with PG (Richards et al, 2002). There were also no negative effects seen in reproduction or offspring that were born from parents taking high concentrations of trehalose (Richards et al, 2002).

There are many antifreeze compounds that can be used to prevent ice formation in cryogenic samples. A vast majority of these are antifreeze proteins (AFPs) and antifreeze glycoproteins (AFGPs). While a large amount of these have been found naturally in

organisms that have adapted to low temperature conditions via ice avoidance, the exact mechanisms they work through and even some of their compositions are yet unknown. However, the limited information available has been enough for some of these proteins to be considered as possible solutions to the preservation of tissue. Furthermore, while the addition of AFPs and AFGPs to cryoprotectant solutions has been shown to reduce the crystallization of water, the mechanism of binding doesn't prove to be effective on all planes of growth. This results in the formation of spicules, long sharp needles, that can result in even more damage.

Scientists have also considered the fact that by chemically binding the water molecules it should be possible to limit the creation of nucleation points and further crystal growth. A study by Fahy in 2001 used molecular modeling techniques in order to identify molecules, now known as synthetic ice blockers (SIBs), that would be able to complement the hydrogen bonding spaces in water. His hypothesis that 1,3,5-cyclohexanetriol and its derivative diols posses the specific conformation, size and shape proved to be correct. However, further testing showed that the triol was too toxic and impractical to use at concentrations above 3% (Fuller, 2004). Trials made with 1,3-cyclohexanediol and 1,4-cyclohexanediol, also known as 1,3 CHD and 1,4 CHD, in combination with other CPAs have shown consistent effective prevention against nucleation during vitrification procedures, even at low concentrations.

It should also be noted that while combinations of different CPAs have proven to be more effective and less (Best, 2015). Correlation between efficacy of mixtures and the number of components have been found in testing, but these vary by cell type and species. A more in depth knowledge of what the mechanisms causing these differences are would result in a more efficient way to determine the correct combination of CPAs.

2.4 Natural Models that use Antifreeze Agents

A variety of natural models exist that show either freeze avoidance or freeze tolerance. Freeze avoidance are organisms that prevent ice from forming around their cells while freeze tolerance is organisms that allow ice formation to happen and survive the process. Organisms with these capabilities range from bacteria to fish to reptiles.

2.4.1 Ice nucleators preventing ice formation

Bacteria that exhibit ice nucleating properties extracellularly can be found in multiple environments (Obata et al, 1999). The controlled nucleation results in their ability to survive under harsh conditions. However, their presence in both plants and other organisms can lead to freeze damage on their host (Orser et al, 1985).

A similar behavior can be found in more complex organisms due to ice nucleating proteins. Tardigrades, insects, and molluscs can produce this kind of protein as part of their ice tolerance mechanism for survival. Similarly, vertebrates that have adapted to cold environments show ice nucleating proteins in their blood, such as some lizards and snakes (Constanzo and Lee, 1996; Obata et al., 1999; Fuller et al., 2004). While the full mechanisms surrounding this method for cryoprotection has yet to be fully understood, it seems likely that

the concentration of nucleating agents in particular areas reduces the total amount of ice growing inside the cells and damaging the integrity of the tissue. While effective for natural models, controlling where nucleating agents concentrate is difficult to induce in other organisms and therefore not an applicable approach for cryopreservation of tissue and organs and will not be used in this study.

2.4.2 Models using non-protein CPAs: xylomannan, glucose, and urea

Natural models such as the Alaskan wood frog give insight into important components for cryopreservation. The wood frogs are a model that are freeze tolerant instead of practicing freeze avoidance, which means that their system is capable of handling complete freezing instead of trying to prevent freezing from occurring. The Alaskan wood frog is similar to the *Upis ceramboides* in that it uses an antifreeze agent that is not a protein yet it acts like one (Larson et al, 2014). Generally, natural models will implement compounds like urea or glucose to assist with freezing, which are readily available in the system. However, the Alaskan wood frog will start secreting an AFGL that is xylomannan-based in its muscles and organs (Larson et al, 2014). Skin, however, does not receive any of this AFGL (Larson et al, 2014). Xylomannan has not been widely tested in other organisms either, so it is unknown if it would be a toxic CPA or even effective when it is not naturally occurring.

Each of these agents have a specific and unique purpose for the freeze tolerance of the frog. Increased glucose and urea in the system prevents desiccation while urea is also a metabolic suppressant. The AFGL binds to the cell membrane to prevent propagation of ice along the membrane and into the cytoplasm. The combination of these effects is what allows the frogs to survive below freezing temperatures and is the basis for the chemicals chosen in this experiment.

Urea as a CPA is only found in frogs, and it is also not universally used for hibernating and freezing frogs (Higgins and Swanson, 2013). However, it is commonly used as a mechanism to resolve stress from imbalances of water in amphibians, including multiple species of frogs (Higgins and Swanson, 2013). The rarity of urea's natural appearance in animals could be due to urea being highly toxic to some species, such as dogs where it will induce anorexia, vomiting, and diarrhea, among other symptoms, that eventually led to coma (Anderson, F, 2005).

In experiments performed with the Alaskan wood frogs, animals living in natural habitats over the course of two years had 100% survival rates, though artificially frozen frogs had lower survival rates of about 50% (Larson et al, 2014). When compared with specimens frozen in lab, the wild frogs had higher levels of glucose than frogs that were artificially frozen (Larson et al, 2014). Both wild and lab frogs endured temperatures to -18°C and it is believed that they could survive past -22°C without any complications (Larson et al, 2014).

The wood frog is not the only animal to use xylomannan; the Alaskan beetle, *Upis ceramboides*, is another natural model containing a xylomannan-based AFGL. These beetles are capable of freezing to -60°C (K.R. Walters, 2009). It is suggested that there is a lipid bond between the xylomannan and cell membrane which protects the cells by preventing extracellular ice from entering the cell and stabilizing the plasma membrane of the *U.*

ceramboides cells (K.R. Walters, 2009). This results in thermal hysteresis, which in these beetles is 3.7 ± 0.3°C at 5mg/mL, and allows for their antifreeze capabilities (K.R. Walters, 2009).

Xylomannan is a difficult compound to synthesize, taking up to seventeen steps (Crich, 2011). Due to the cost and potentially low yield of synthesizing this compound, it is not reasonable for this study. However, the mechanism of how it works in tandem with sugars, such as glucose and trehalose, and urea are a basis of the combination of cryoprotectants chosen for this research.

2.5 Diffusion Models of CPAs in Tissue

Knowledge of the diffusion rate of CPAs through cell membranes and between cells is vital to the successful application of a CPA or mixture of CPAs. The diffusion rate is necessary to know how long tissue must sit in the solution of CPAs and media before it can be frozen. In instances where toxic CPAs are used, the diffusion rate can be used as an elimination factor, since having a slow diffusion rate resulting in requiring the tissue to soak in them for a long time would be counterproductive to successfully freezing and thawing the sample.

A study done in 2009 by Bashkatov and Genina used a one-dimensional diffusion equation for drug transport to determine the rate of diffusion that glucose can achieve in human tissue. They used a variety of methods to determine the diffusion rates, including spectroscopic, photoacoustic, using radioactive labels, and looking at light scattering measurements. The following equation is from the spectroscopic method and uses D as the diffusion coefficient, C(x, t) as the chemical concentration, t for time, and x as the spatial coordinate (Tuchin, 2008):

$$\frac{dC(x,t)}{dt} = D\frac{d^2C(x,t)}{dx^2}$$

Not only is the diffusion coefficient for glucose known, but so is the coefficient for trehalose. In 2015, Abazari et al. did a study on how engineered trehalose permeates through mammalian cell lines, providing the information needed to understand diffusion rates of trehalose. The purpose of their study also focused on using trehalose as a CPA and therefore the results of their diffusion rates directly correlate towards that application of trehalose. Abazari et al. recommend using trehalose hexaacetate for cryopreservation, as the hexaacetate improves permeability through the cell membrane, inducing up to ten times the amount of trehalose intracellularly than extracellularly.

The diffusion models that exist for other cryoprotectants that are being used in this experiment have diffusion coefficients based upon research conducted in aqueous solution and not through cell membranes, and thus will have to be extrapolated for modeled use in cultures such as rat smooth muscle tissue.

2.6 Biomedical Engineering Background and Techniques

When freezing tissue in a laboratory setting, there are specific tests performed in order to determine if the cryopreservation was successful. While the testing varies depending on the tissue type used, cell viability count, mechanical testing, and visual inspection have been tests performed for post-cryopreserved tissues.

To measure cell viability, or the amount of cells left alive after cryopreservation, a technique called an alamarBlue assay is performed. AlamarBlue contains a non-toxic dye called resazurin, which turns from blue to pink in the presence of chemicals released during normal cell metabolic functions. The dye is put in culture with the sample, and the more pink color that is observed after a specified period of time, the percentage of cells left alive (viable) can be calculated. It is a simple and effective way of measuring the survival rate. This can be quantified by creating a standard curve and comparing spectrophotometer results of each test to the standard curve to approximate the number of cells alive.

The mechanical testing performed on cryopreserved tissue completely depends on the tissue type and shape of the sample. It is important to test for differences in mechanical properties, because simple visual inspection does not always show potential structural damage. For tissues like bone, cartilage, or muscle, tensile or compressive tests are effective, giving maximum stress and strain and other useful physiological information. More complex tissues require more specific tests. For example, a study researching the effects of cryogenic preservation on rabbit carotid arteries measured the percent elasticity of the blood vessels, because they believed this property may have been compromised in the process. (Song, 1995) The mechanical testing done on any tissue sample should be closely related to that tissue’s function.

Chapter 3: Project Strategy and Experimental Design

The initial goal of this project was fulfill the following client statement: Develop cryopreservation methodologies to prevent the negative effects of the current process. The main issues with the commonly used method the team chose to focus on finding solutions for were the toxicity of the CPA, the dehydration of the cells during the freezing process, and the nucleation of ice crystals, both intracellularly and extracellularly.

To combat these issues, the team came up with the following design objectives.

Design Objectives:

- Prevent ice crystal formation (intracellular and extracellular)
- Contain no toxic elements for mammalian cells
- Sustain tissue viability post freeze-thaw
- Maintain tissue integrity post freeze-thaw

These objectives, background research, and analysis of our project design requirements helped to reform and focus the client statement to the following: formulate custom cryomedia from non-toxic cryopreservation agents (CPAs) that maintain post freeze-thaw viability and function in living tissue.

3.1 Project Approach

3.1.1 Engineering Standards

In order to discover and develop an approach to create a custom cryomedia and maintain both viability and function, each variable in every permutation of the cryopreservation process must be observed to have reliable findings.

The design of the cryomedia should be reproducible, detailed in the methodology. The cell culture media was formulated by combining DMEM 1X (Mediatech Inc., Manassas VA), GlutaMAX (100X) (Life Technologies Corp., Grand Island, NY), Fetal Bovine Serum, Penn/Strep, and NEAA. Chemicals used included 1-3 Cyclohexanediol (Tokyo Chemical Industry, Portland OR), Hydroxyethyl starch (Santa Cruz Biotechnology, Dallas TX), and D-(+)-Trehalose dihydrate (Alfa Aesar, Ward Hill MA) and DMSO (Mediatech, Inc., Manassas VA) in prepared cell media. Agarose molds were formulated following the procedure laid out in accordance with the protocol defined in Appendix B.3 with Agarose (Fisher Scientific, Fair Lawn NJ) and the previously made cell media. Cell detachment from the plate was achieved by using 0.25% Trypsin (Mediatech Inc., Manassas, VA), which was diluted down to 0.05%.

AlamarBlue Cell Viability Reagent (Life Technologies Corp,. Eugene, OR) was used to determine the viability of cells.

Rat aortic smooth muscle cells, obtained from the graduate labs of Professor Rolle were grown in Celtreat 75 cm^2 Tissue Culture Flask Vent Cap and Celltreat 96 Well Tissue Culture Plates. The Celltreat flasks and plates satisfy the standardization of the sterilization of plates defined by the International Organization for Standardization (ISO), ISO 11137-2:2017 as well as the standard of the cell plate, ISO 24998:2008. The standard of cell counting methods, ISO 20391-1:2018, was followed.

The experiments were performed in a Nuaire Class II Type A/B3 Biological Safety Cabinet. This satisfies the need to have a sterile environment, following ISO 14937:2009.

3.1.2 Experimental Approach

Keeping these standards and project objectives in mind, three design criteria were created. First, the custom cryomedia produced must have similar or better cytotoxicity results than 10% DMSO. Second, the use of this custom cryomedia must results in equal or greater viability values than 10% DMSO. Finally, the custom cryomedia must not impede normal call or tissue function. With these design criteria in mind, the experimental design was tailored to address these needs and find the most ideal CPA solution.

For the scope of this experiment, five individual elements of the cryogenic process were chosen: Sample Type, Preparation Method, Freezing Method, Time Frozen, and Thawing Method. Variables for the elements of the experimental process can be seen in the Table 1 below.

	CPA	Freeze Method	Time Frozen
1	10% DMSO	Slow Freeze	24 Hours
2	10% DMSO	Slow Freeze	48 Hours
3	10% DMSO	Vitrification	24 Hours
4	10% DMSO	Vitrification	48 Hours
5	10% DMSO	Vitrification	24 Hours
6	6% 1-3 CHD + 15% Trehalose	Vitrification	24 Hours
7	6% 1-3 CHD, 6% Hes, 15% Trehalose	Vitrification	24 Hours

Table 1. **Plan to carry out the series of trials.** There are three variable elements; cryopreservant, freezing procedure, and freezing time. There are two tests: the first (rows 1-4) is the control trials to determine significance between freezing method and freezing time. The second (rows 5-7)is the trial using the new CPAs in this experiment.

When picking a sample to test on, both availability and similarity to human tissue were considered. The sample type ultimately chosen to test the proposed cryopreservation method was rat aortic smooth muscle rings. In order to account for delayed cellular death, all testing was performed on the samples 24 hours after the thawing method was completed. For tissue ring samples, an alamarBlue assay was conducted to determine cell viability, followed by a tensile test to measure maximum tensile strength in order to compare to pre-existing data for non-cryogenically preserved samples. The protocol for the alamarBlue assay can be found in Appendix B.1.

Each combination of variables being tested had been given an identifier, T#, denoted in the first column of Table 1. Appendix C contains a list of all combinations to be tested with their own unique identifiers. Each combination was conducted in triplicate for each experiment and repeated on three separate occasions. This was done to avoid errors caused by uncontrollable environmental factors. Samples were prepared, frozen, thawed, and tested in groups based on their sample type, time frozen, and thawing method. A detailed log of group composition and when they were tested can also be found in Appendix C.

The primary control was a commonly used CPA for freezing samples: 10% DMSO. They were used to determine any difference between 24 and 48 hours of freezing along with vitrification and slow freezing. The experimental CPAs, CHD, HES, and trehalose, were compared against samples vitrified with 10% DMSO to test for significance. The remainder of this chapter will serve to explain and justify the variables chosen for each element of the cryogenic process.

3.2 Experimental variables

3.2.1 Sample Type

In order to one day cryopreserve entire organs, the cryogenic process' effect on smaller tissue samples must be understood first. Therefore, for our experiments, smooth muscle tissue rings were chosen with the rationale that by testing on dense tissue, findings will be easily translatable to more complex tissue. Aortic rat smooth muscle cells, when seeded into custom made agarose molds (Figure 2), aggregate into smooth muscle rings (Appendix B.3 - B.4). These rings take around seven days to reach maturity, and can be kept alive far beyond that time point. This resilience and ease of creation led to the decision to use these rings as the main sample type for the freezing experiments. Factors like cryopreservant diffusivity in the sample are harder to control, as it is a three dimensional ring, so data analysis from preexisting experiments provided data for that variable. If a trial shows promising results, that data could then be used for a variety of organ-like samples.

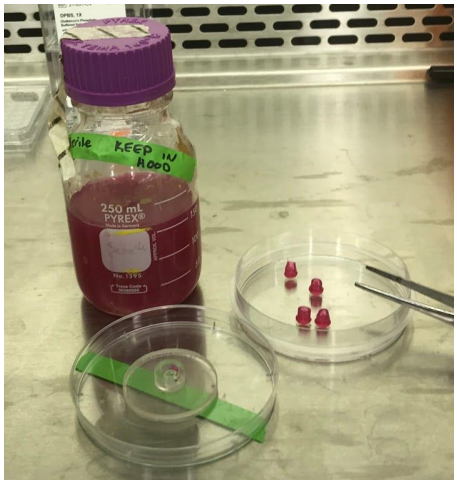


Figure 2. PDMS, agar, and agarose mold for making tissue rings.

3.2.2 Proof of Concept

In order to demonstrate that the proposed CPA solution serves as a non toxic alternative, a batch of cells were grown and exposed to half of the concentration of cryomedia used for freezing the tissue rings for a 24 hour period. The CPA combination will then be removed and the cells flushed with fresh culture media. Viability of the cells will be measured by obtaining alamarBlue readings before the cells are exposed to the CPAs and 24 hours after the CPA solutions have been replaced by regular cell media. This experiment was designed to test the cytotoxicity of the CPA candidates.

3.2.3 Chemical Selection and Preparation Method

The control preparation methods used only 10% DMSO. 10% DMSO was chosen as a control because of its popularity and staple use in current cryobiology research. Thermo Fisher Scientific, a leading biotech company focused on lab equipment, uses DMSO in all cell freezing media sold. Research on antifreeze proteins was performed, however it was decided that this would not be the best approach. Instead, trehalose, 1-3 cyclohexanediol, and hydroxyethyl starch were considered.

Trehalose was chosen as a potentially better alternative to glycerol. In high concentrations, glycerol functions to help stop osmotic stress which leads to cell death (Reyes et al, 2002). Trehalose has been regarded by multiple sources as a leading method of prevention for negative dehydration effects during the cryopreservation process (Fuller et al, 2004). As such, most of its mechanisms of action and properties have been studied; any remaining missing information was extrapolated from glucose.

In order to prevent ice nucleation in both the media and inside the cells, the water binding properties of 1-3 cyclohexanediol were tested. Many tests have been conducted with this agent, and its usefulness in the cryogenic process has been recorded (Fahy, 2001). 1-3 CHD will not be effective alone, so our experimental design paired it first with only Trehalose and then a combination of Trehalose and Hydroxyethyl starch (HES). The water binding properties paired with the cryoprotective properties of the trehalose and the HES were tested. Testing the cryopreservation capabilities of a mixture of CHD and Trehalose, with and without

the presence of HES, was intended to provide more information of the exact effects of each individual agent (Fuller et al, 2004). The molecular structure of all three CPAs can be seen in Figure 3.

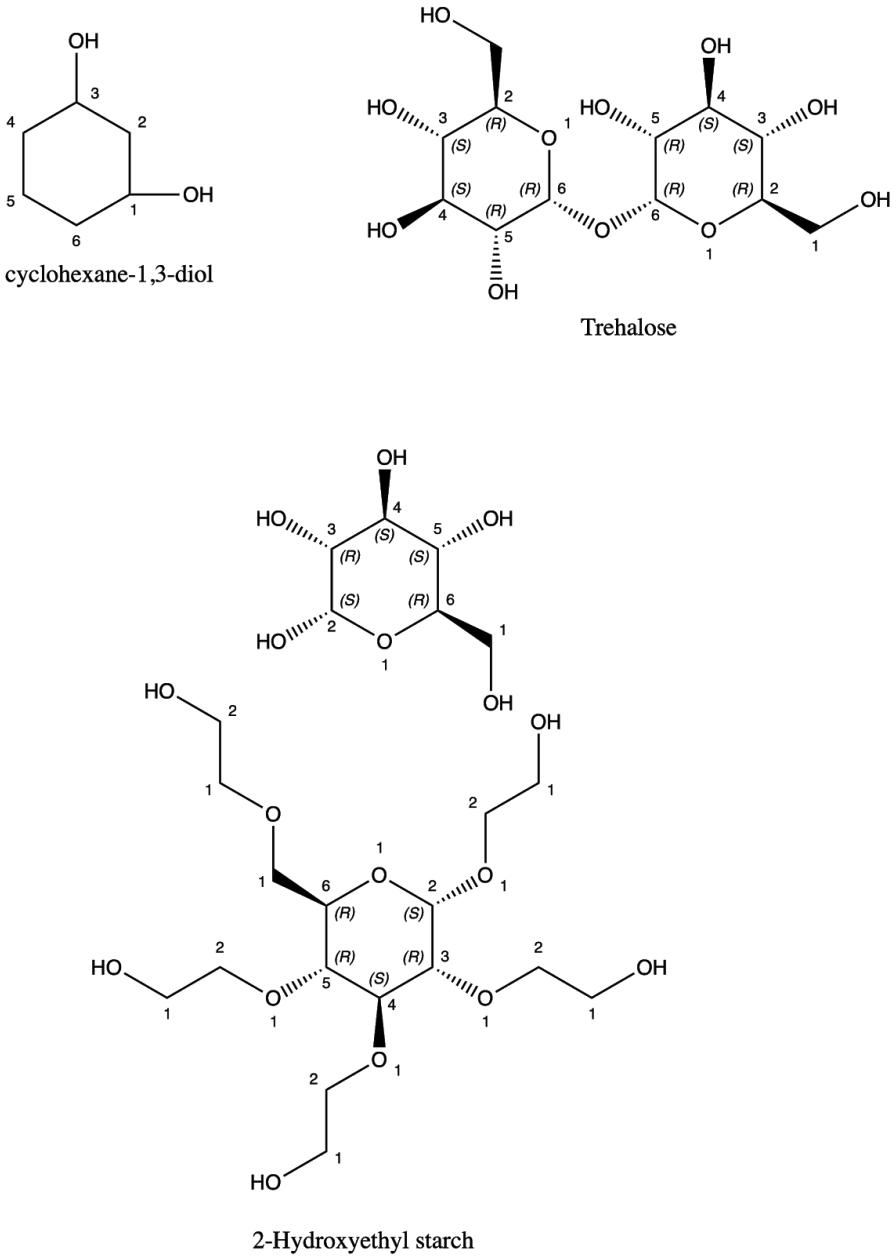


Figure 3. Molecular structures of the selected CPAs. The 1,3-cyclohexanediol used included both *cis* and *trans* isomers of the molecule. Figures made by Alessandra Torres using ChemDraw.

3.2.4 Freezing Method

Two freezing methods were tested: standard freeze and vitrification. Standard freeze refers to the industry standard of using a cooler to slowly chill the sample to -80°C, where it

was then stored (see Appendix B.5). The structure of water molecules when frozen in the different methods can be seen in Figure 4. Vitrification was the freezing method used in the remaining experiments, as it results in a comparable post thaw viability (Achim, 2016). The controls will act to confirm this, and experiments moved forward using vitrification as the freezing method. Vitrification protocol can be found in Appendix B.6.

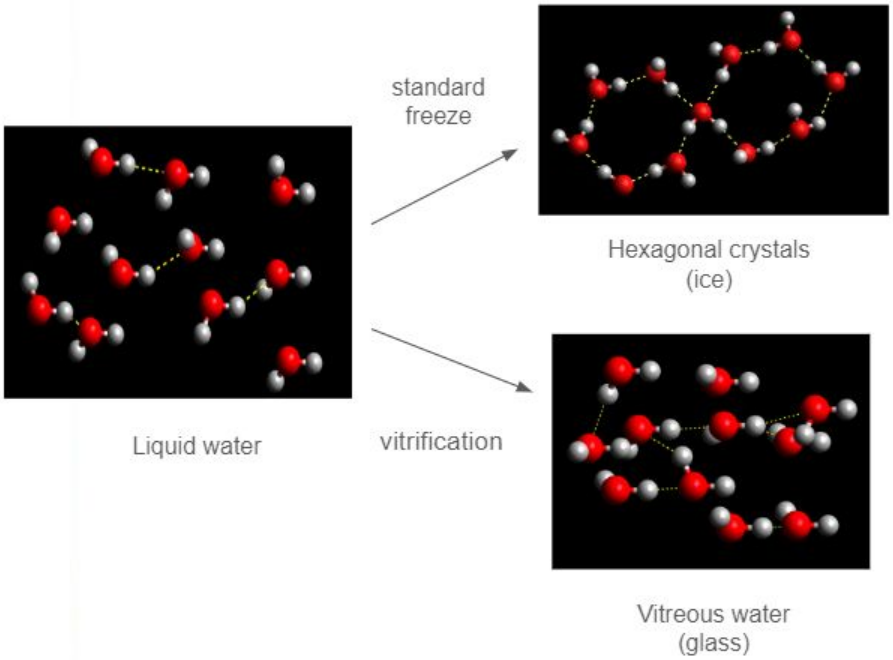


Fig. 4: Resulting H_2O structures of different freeze methods. Standard freeze results in the crystallization and formation of hexagonal crystals whereas vitrification results in water molecules arrested in a glassy state. Figures made by Alessandra Torres using Avogadro.

3.2.5 Time Frozen

The two experimental timeframes chosen to test the controls (24 hours and 48 hours) were chosen with the scope of the overall project in mind. The time frozen refers to the time the sample stayed at the minimum temperature of it's freezing method. For standard freeze samples, the timer should in theory begin 1 hour and 45 minutes after they are placed in the -80 freezer (at a rate of approximately -1°C/minute, the difference between a room temperature of 25°C to -80°C is a 105°C difference and would therefore require 105 minutes); however, due to variations in the use of Mr. Frosty units, we froze the standard freeze for 24 hours before starting the official freezing time. We did not precool the Mr. Frosty at all and inserted our samples at room temperature, which was why we allowed a 24 hour period before starting the freeze timer as other labs sometimes precool between 4°C and -20°C for 24 hours to quicken freeze time for samples (JES 2009). For vitrification samples, rings are at the minimum temperature immediately after submersion in liquid nitrogen. The trials will be frozen for 24 hours in order to complete everything in the allotted time frame, as well as to record the effects of the other combinations of elements. The exact same trials were duplicated and frozen for 48 hours in order to observe if the period of time frozen had

any substantial effect on viability. Once data from the controls was analyzed, only one of these times was chosen..

3.3 Chemical studies

3.3.1 Diffusion

The values we planned to obtain from the equation based simulations of diffusion would be further backed by an experimental measurement of the diffusion through the tissue rings. An initial experiment would be performed to check the influence of the agarose on the diffusion, if it is confirmed that the agarose mold is not the determining factor, a variety of dyes, of similar size to the proposed CPA chemicals, would be used to get an estimated rate of diffusion through the rings. Further gas chromatography (GC) and high pressure liquid chromatography (HPLC) would be performed if data gathered from experimental diffusion is unsatisfactory.

3.3.2 Crystallization studies

In order to prove that the vitrification was taking place as expected, we planned to conduct low-temperature microscopic studies. Identifying the formed ice as hexagonal, cubic or amorphous would ensure that the protocol and chemicals employed were working as theoretically expected. It would also show the presence of microcracking on the crystalline structure. The possibility of modifying the rate of freezing of the sample would help identify transition temperatures that would allow for any modifications in the protocol that would ensure a better result. These experiments were designed to be conducted by adapting a single crystal diffractometer. The solid state could be further characterized by microscopy studies.

3.3.3 Chemical characterization

Since there is little existing literature on the chemical interactions of the proposed mixture, a series of Infrared Spectroscopy (IR) and Nuclear Magnetic Resonance (NMR) studies will be conducted on pure samples and mixtures of the chemicals (HES + 1,3-CHD, HES + trehalose, 1,3-CHD + trehalose). This will help the efforts to have a better chemical understanding of the mechanisms in action during the cryogenic process. The IR graphs obtained from the samples will show peaks of percent light transmission (%T) at different wavenumbers, which can be correlated to the vibrational and rotational motions in the molecules. This in turn can be translated to different bond and structures present and can corroborate that the material we have is what we think it is. The NMR spectroscopy analysis is deeply connected to the atomic spin. The process involved subjecting a sample to a magnetic field, which results in changes in the resonance frequency of the atoms. This study allows for identification of the electronic structure of the molecules, as well as the functional groups that are present on it. The chemical shift and the couplings shown on the obtained NMR spectra should be fairly unique to the molecules employed.

3.3.4 Freezing point depression

Since the original intent was to gather as much information about the action of the chemicals employed in the experiment, an experiment to determine the freezing point depression caused by the CPAs was designed. By slowly freezing mixtures of cell media with the CPAs in different concentrations and comparing their freezing point to that of pure cell media and water we would be able to determine how much the CPAs added help depress the freezing point. Being able to identify the crystallization point and the melting point of the mixtures would enable us to know the amount of thermal hysteresis caused in each case. This should also give some clues to the underlying interactions happening on the mixtures during the process. Ideally, this experiment would be done with a nanoliter osmometer.

Chapter 4: Methodology

Given the broad scope of this project, the experiment was broken down into four separate sections: Tissue Growth & Engineering, Chemical Analysis, Cryopreservation, and Post-Cryopreservation Tissue Analysis. The following chapter serves to explain in detail the steps undergone in each of these sections.

4.1 Tissue Growth and Engineering

4.1.1 Rat aortic smooth muscle cell culture

All protocols for handling rat smooth muscle cells were derived from the Rolle Lab Rat Aortic Smooth Muscle Cell Culture Manual. The cells were plated in a 75 mm^2 cell culture flask with media (DMEM + 10% Fetal Bovine Serum (FBS), 1% Penn/Strep, 1% GlutaMAX, 1% NEAA). Media was prepared in 500 mL batches. Media was changed every two days. After aspirating the old media, 10 mL of new, pre-warmed media was added.

4.1.2 Splitting cells

Once cells were 70% - 90% confluent, they were split. To do this, the old media was aspirated from the culture flask, followed by two washes with DPBS (-). The flask was then trypsinized with 3mL 0.05% Trypsin and placed in the incubator for five minutes. After that time, the cells were checked under the microscope to see if the cells were detached: if not they were allowed additional time in the incubator (approximately one to two more minutes), and if they were 3 mL of new pre-warmed media was added to the flask to neutralize the trypsin. The cell suspension was then transferred to a 15 mL conical tube where it was spun at 200 rcf for five minutes to pelletize the cells. Following that, the media was aspirated and 5 mL of pre-warmed fresh media was added, and the cells were mixed. A count of the cells was then performed using a hemocytometer, followed by the replating of the cells at the desired concentration.

4.1.3 Smooth muscle ring creation

The process of creating smooth muscle rings was derived from the JOVE protocol, which can be found in Appendix B.4. The agarose molds which held the growing tissue rings were formed. The agarose wells themselves were made of a 2% agarose-98% DMEM solution. This solution, along with the PDMS negative for the molds was then autoclaved to sterilize. Once sterile, these components were move to a lab hood. 250 uL of the molten agarose solution was pipetted into the PDMS negative and allowed to cool for approximately 2 minutes. With gentle manipulation, the solid agarose molds were “popped” out of the PDMS negative and placed into a 48-well plate. These fresh molds were equilibrated in the hood overnight in 0.3 mL of media.

Once equilibrated, the molds were ready to have cells seeded into them. A solution of $10x10^6$ cells/mL was prepared for seeding rings and 50 uL of this solution was gently pipetted into each mold. Roughly 0.2 mL of fresh media was placed around the mold, without letting it spill into the mold. This plate was placed in the incubator at 37°C, 5% CO_2 overnight. The next day the excess media was aspirated, and 0.45 mL of fresh media was added to completely cover the mold. This media was changed daily until rings were ready for testing.

4.1.4 Preparing stock solutions

To optimally prepare stock solutions, chemicals were added to the conical tubes first, then pre-warmed media was pipetted into the conical tube. Pipette tips were exchanged with new ones after each new chemical was introduced. Next, the conical tubes were vigorously shook by hand. If the chemicals did not completely dissolve, they were then placed in the centrifuge. No ideal centrifuge speed or time was determined for this process. The only chemical that underwent centrifuging was HES, as it was the most difficult to dissolve. Another method used when vigorous shaking did not work was to quickly pipette the solution up and down to mix the chemical into the media.

4.2 Chemical Analysis

4.2.1 Cytotoxicity and precipitation tests on the CPAs

As the goal of this project was to find a more successful alternative to the commonly used 10% DMSO, all alternative chemicals that showed theoretical potential were tested to check for any adverse effects. To test for this, two experiments were conducted: a precipitation test to ensure the CPA/media solutions had no unforeseen chemical interactions and a cytotoxicity test to ensure the CPA's did not harm the cells or affect cellular behavior.

The precipitation test was conducted by adding each chemical to cell media without cells, then adding combinations of chemicals together with cell media without cells, and also pairing each chemical with cell media and alamarBlue without cells. The control kept for this test was cell media without cells or additional chemicals.The CPA solutions including the cell media totaled 0.9mL for wells including alamarBlue, which totaled 0.1mL. These solutions were plated in a 12 well plate. These plates were then placed in an incubator overnight. The next day, the wells were inspected under a microscope to check to see if any of the chemicals precipitated out of the solutions..

To begin the cytotoxicity tests, the various CPA concentrations were diluted to half of the concentration than would be used for cryopreservation: 3% CHD and HES, and 7.5% TRE. This gives the same ratio between chemicals as the final concentration: 1:1:2. Table 2 below shows which CPA's were tested in this experiment and at what concentrations.

Table 2	CHD	HES	TRE

Solution 1	3%	0%	0%
Solution 2	0%	3%	0%
Solution 3	0%	0%	7.5%
Solution 4	3%	3%	0%
Solution 5	3%	0%	7.5%
Solution 6	0%	3%	7.5%
Solution 7	3%	3%	7.5%

One day prior to the cytotoxicity experiment, cultures were started in 96-well plates. Cells were seeded at 10,000 cells/well and given 24 hours in the incubator to allow for some proliferation and adhesion to the plates. The media from each plate was aspirated, and 3 mL of the CPA solutions were added. These plates were placed back into the incubator overnight. After 24 hours, an alamarBlue assay was conducted as described in the Tissue Analysis section.

4.2.2 Spectroscopy studies

In order to know more about the chemicals used for this experiment, IR spectroscopy of the individual compounds were taken. Comparison of the expected graphs and the obtained ones, will help identify unexpected reactivity between the compounds and fill out gaps in the currently available data.

4.2.3 Freezing point depression

A simple procedure was created for this experiment, due to the lack of access to machinery necessary for a more accurate study. 1ml of cell media, 1ml of water and 1ml of each CPA stock solution were placed on individual cryovials and properly labeled. The cryovials were then placed in the Mr. Frosty and into the -80˚C freezer. The vials were checked every 3-5 minutes to check for freezing and any other visible changes in state, including viscosity. The samples were then analyzed under a microscope.

4.3 Cryopreservation Process

4.3.1 Freezing smooth muscle rings

The standard freeze protocol was derived from “The Freezing of Mammalian Cells,” a protocol used by the Biomedical Engineering Department at WPI. This protocol can be found in Appendix B.5. To begin, using forceps, an agarose well containing a full tissue ring was moved into a cryovial. This cryovial was filled with 1 mL of the desire CPA. If the CPA was 10% DMSO, it was immediately transferred to Mr. Frosty (Figure 5) and put into the -80

freezer. If the CPA was a combination of CHD, TRE, and HES, we waited approximately twenty-minutes for the CPA to soak into the tissue ring before placing the cryovial in the Mr. Frosty and then the -80 freezer.

Figure 5. Mr. Frosty device used in the standard freeze procedure. Cryovials are inserted into the styrofoam container that then insulates it from the freezer. This regulates the sample to not experience shock from freezing too quickly, as it decrease by approximately -1°C/min.

The vitrification method was similar to the standard freeze. Follow the above protocol, but instead of transferring to the Mr. Frosty unit and then a -80 freezer, we placed the cryovials into a liquid N_2 tank. For this study, cryovials were placed in styrofoam boxes and then placed in the bottom of the cryotank, ensuring they were always in liquid N_2 and not in the vapor phase in the upper portion of the cryotanks.

4.3.2 Thawing muscle rings

The thawing protocol was derived from the Rolle Lab Rat Aortic Smooth Muscle Cell Culture Manual. We filled a fresh well-plate with 3 mL of pre-warmed media. Then, the cryovial was removed from the cryotank or freezer and placed in a small beaker filled with warm tap water (~ 37°C). The solution surrounding the agarose mold completely thawed in 1-2 minutes. Using forceps, we transferred the ring and mold into the well-plate and then placed it into the incubator. We allowed it to incubate for 24 hours before starting the test.

4.4 Tissue Analysis

4.4.1 Ring viability testing

An alamarBlue assay was conducted both before and after freezing, and the percent viability was then calculated. The protocol for the alamarBlue assay was derived from ThermoFisher Scientific and was slightly modified to work with our 3D tissue samples. AlamarBlue reagent is pipetted directly onto the rings in the volume of one tenth the total volume of media. This is allowed to incubate for four hours, protected from light by wrapping the plate in aluminum foil. After the incubation period. the samples are then brought to the spectrophotometer, and the absorbance values are read using a 600 nm reference wavelength.

At times, this was conducted by pipetting out the media from the tissue ring into a sterile 96-well plate then added fresh, pre-warmed media into the plates with the tissue rings. This was done so the rings did not have to sit in the media for longer than necessary and it allowed us to pipette fresh media to the rings.

4.4.2 Ring tensile strength testing

The rings were removed from the agarose wells very carefully with forceps and placed on special wire fixtures. Weight was incrementally added and a photograph was taken after each. The total elongation and weight on the sample allows for the calculation of maximum stress and strain on the ring.

Chapter 5: Results

The reasoning behind the experimental series was to test whether the selected non-toxic CPAs and freezing/thawing methodology resulted in viability comparable to that of the commonly used 10% DMSO. In order to test the validity of our hypothesis we conducted a series of experiments, including testing the cytotoxicity of the chemicals used and comparing the viability of the rat smooth muscle tissue rings frozen with each of the CPAs. Workflows of the experimental series can be found in Appendix E. The following sections detail the results obtained from each part of the experimental series.

The completed workflow for tissue ring production is displayed in Figure 6. A more descriptive version of this diagram can be found in Appendix E.1. This workflow diagram for the entire experiment was divided into three sections: preparation, freezing, and thawing. This was due to the length of the workflow along with the different procedures involved for each section.

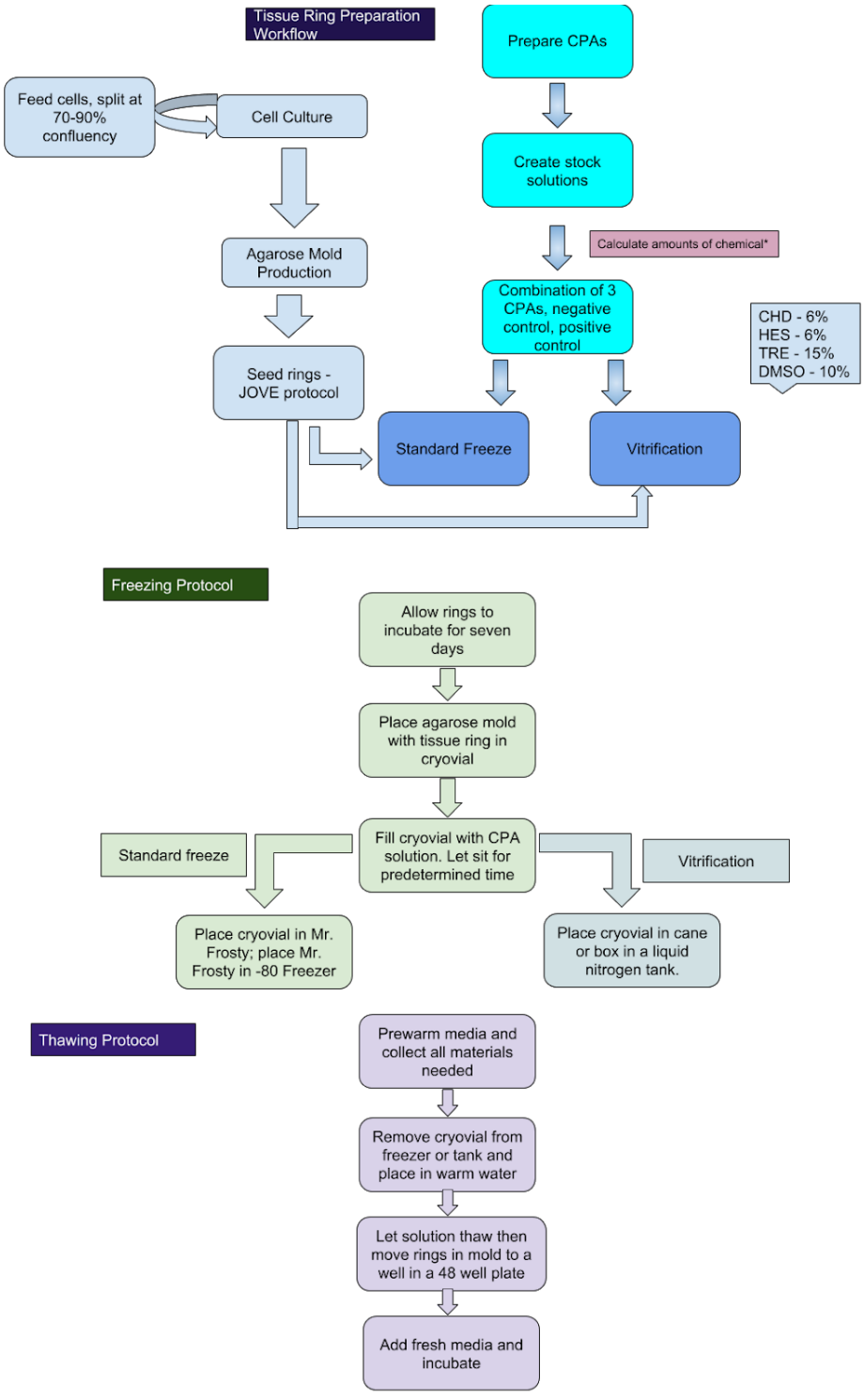


Figure 6. Ring creation, freezing, and thawing workflow diagram. The blue sections shows the tissue ring creation, the green shows the freezing procedures, and the purple shows the thawing procedures.

5.1 Final chemical concentrations and properties

5.1.1 Rationale for final CPA concentrations: 6% HES, CHD, and 15% Trehalose

The concentrations chosen were 6% of HES and CHD and 15% of trehalose in the final mixture. These were all selected due to previous studies and their results. We related 15% trehalose to studies that use glycerol or glucose, notably the study in 2018 by Ahn, Park, and Lim where they studied different concentrations of glycerol and determined that 15% was the most effective. 6% CHD was chosen due to studies done by cryobiologist Greg Fahy in 1995, which showed the reduced ice crystals formed when using CHD at this concentration. 6% HES was selected also based on previous studies of its uses as a cryopreservation agent; while there was not a consensus of what the optimal concentration of HES is, 6% has already been shown to have a positive effect in cryopreservation of blood stem cells (Chan, et al., 2007).

5.1.2 No visible chemical reactions and no precipitates formed on the solutions

CHD, HES, and TRE do not visibly react with each other, cell culture media, or alamarBlue to form precipitates. The reaction between chemicals with each other and cell media was tested by forming stock solutions of the media and letting sit for 24 hours, and the resulting solutions are shown in Figure 7. Then it was tested with alamarBlue by incubating each chemical in cell culture media and alamarBlue for 24 hours, shown in Figure 8.

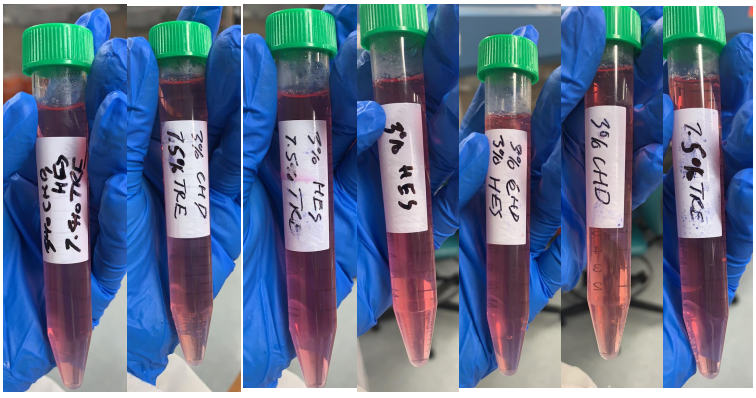


Figure 7. No precipitates in initial stock solution for cytotoxicity. The seven stock solutions prepared for cytotoxicity testing and to also test for precipitates that form when the chemicals are mixed together with cell media. No precipitates formed and the media remained clear.

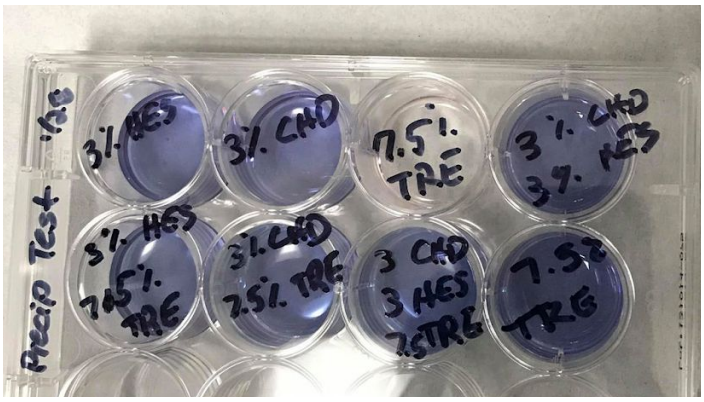


Figure 8. No precipitates formed with CPAs and alamarBlue. The seven stock solutions from Figure 8 mixed with alamarBlue then left to incubate for 24 hours. This test shows that no precipitates form with the alamarBlue and that the chemicals will not turn the alamarBlue dye to pink, and therefore will not change the viability results.

5.1.5 Spectroscopy studies

In order to study the chemical interactions between the selected CPA agents, a series of IR and NMR studies were conducted. This allowed us a better understanding of the underlying reactions happening in the final chemical solution. Because there is limited information on the chemical and physical properties of the CHD and the HES, these studies were particularly important. The IR spectra showed that the chemicals used were largely pure. We also gathered one of the few IR spectra available for 2-hydroxyethyl. The IR spectra can be found in Appendix D.3.

5.2 1,3- CHD, HES, and Trehalose are not more toxic than 10% DMSO over extended exposure in culture

The cytotoxicity of the proposed CPAs, Hydroxyethyl Starch, 1,3-Cyclohexanediol and Trehalose, as well as their combinations was tested to ensure that the proposed mixture was non-toxic to the tissue. The cell culture was exposed to high concentrations of these chemicals for long exposure times to show if the CPAs could be left in thawed samples or if it needed to be removed quickly like 10% DMSO. The alamarBlue assay conducted on the cells after their exposure to the different solutions suggested that the CPAs proposed in this paper had a similar impact on the cells as 10% DMSO after 6-24 hours of exposure. Further testing with lower concentrations and different exposure times to the chemicals should be done. This rationale and other suggestions are included in Section 6.1.1 and 6.2.2.

5.2.1 Visual data of cell culture for toxicity in cryoprotectants

Four cytotoxicity tests were run. A variety of experimental procedures were used among the cytotoxicity tests, and due to this, only one test was pertinent for analysis. The final workflow for the cytotoxicity test is in Figure 9. It shows the steps taken during the finalized procedure to perform cytotoxicity tests with new CPAs. A more in-depth analysis of this workflow is in Appendix E.2. As mentioned on the methodology section, the cells were

incubated for 24 hours in the cell media, then incubated for 6-24 hours on the cell media and CPAs mixtures. Cultures then underwent an AlamarBlue assay for viability.

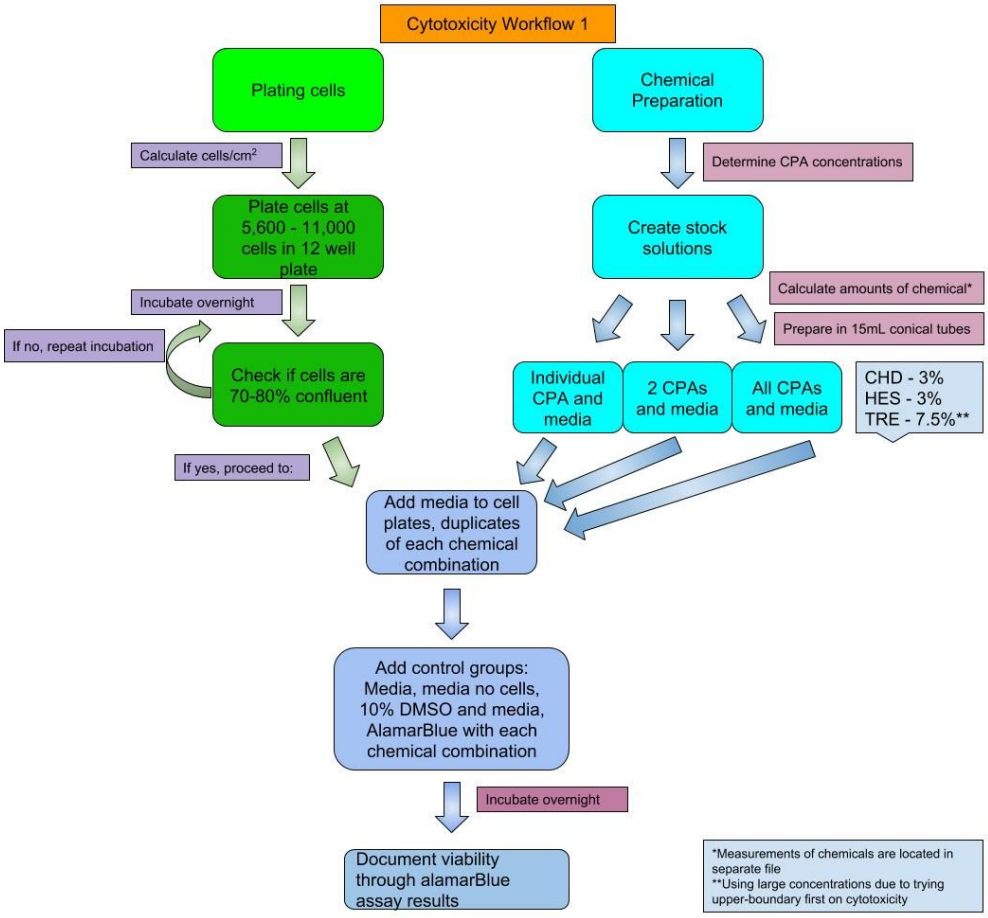


Figure 9. Cytotoxicity workflow diagram.

5.2.2 AlamarBlue results of cytotoxicity culture tests

To test if the CPAs are toxic, cells were cultured for 6-24 hours in media containing half the concentration of the CPA as used for freezing protocols. Then the cells were incubated with 10% alamarBlue for approximately 3 hours before taking a sample of the media and analyzing it at a different facility. The cytotoxicity test was also compared to a standard curve to ensure that the assay was run in the linear part of the curve. Results are

shown in Figure 10.

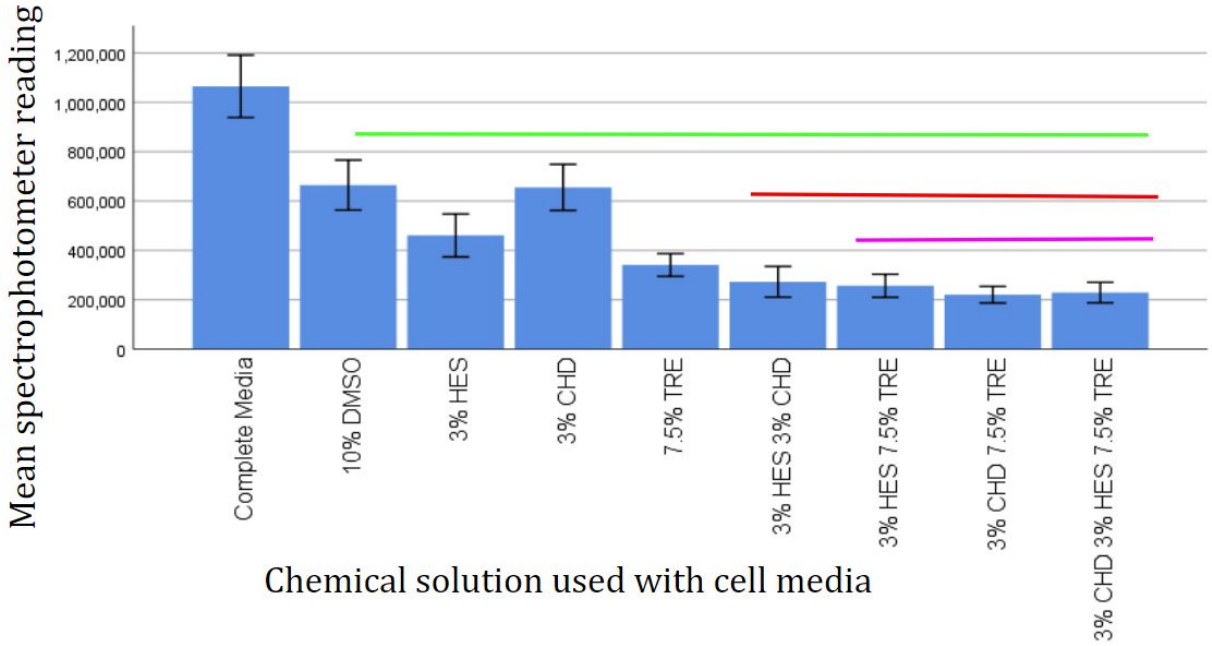


Figure 10. CPAs are toxic at high concentrations in culture. AlamarBlue assays were performed on cells with CPA combinations and concentrations shown. The green line shows significant difference from the positive control, complete media. The red line shows significant difference from 10% DMSO, and the pink link shows significant difference from 3% 1-3 CHD. No other significant differences were seen. Significance was determined through use of ANOVA followed by a Tukey test.

These findings show that the CPAs when isolated are not different from 10% DMSO in terms of toxicity. All samples had significantly lower viability than completed media, and only 3% HES, 3% CHD, and 7.5% Trehalose when used individually were not significantly different than 10% DMSO (Figure 10). However, once any of the chemicals were combined together, they became significantly more toxic than 10% DMSO. This could be caused by the high concentration of chemicals in the culture media along with unusually long exposure times for a CPA solution, and not due to the chemicals themselves being toxic.

5.3 No significant difference between standard freezing and vitrification with 10% DMSO

5.3.1 Visual inspection of tissue rings after thawing

After thawing, most of the rings appeared to remain intact and similar looking to the rings before the freeze thaw cycle. There was no significant visual difference in the thawed rings. The process of removing them from the cryovials resulted in some of the outside of the

agarose walls ripping. This was not considered an issue due to the fact that the central post of the mold was left intact.

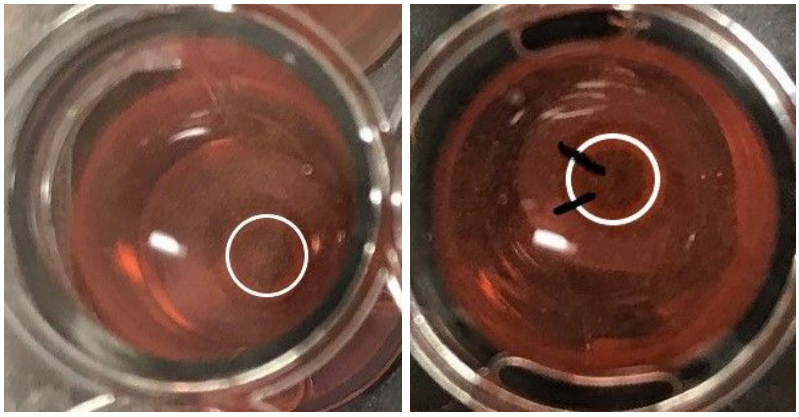


Figure 11. **Structural differences between healthy and damaged tissue rings.** A healthy ring (left) highlighted to show full ring integrity. An unhealthy ring (right) with section in between black lines not solid enough to maintain ring integrity.

5.3.2 Viability results of trials testing freezing methods and time not significantly different

We next examined the effects of proposed freezing methods on tissues, using smooth muscle rings as our model tissue. To compare vitrification with the standard slow freezing protocol for tissues, ring samples were frozen using 10% DMSO as the only cryoprotective agent. Two different freezing procedures were used: the standard slow freeze with a Mr. Frosty in a -80˚C Freezer, and vitrifying by submerging the cryovials in a LN_2 tank. Additionally, samples were frozen for both 24 and 48 hours in order to determine whether the time frozen made a difference on the results. The viability measurement was determined by conducting an alamarBlue assay directly before freezing the tissue rings and 24 hours after thawing the tissue rings. The data shown are post-thaw viability normalized for pre-freeze viability by dividing the post-thaw value by the pre-freeze value (Figures 12).

There were no differences in tissue ring viability when comparing 24 hour to 48 hour freezing time and vitrification to standard freezing methods. Therefore, it was determined that vitrification was as equally powerful as standard freezing when paired with a CPA, and that 24 hours was a long enough freeze time to gather results for future tests. However, there was significance across each trial performed, and therefore the data is graphed by trial in Figure 12 instead of averaged together.

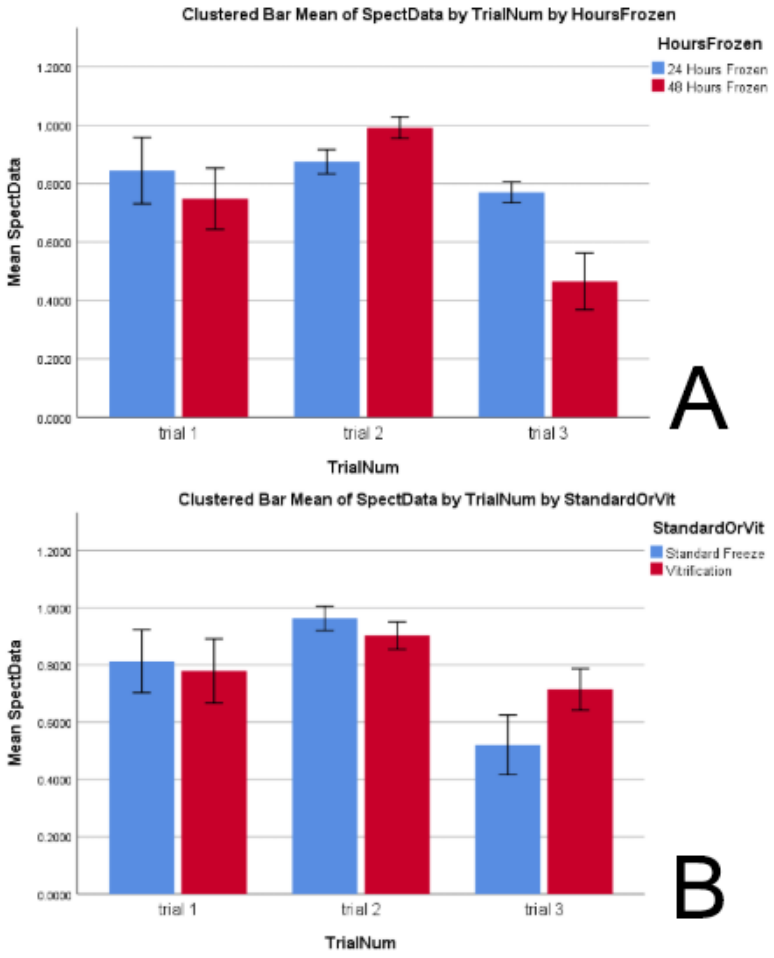


Figure 12. alamarBlue results shown by trial for comparison of freezing time and method. Tested for difference between freezing for 24 hours and freezing for 48 hours along with a difference between vitrification and standard freezing using the same data set. There was no significant difference for freezing times or freezing methods, but there is significance between the trials, therefore the trials have not been averaged together. To determine significance, an ANOVA test was run followed by a Tukey test.

5.4 Post-thaw data of experimental CPAs was highly variable

Tissue rings grown in agarose molds were vitrified for 24 hours. They were then incubated for another 24 hours before viability tests were conducted. Viability was determined by taking the ratio of a pre-freeze and post-freeze spectrophotometer reading after incubating the tissue rings with 10% alamarBlue for 4 hours.

5.4.1 Visual inspection of rings frozen and thawed in HES, 1,3-CHD, and Trehalose

Similar to the control rings, most of the thawed rings appeared to be intact and similar to the pre-freeze thaw rings. One thing that differed was the finding of the destruction of the agarose molds of some of the samples, which is discussed in the next section. The deteriorated molds made it difficult to locate the rings, as they were not sitting in the well of the mold and were rather amongst the pieces of the broken-down agarose. In addition, the media in these wells was rather cloudy. The rings in the molds that were not destroyed were

intact. Again, like the control, removal of the rings from the cryovials managed to be a difficult task that sometimes resulted in the tearing of the outer wall of the mold. The center posts of the molds were not disturbed, however, so this was not an issue in these samples.

5.4.2 Reaction of Agarose with Disaccharides

While extracting the tissue rings frozen with the custom CPAs, there were instances of the agarose molds falling apart while being grabbed with the forceps. Some of the molds also appeared to be in pieces before being handled by the forceps. This had been seen previously in the control series with 10% DMSO at a less frequent rate. The molds that had been affected with 10% DMSO were still more structurally stable than the samples affected by solutions including 15% trehalose. This showed that trehalose and other disaccharides interact with the sugar in agarose to dissolve it, which is consistent with other researchers' findings (Russ). Disaccharides like trehalose have an effect on hydrocolloids which increases the residual moisture content (Russ).

5.4.3 Custom CPAs post thawing shows inconclusive data

The viability ratio was plotted in a scatter plot to show the distribution of each CPA composition used: 10% DMSO as the negative control, 6% CHD and 15% Trehalose, and 6% CHD, 6% HES, and 15% Trehalose. As seen in Figure 13, the distribution for the negative control had high variation, ranging from a ratio of 0.169 to 1.15. Due to this variation, the data was not analysed using any statistical method, and was determined to be inconclusive. Reviewing and performing this test in the future is recommended for any significant results to be obtained.

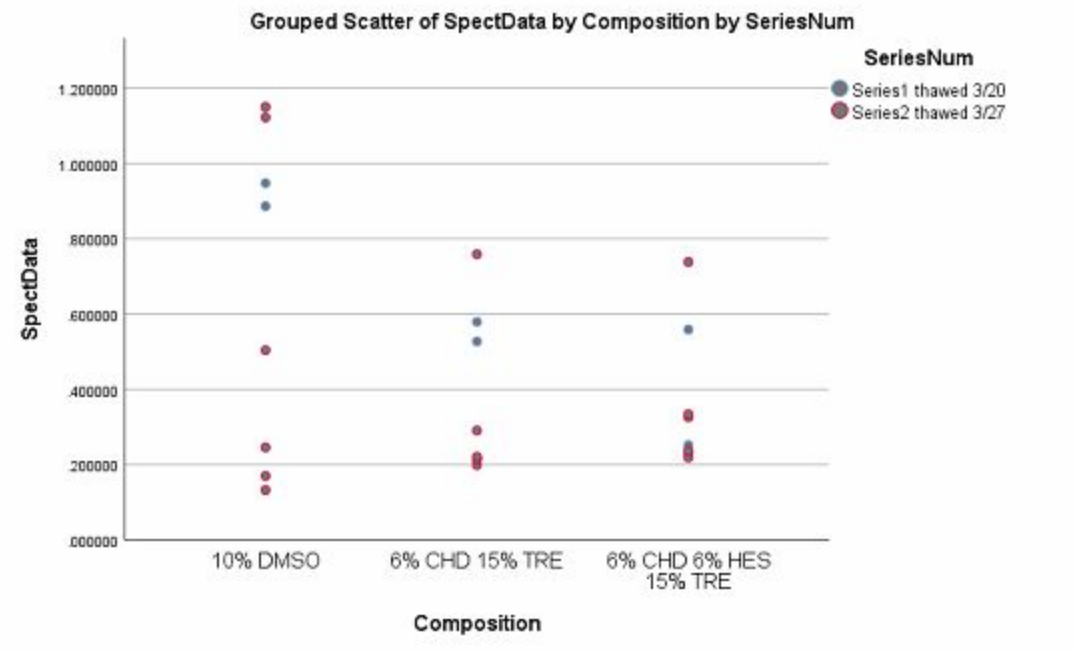


Figure 13 . Scatter plot depicting inconclusive custom CPA viability results.

5.5 Freezing point depression of cell media due to CPAs

5.5.1 Cell media mixtures containing only CHD and Trehalose froze after the mixtures with the CPA combinations

The experiment to determine the freezing rate of the different mixtures showed that the cell media combination with 3% CHD and the one with 7.5% Trehalose froze much later than the mixtures that contained the 3% HES as well as the combinations (3% CHD + 7.5% Trehalose, 3% CHD + 3% HES + 7.5% Trehalose, 6% HES + 6% CHD + 15% Trehalose). This result was unexpected, since the freezing point depression of the mixture is usually directly affected by the concentration of the foreign substance on the mixture (in this case the CPAs in the cell media). The order in which the different mixtures froze can be found in Figure 14. The mixture with the concentrations used during the tissue freezing experiment froze before the 3% CHD, the 7.5% Trehalose and the 10% DMSO mixtures.

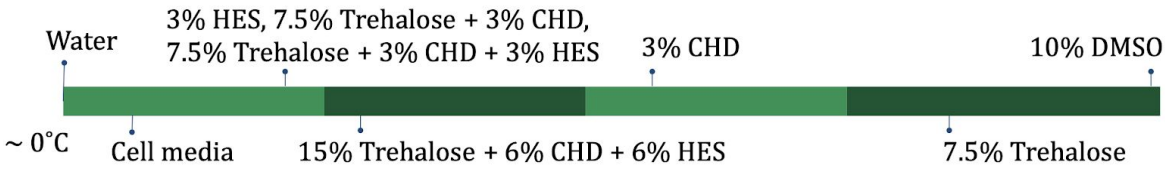


Fig. 14. Order in which the CPA mixtures froze during the freezing point depression experiment. A significant amount of time passed between the 3% CHD freezing and the 10% DMSO freezing. Each color change is approximately 10 minutes.

5.6 Different structures across antifreeze proteins

Antifreeze proteins (AFP) were another method analyzed to find a molecule to use in a CPA solution due to their natural occurrence. While no antifreeze protein has been confirmed to be universally effective, a few similarities have been noted. In Figure 15, the snow flea, mealworm, and ribbed pine borer beetle all show a condensed, repeated structure. While they each have different secondary folding, their tertiary structures are similar and further research into these proteins would be needed to confirm their similarity and if they are usable in other organism. A possible method to determine this is to continue working with the models from UCSF Chimera, to find overlapping domains, mechanisms of action specifically relating to antifreeze properties, and highly conserved regions for each species.

We selected a small sample of five different organisms that had an AFP to show the differences between their structures, specifically size and secondary folding. The first three proteins are relatively small while the last was rather large and has quaternary folding. The snow flea AFP has no alpha helices or beta sheets, the mealworm AFP was mostly alpha helices, and the beetle was mostly beta sheets. Despite this non-conservation of secondary

folding, all of these AFPs work to prevent organisms from dying due to low temperatures, indicating similarities may lie elsewhere.

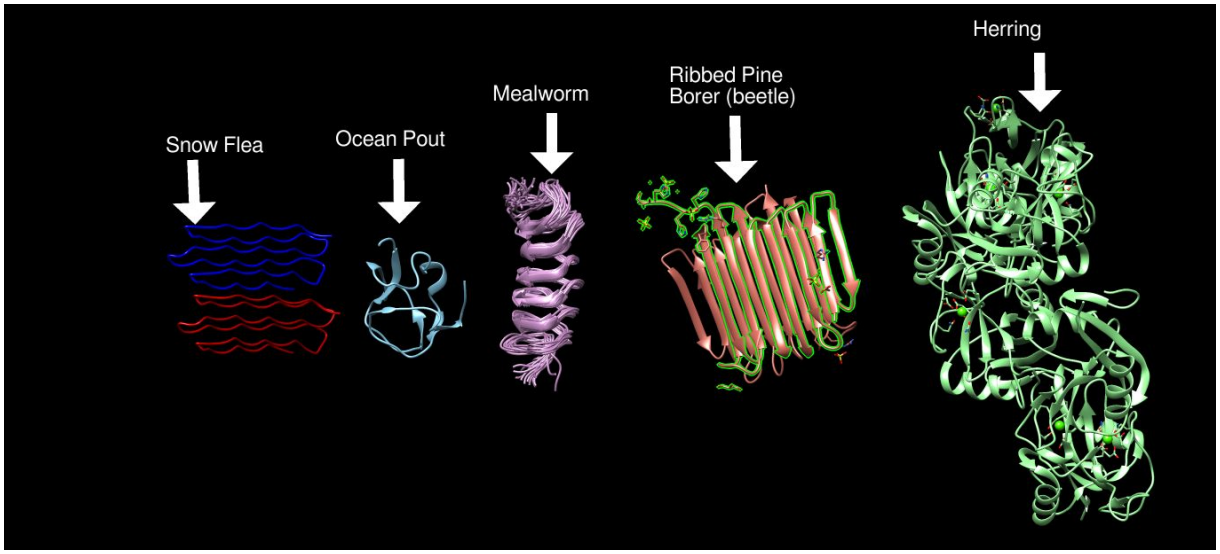


Figure 15: Lack of structural similarity between antifreeze proteins modeled in Chimera. PDB files of the antifreeze proteins from 3BOI (Snow Flea, red and blue), 5MSI (Ocean Pout fish, light blue), 1L1I (Mealworm, purple), 4DT5 (Ribbed Pine Borer beetle, pink), and 2PY2 (Herring, teal) are shown here to display the variety between antifreeze proteins. No manipulation or comparison between proteins were conducted except visually. Models visualized in UCSF Chimera by Alicia Howell.

5.6.1 Insulin-like growth factor with antifreeze properties

Unlike the previous models, *C. elegans* are freeze tolerant because of a specific allele they carry, which also means that not all *C. elegans* are freeze tolerant. This allele is *daf-2(e1370)* and is categorized as an insulin/insulin-like growth factor (Hu et al, 2015). A search using UniProt shows that the closest related animal between humans and *C. elegans* to have this specific gene was mice, and there are no conserved regions. The *C. elegans'* protein was 1,846 amino acids long, whereas the mouse was 407 amino acids. Humans share a similar gene to mice, but the protein was even less similar to that of the *C. elegans* since it was 381 amino acids long. It is likely that this gene in humans and mice is solely for insulin purposes, while in *C. elegans* it is a multifunctional protein, coding for other attributes such as life span (Hu et al, 2015). Figure 16 shows the structures for the protein in each of the three species and their structural differences.

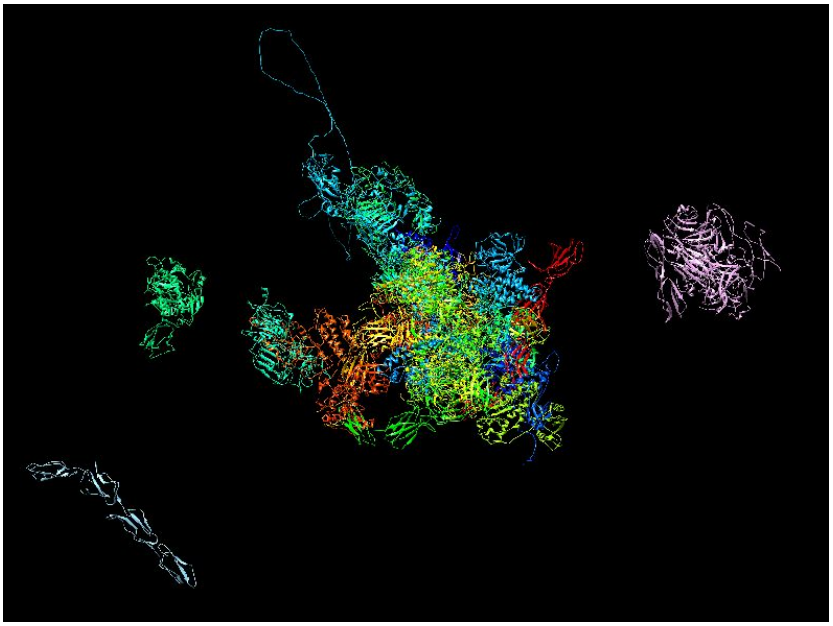


Figure 16 . Lack of structural similarities for the daf-2 protein in a mouse, human, and C. elegans. Taken from Chimera with fetch IDs of Q61476.1 from modbase for the mouse protein (blue, bottom left), Q968Y9 from modbase for the C. elegans protein (multicolored, center), and 1UPN from PDB for the human protein (purple, far right). Models visualized in UCSF Chimera by Alicia Howell.

Chapter 6. Discussion and Final Design

This project was started with the intention to come up with a mixture of cryoprotectant agents and a freeze-thaw methodology that would improve on the commonly used 10% DMSO. The goals for the CPA mixture were: to prevent the formation of ice crystals during the freeze-thaw procedure, to be non-toxic to the tissue, and to maintain tissue integrity and viability. The final design will be described in detail in this chapter.

6.1 Analysis of experimental results

This project took the first steps towards designing a novel cryopreservation method. The results from the experiments performed during this study can be used as a foundation for future work in the field. The most important conclusions are discussed in the following sections, as well as how they impacted the final design.

6.1.1 Cytotoxicity results show that individual CPAs no more toxic to cells than 10% DMSO

The cytotoxicity tests performed suggested that at the concentrations tested there were some toxic effect on the cells in suspension after 24 hours. However, the mixtures that included each CPA on their own seemed promising, since none of them were significantly more toxic than 10% DMSO. Further research with a shorter time of the media in the cells could be conducted, as a hypothesis we had was that the cells exhaust the media at a faster rate than we were replacing it, since there were lesser amounts of media due to the high concentrations of chemicals. Additional research can be performed using the tissue rings rather than cells, as there was a greater density of cells in the rings which may affect how the chemicals react. Variation of the CPA concentrations is also a possible solution, and the easiest route to test for following experiments.

The results gathered in this experiment were not what originally hypothesized; all of the combinations of CPAs had significantly less cell viability, indicating that it was negatively affecting the metabolism of the cells in media which was likely due to the death of the cells. The interactions between the chemicals in the solutions making an impact on the toxicity of the mixture is still a possibility. Further studies on these particular interactions should be conducted in order to come up with a better combination.

6.1.2 Comparison of slow freeze and vitrification with 10% DMSO show no significant difference between methods

Slow freezing and vitrifying with 10% DMSO resulted in similar results. While this was originally intended to be a control model for the rest of the experiments done on tissue, it also aided us in proving that vitrifying the samples was a viable solution. The results obtained from these tests can also be used as a basis for further experimentation. Following the

results obtained in the freezing point depression experiment, which is analyzed in more detail in the following section 6.1.3., more research was conducted on the specific mechanisms and interactions of DMSO and water. A study conducted by Kirchner and Reiher in 2002 shows that DMSO-water clusters were formed when these compounds are mixed. This accounts for not only a significant freezing point depression, but also results in a predilection to form amorphous water crystals (Havemeyer, 1966). Since this was the intended purpose of the water binding CPAs employed in our study, it would also be possible to replace them with DMSO. Designing and running experiments to determine the minimum amount of DMSO necessary to prevent nucleation during vitrification would, perhaps, allow us to decrease the concentration to a less toxic amount. Mixing the 10% DMSO with either the 1,3-CHD or small amounts of sugar for the osmotic imbalance could also be a possible combination to explore.

None of the testing performed using the CPA combinations was able to provide adequate data to measure viability results. There are many potential reasons for this, including immature rings, non-uniform transport from cryovials to plates, degradation of the agarose rings which may have affected alamarBlue readings, as well as too small a sample size. In order to change the concentrations or fully discard the proposed CPA mixtures, further testing would be necessary.

6.1.3 Freezing point depression results show probable interaction between chemicals

As mentioned in the previous chapter, the freezing point depression experiment resulted in unexpected freezing patterns. The combinations of CPAs froze before the samples that had only 3% CHD and 7.5% Trehalose. Since the freezing point depression is usually directly related to the concentration of the chemicals in the solution, this points towards some unexpected interactions between the molecules that affect the abilities of the CPAs to properly bind to the water. This could explain why there seemed to be a downwards trend on the vitrification experiments using our custom CPA mixtures. However, due to the variability on the data gathered, more iterations of the vitrification experiment would be necessary for further analysis. The other unexpected result was the long time that passed before the 10% DMSO sample froze. There is a significant decrease of the freezing point caused by the 10% DMSO, and a predilection towards vitrification after a period of supercooling (Havemeyer, 1966). A more detailed experiment to identify the freezing and melting points of the mixtures would also help finalize an ideal methodology for freezing.

6.2 Future work

A variety of different experiments could follow this work. These experiments can include other antifreeze compounds, different thawing methods, and what types of tissue are used in testing. Below are our recommendations for starting points for further experimentation.

6.2.1 Alternative test methods

6.2.1.1 Checkpoint thawing

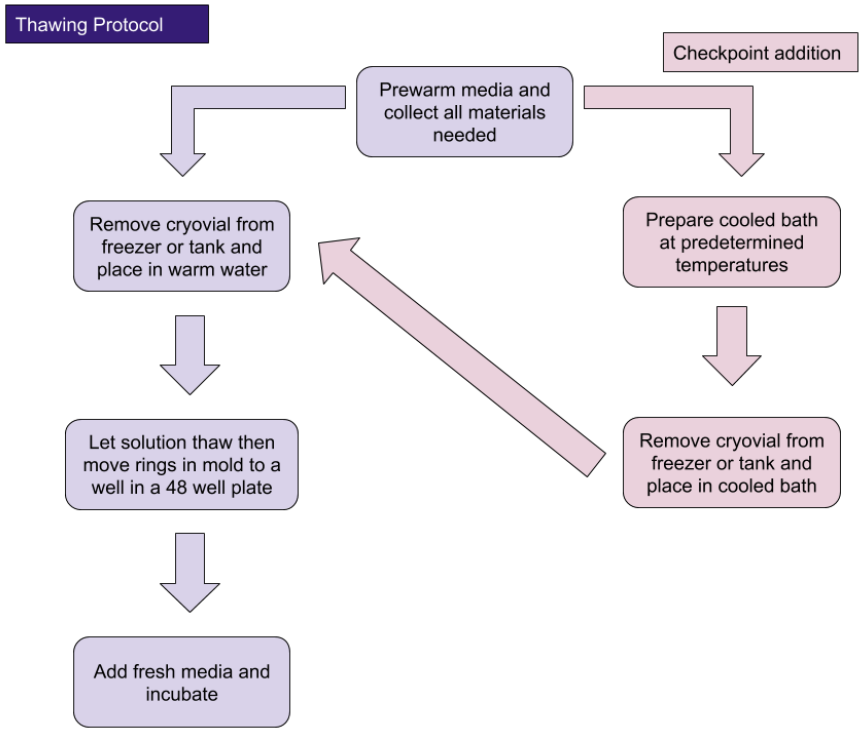


Figure 17. Thawing Workflow

As mentioned in Section 2.2.4, another step to prevent re-crystallization upon thawing the samples would be to implement a checkpoint thaw (Figure 17). This would be achieved by placing the samples in cooling baths or in freezers that have been set to the appropriate temperatures, estimated at: -135˚C (Tg) and -4˚C. These values can be modified upon further studies to determine the exact glass transition temperature and the freezing point of the mixture. This would help soften the glass and reduce the amount of nucleation that could potentially occur during the thawing procedure. Potential methods for this experiment include the use of cooling baths of n-pentane and LN2 for the ~ -135˚C bath and ice and sodium chloride (or similar salt) for the ~ -4˚C cooling bath. Lacking the resources for the full test, it would also be possible to run a test trying only the -4˚C checkpoint, by using a freezer, the aforementioned cooling bath or a Peltier module attached to a thermocouple and a temperature controller. A small platform of Aluminum or Copper could be used to hold and ensure cooling of all samples. For this method a heatsink and appropriate power supply would be needed.

6.2.2 Retesting CPA Cytotoxicity with Tissue

The results obtained from the cytotoxicity tests were unexpected to the team. This was because the CPA mixtures that showed the lowest viability after both 6 and 12 hours in solution were the ones that included Trehalose which is a food grade chemical and HES which is an approved pharmaceutical drug. It was hypothesized that the low viability was just as a result of lack of regular media to the sample, and the cells were starved out rather than killed by the chemicals themselves. To prove this, the team recommends running another cytotoxicity test but this time on tissue rings themselves rather than just rat cell culture. This would allow for more media changes, and any negative effects could be more accurately proven to be caused by the chemicals. Another possible causes for this damage are the high concentrations tested on cells and the amount of time the cells were exposed to the chemicals, modifying these variables could also result in viability changes.

6.2.3 Testing Different Sample Types

Another future step the team recommends taking is testing different tissue types with the same CPAs used. While the results for the custom CPAs were not hugely successful, there was some success in freezing this thick tissue and being able to thaw it back into healthy tissue. We believe that with some refinement to the protocols used in this experiment, the CPAs used could provide promising results when tested with complex tissue such as cartilage or heart muscle. Even though DMSO had better viability results overall, if the custom CPAs used could be modified to get slightly better post-thaw viability, the fact that they are not carcinogenic like DMSO means they could be a promising alternative for freezing tissues intended for transplantation.

6.2.4 Alternative tests on thawed rings

6.2.4.1 Extensive Mechanical Testing

With the failure to obtain conclusive data with the mechanical testing performed in this experiment, the team recommends performing tensile testing on both healthy and post-thawed samples on an Instron with the proper load cell (1N). This will provide better, more precise data that more conclusions will be able to draw from. Along with this, the team recommends performing histology on the tissue ring samples. This will allow for observations to be made about how well the different CPAs diffuse through thick tissue, as well as if ice crystals form and if so, their effect on the sample.

6.2.5 Crystallography Studies

In order to obtain better data regarding the actual state of the mixtures that are frozen, we would recommend performing crystallography studies. A single crystal X-ray crystallography study in the presence of a LN2 line should be conducted in order to analyze whether the proposed chemicals aided with the formation of vitreous ice. The lack of a regular diffraction pattern would signify that the water was taking an amorphous solid form,

confirming our hypothesis that this combination of chemicals would reduce the possibility of damage to the tissue due to ice formation. This study could also show the exact configuration of the ice that may form on the sample, which would allow us to gather more data regarding the freezing mechanisms at play. Furthermore, using a temperature ramp would allow us to identify the state transition temperatures of the chemicals and the different mixtures, as well as let us identify if there was a more adequate rate of cooling. The samples to be used should include controls: water, cell media and 10% DMSO in cell media, as well as the samples to be evaluated: 6% CHD, 6% HES, 15% Trehalose, and the different combinations between them in cell media.

Further microscopy studies could also be performed to confirm and observe the crystallization patterns as well as the presence of imperfections, fractures and cracks in the solid state. This information could then be used to make adjustments to parts of the methodology or the chemicals involved.

6.2.6 Xylomannan

Xylomannan, which is the basis of the AFGL secreted by the Alaskan wood frog and *Upis ceramboides*, was previously discussed in Section 2.4.2. The utilization of xylomannan as a CPA would require its synthesis, followed by cytotoxicity tests, then efficacy tests to see if the synthesized version was as effective as the natural occurring counterpart. An important factor of this would be the ability to freeze to temperatures such as -80°C for the standard freeze procedure as well as below -150°C for vitrification. The previous research performed only mentioned the ability to reach temperatures as low as -60°C.6.3 Antifreeze proteins could not work as a model system

Antifreeze proteins were an ideal candidate for use in cryopreservation as they are naturally occurring and likely to be safer to use in living systems. However, there was no research showing an antifreeze protein that had the potential to work universally across different species.

6.4 Final Design

While there is much further experimentation necessary, the team recommends a focus on the use of 1-3 cyclohexanediol as a cryopreservation agent. As a result of it being cell permeable, stopping ice crystal nucleation, as well as the results obtained from the cytotoxicity test and potential antibacterial properties lead the team to believe that CHD could be a component in a CPA that could successfully allow for the cryopreservation and recovery of tissue samples. We recommend a concentration of 6% and for it to be paired with vitrification methods by flash-freezing in liquid nitrogen. If further testing was conducted and results were positive, the creation of a marketable cryomedia would be assisted by our pre-existing methodologies, and the societal impact of being able to freeze and preserve tissues with no negative drawbacks would be immeasurable, as currently 50% of all organs harvested are unable to be used due to poor preservation (Transplant Safety, CDC). This goal is what motivates the team into pursuing future research in the field of cryopreservation.

Chapter 7. Conclusion

The goal of this project was to create a custom cryomedia by looking at that would be nontoxic, prevent crystal formation, maintain tissue integrity, and result in post-thaw tissue that was as viable as tissue frozen with 10% DMSO.

One of the main conclusions we were able to draw from our project was that vitrification of the rings was not significantly different than the slow freeze method.

These results of the cytotoxicity tests of our cryomedia tests confirmed that after 24 hours, the CPAs had a toxic effect on the cells in suspension. Every combination of our CPAs had significantly less cell viability than the 10% DMSO control. This indicated that the CPAs were likely to be resulting in the death of the cells. We hypothesized that this could have been due to the high percentage of the chemicals in the media which may have resulted in detrimental conditions of osmotic imbalance. The cells may have not had enough media and exhausted the media too quickly.

The results of the ring viability experiments run did not provide any data we could work with about our custom CPAs. The team believes that there is a lot of potential, and that additional testing should be performed. While our ring viability results did not look promising, the data from the 10 % DMSO treated rings was widely varied, with some rings thriving while others failed. Due to this, we believe the limited success of our freezing trials with the custom CPAs calls for extensive further research to be conducted.

We were able to develop methodologies that can be used in the future. Additional testing on our custom cryomedia would be required to determine its effectiveness of achieving our original goals. Since the individual chemicals used in our custom cryomedia showed to not have significant difference in viability than 10% DMSO, we hypothesized that altering their concentrations and using them in addition to a lower concentration of DMSO may optimize the properties that we sought in them for being effective CPAs. We also proposed future methodologies and suggested experiments that may be performed in future work.

Bibliography

Abazari, A., Meimetis, L. G., Budin, G., Bale, S. S., Weissleder, R., & Toner, M. (2015). Engineered Trehalose Permeable to Mammalian Cells. Plos One,10(6). doi:10.1371/journal.pone.0130323

Achim, B. *et al*, "Cryopreservation of Endothelial Cells in Various Cryoprotective Agents and Media – Vitrification versus Slow Freezing Methods," PLos One, 11*(2),* 2016. DOI: http://dx.doi.org/10.1371/journal.pone.0149660.

Ahn, J. Y., Park, J. Y., & Lim, H. K. (2018). Effects of different diluents, cryoprotective agents, and freezing rates on sperm cryopreservation in Epinephelus akaara. Cryobiology,83, 60-64. doi:10.1016/j.cryobiol.2018.06.003

AlamarBlue® Cell Viability Assay Protocol. (n.d.). Retrieved from https://www.thermofisher.com/us/en/home/references/protocols/cell-and-tissue-analysis/cell-profilteration-assay-protocols/cell-viability-with-alamarblue.html

Alcor. (n.d.). Cryopreservation and Fracturing. Retrieved December 16, 2018, from https://alcor.org/Library/html/CryopreservationAndFracturing.html

Anderson, F. (2005). Final report of the safety assessment of Urea. International Journal of Toxicology, 24 Suppl 3:1-56. PMID: 16422263

Bakhach, J. (2009). The cryopreservation of composite tissues. Organogenesis, 5(3), 119-126. doi:10.4161/org.5.3.9583

Best, B (2015) Cryoprotectant toxicity: facts, issues and questions. Rejuvenation Res., 18 (5), 422-436 doi:10.1089/rej.2014.1656

Buitink, J., Claessens, M., Hemminga, M., Hoekstra, F. (1998) Influence of water content and temperature on molecular mobility and intracellular glasses in seeds and pollen, Plant Physiol. 118, 531-541

Chan, W. S., Ma, M., Bhatty, M. U., Preti, R., DeVito, M., Pecora, A. L., Donato, M. L., Feldman, T., Goldberg, S. L., Goy, A., Siegel, D. S., & Rowley, S. D.(2007). Cryopreservation of Peripheral Blood Stem Cells (PBSC) with Hydroxyethylstarch (HES) and Dimethylsulfoxide (DMSO) Results in Faster Granulocyte Recovery Than Using Dimethylsulfoxide Alone.. Blood, 110(11),3287. Accessed March 03, 2019. Retrieved from http://www.bloodjournal.org/content/110/11/3287

Clapisson G, Salinas C, Malacher P, Michallet M, Philip I, and Philip T. (2004) Cryopreservation with hydroxyethylstarch (HES) + dimethylsulfoxide (DMSO) gives better results than DMSO alone. *Bulletin du Cance*r, 91(4):E97-102.

Clarke, A. (2009). Faculty of 1000 evaluation for A nonprotein thermal hysteresis-producing xylomannan antifreeze in the freeze-tolerant Alaskan beetle Upis ceramboides. F1000 - Post-publication Peer Review of the Biomedical Literature. doi:10.3410/f.1282034.749127

Constanzo, J., Lee, R. (1996) Mini-review: ice nucleation in freeze-tolerant vertebrates, *Cryo-Letters*, 17, 111-118.

Crich, D., & Rahaman, M. Y. (2011). Synthesis and Structural Verification of the Xylomannan Antifreeze Substance from the Freeze-Tolerant Alaskan Beetle Upis ceramboides. *The Journal of Organic Chemistry,76*(21), 8611-8620. doi:10.1021/jo201780e

Crowe, J, Carpenter, J, Crowe, L (1998) The role of vitrification in anhydrobiosis, Annu Rev Physiol, 60, 73-103

Cryopreservation of Mammalian Cells. (n.d.). Retrieved from https://www.thermofisher.com/us/en/home/references/gibco-cell-culture-basics/cell-culture-protocols/cryopreservation-of-mammalian-cells.html

Doyle, D. J. (2017). Faculty of 1000 evaluation for Improved tissue cryopreservation using inductive heating of magnetic nanoparticles. F1000 - Post-publication Peer Review of the Biomedical Literature. doi:10.3410/f.727365372.793529503

Echlin, P. “Low-Temperature Microscopy and Analysis” Plenum Press: New York, 1992.

Elford, B. and Walter, C. (1972) Preservation of structure and function of smooth muscle cooled to -79˚C in unfrozen media, Nat. New Biol, 236, 58-60

Fahy, G., DaMouta, C., Tsonev, L., Khirabadi, B., Mehl, P., and Meryman, H. (1995) Cellular Injury associated with organ cryopreservation: Chemical toxicity and cooling injury in *Cell Biology of Trauma*, Lemasters, JJ. and Oliver, C., Eds., CRC Press, Boca Raton, FL, 333-356

Fahy, G. (2001) Process for Preparing Novel Ice-Controlling Molecules, U.S. Patent 6,303,388B1

Fahy, G. and Wowk, B. (2015) Principles of cryopreservation by vitrification, Methods in Molecular Biology, doi: 10.1007/978-1-4939-2193-5_2.

Farrant, J. (1965) Mechanisms of cell damage during freezing and thawing and its prevention, Nature, 205, 1284 - 1287

Fehily, D. (2012). Tissue and cell processing: An essential guide. Chichester, West Sussex: Wiley-Blackwell.

Fuller, B., Lane, N. & Benson E. (2004) Life in the frozen state. London: CRC Press

Gwyther, T., Hu, J., Billiar, K., & Rolle, M. (2011). Directed Cellular Self-Assembly to Fabricate Cell-Derived Tissue Rings for Biomechanical Analysis and Tissue Engineering. *J. Vis. Exp.* (57), e3366. doi:10.3791/3366

Higgins, S. A., & Swanson, D. L. (2013). Urea is not a universal cryoprotectant among hibernating anurans: Evidence from the freeze-tolerant boreal chorus frog (Pseudacris maculata). Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology,164(2), 344-350. doi:10.1016/j.cbpa.2012.11.002

Hu, J., Xu, X., Huang, L., Wang, L., & Fang, N. (2015). Freeze–thaw Caenorhabditis elegans freeze–thaw stress response is regulated by the insulin/IGF-1 receptor daf-2. BMC Genetics,16(1). doi:10.1186/s12863-015-0298-5

Havemeyer, R. N. (1966), Freezing point curve of dimethyl sulfoxide—water solutions. J. Pharm. Sci., 55: 851-853. doi:10.1002/jps.2600550822

Hunt, C. (1984) Studies on cellular structure and ice location in frozen organs and tissues: The use of freeze-substitutions and related techniques, *Cryobiology*, 31, 483-500

Hunt, C., Taylor, M. & Pegg, D. (1982) Freeze substitution and isothermal freeze fixation studies to elucidate the pattern of ice formation on smooth muscle at 252K (-21C), *J. Microscopy,* 125, 177-186.

Huntington, H. B., & MacCrone, R. K. (2014). Thermal hysteresis. *AccessScience*. doi:10.1036/1097-8542.689500

ISO 11137-2:2017. (2017, June). Retrieved from https://www.iso.org/standard/63841.html

ISO 14937:2009. (2015, April). Retrieved from https://www.iso.org/standard/44954.html

ISO 20391-1:2018. (2018, January). Retrieved from https://www.iso.org/standard/68879.html

ISO 24998:2008. (2017, June). Retrieved from https://www.iso.org/standard/42736.html

Jacobsen, Karlsson, J. O., Cravalho, E. G., & Toner, M. (1994). A model of diffusion-limited ice growth inside biological cells during freezing. Journal of Applied Physics, 75(9), 4442-4455. doi:10.1063/1.355959

JES. (2009, October 13). Poll: Mr. Frost, warm or cold? Message posted to https://www.scientistsolutions.com/forum/cell-culture-and-tissue-culture-equipment/poll-mr-frosty-warm-or-cold

Karlsson, J. O., & Toner, M. (1996). Long-term storage of tissues by cryopreservation: Critical issues. Biomaterials, 17(3), 243-256. doi:10.1016/0142-9612(96)85562-1

Kearns, J. Personal communication, December 12, 2018

Key Facts | Overview | Transplant Safety | CDC. (n.d.). Retrieved from https://www.cdc.gov/transplantsafety/overview/key-facts.html

Kirchner, B. and Reiher, M. (2002) The secret of dimethyl sulfoxide-water mixtures. A quantum chemical study of 1DMSO-nwater clusters, *Journal of the American Chemistry Society,* 124 (21), pp 6206–6215

Kline, M., Dreyer, M., Gyring, P., Lifson, M., & Assal, R. E. (2015). 60. Innovative cryoprotectants for tissue and organ preservation. Cryobiology, 71(1), 180. doi:10.1016/j.cryobiol.2015.05.066

Knight, Charles A. (Charles Alfred) & Commission on College Physics (1967). *The freezing of supercooled liquids*. Published for the Commission on College Physics Van Nostrand, Princeton, N.J

Kuleshova, L., Gouk, S., & Hutmacher, D. (2007). Vitrification as a prospect for cryopreservation of tissue-engineered constructs. Biomaterials, 28(9), 1585-1596. doi:10.1016/j.biomaterials.2006.11.047

Larson, D. J., Middle, L., Vu, H., Zhang, W., Serianni, A. S., Duman, J., & Barnes, B. M. (2014). Wood frog adaptations to overwintering in Alaska: New limits to freezing tolerance. Journal of Experimental Biology,217(12), 2193-2200. doi:10.1242/jeb.101931

Liu, Y., Xu, X., Ma, X., Martin-Rendon, E., Watt, S., & Cui, Z. (2010). Cryopreservation of human bone marrow-derived mesenchymal stem cells with reduced dimethylsulfoxide and well-defined freezing solutions. Biotechnology Progress, 26(6), 1635-1643. doi:10.1002/btpr.464

Luyet, B. (1937) The vitrification of organic colloids and of protoplasm. *Biodynamica*, 10, 47-52

Luyet, B. & Gehenio, P. (1940) Life and death at low temperatures. *Biodynamica,* 3, 33-99

Mathew, A. (2018) 12th International Conference & Exhibition on Tissue Preservation,Lifecare and Biobanking November 10, 2018, Atlanta, Georgia

Meryman, H. T. (2007). Cryopreservation of living cells: Principles and practice. Transfusion, 47(5), 935-945. doi:10.1111/j.1537-2995.2007.01212.x

Muldrew, K. and McGann, L. (1990) Mechanisms of intracellular ice formation, *Biophys J., 57, 525 - 532*

Obata, H., Muryoi, N., Kawahara, H., Yamade, K., Nishikawa, J. (1999) Identification of a novel nicleating bacterium of Antarctic origin and its ice nucleation properties, *Cryobiology,* 38, 131-139

Orser, C., Staskawics, B., Panapoulos, N., Dahlbeck, J., Lindow, S. (1985) Cloning and expression of bacterial ice nucleation genes in Escherichia coli, *J. Bacteriology*, 164, 359-366

Pang, Xiao-Feng (2014) Water: Molecular Structure and Properties. World Scientific, 9814440434

Pegg, D. (1987) Ice Crystals in tissues and organs, in The Biophysics of organ preservation, Pedd D. and Karow, A. Eds., Plenum, New York, pp 117-140

Pegg, D. & Diaper, M. (1990) Freezing versus vitrification: basic principles, found in *Cryopreservation and low temperature biology in blood transfusion,* Vol 24. Kluwer Academic Publishers, 55-69

Reyes, M. D., Saenz, L., Lapierre, L., Crosby, J., & Barros, C. (2002). Evaluation of glucose as a cryoprotectant for boar semen. Veterinary Record,151(16), 477-480. doi:10.1136/vr.151.16.477

Richards, A., Krakowka, S., Dexter, L., Schmid, H., Wolterbeek, A., Waalkens-Berendsen, D., . . . Kurimoto, M. (2002). Trehalose: A review of properties, history of use and human tolerance, and results of multiple safety studies. Food and Chemical Toxicology,40(7), 871-898. doi:10.1016/s0278-6915(02)00011-x

Russ, N, Zielbauer, B., & Vilgis, T. (2014). Impact of sucrose and trehalose on different agarose-hydrocolloid systems. Food Hydrocolloids. 41, 44-52. doi:10.1016/j.foodhyd.2014.03.020

Smith, A. (1991) Biological Effects of Freezing and Supercooling, London: Edward Arnold

Song, Y., Pegg, D., & Hunt, C. (1995). Cryopreservation of the Common Carotid Artery of the Rabbit: Optimization of Dimethyl Sulfoxide Concentration and Cooling Rate. Cryobiology, 32(5), 405-421. doi:10.1006/cryo.1995.1040

Stolzing A, Naaldijk Y, Fedorova V, and Sethe S. (2012) Hydroxyethylstarch in cryopreservation - mechanisms, benefits and problems. *Transfus. Apher. Sci.,* 46(2):137-47. doi: 10.1016/j.transci.2012.01.007.

Strobel, H. A., Hookway, T. A., Piola, M., Fiore, G. B., Soncini, M., Alsberg, E., & Rolle, M. W. (2018). Assembly of Tissue-Engineered Blood Vessels with Spatially Controlled Heterogeneities. Tissue Engineering Part A, 24(19-20), 1492-1503. doi:10.1089/ten.tea.2017.0492

Taylor, M. (1982) The role of pH* and buffer capacity in the recovery of function of smooth muscle cooled to -13˚C in unfrozen media, Cryobiology, 29, 585 - 598

Taylor, M (1984) Sub-zero preservation and the prospect of long term storage of multi-cellular tissues and organs. Transplantation Immunology: Clinical and Experimental by Calne, R. Oxford University, Oxford pp 360-390

Taylor, M, Walter, C. and Elford, C (1978) the pH dependency recovery of smooth muscle from storage at -13˚C in unfrozen media, Cryobiology 15, 452 -460

Tuchin, V., Genina, E., & Larin, K. (2008). Measurement of Glucose Diffusion Coefficients in Human Tissues. *Handbook of Optical Sensing of Glucose in Biological Fluids and Tissues Series in Medical Physics and Biomedical Engineering,*587-621. doi:10.1201/9781584889755.ch19

Vali, G. (n.d.). Stability of nucleation sites for ice. *Atmospheric Aerosols and Nucleation Lecture Notes in Physics,* 645-648. doi:10.1007/3-540-50108-8_1148

Wolf, J., & Bryant, G. (1999). Freezing, drying and/or vitrification of membrane-solute-water systems. *Cryobiology,* 39, 103-129.

Appendix A: Lab Notebooks

A detailed recount of the experiments performed, including any events, timing and relevant notes can be found in two lab notebooks submitted to the advising faculty with this paper.

Appendix B: Resources Used

Appendix B.1 ThermoFischer AlamarBlue Assay Protocol

Retrieved from:
https://www.thermofisher.com/us/en/home/references/protocols/cell-and-tissue-analysis/cell-profilteration-assay-protocols/cell-viability-with-alamarblue.html

Optional: Treat cells with the test compound 24–72 hours prior to performing the alamarBlue® cytotoxicity assay.

1. Add 1/10th volume of alamarBlue® reagent directly to cells in culture medium as described in Table 1.

Format	Volume of cells + medium	Volume of 10x alamarBlue to add
Cuvette	1 mL	100 µL
96-well plate	100 µL	10 µL
384-well plate	40 µL	4 µL

1. Incubate for 1 to 4 hours at 37°C in a cell culture incubator, **protected from direct light**.
2. **Note:** Sensitivity of detection increases with longer incubation times. For samples with fewer cells, use longer incubation times of up to 24 hours.
3. Record results using fluorescence or absorbance as follows:
4. **Fluorescence:** Read fluorescence using a fluorescence excitation wavelength of 540–570 nm (peak excitation is 570 nm). Read fluorescence emission at 580–610 nm (peak emission is 585 nm).
5. **Absorbance:** Monitor the absorbance of alamarBlue® at 570 nm, using 600 nm as a reference wavelength (normalized to the 600 nm value).
6. **Note:** Fluorescence mode measurements are more sensitive. When fluorescence instrumentation is unavailable, monitor the absorbance of alamarBlue® reagent. Assay plates or tubes can be wrapped in foil, stored at 4°C, and read within 1–3 days without affecting the fluorescence or absorbance values.
7. **Optional:** Add 50 µL 3% SDS directly to 100 µL of cells in alamarBlue® reagent to stop the reaction.

Appendix B.2 Rolle Lab Rat Aortic Smooth Muscle Cell Culture Protocol

Medium Components:
Medium should be used within one month of preparation, as some components (particularly L-glutamine) degrade over time.
Rat SMC Growth Medium:

- DMEM
- 10% FBS
- 1% L-glutamine
- 1% Pen-strep
- 1% NEAA
- 1% sodium pyruvate

Thawing Cells:

1. Cells are stored in cryovials in liquid nitrogen when not in culture.
2. Pre-warm the volume of medium needed to 37°C in a water bath prior to adding it to the cells. For rat aortic SMCs, cells are thawed into a 15 cm dish with 17 ml medium (from vials of 1-2 million cells/vial). When different cell numbers are used, see the Seeding Density Table below to find the appropriate sized dish and medium volume.
3. Do not remove cells from cryotank until ready to thaw; after removal place vials in water bath at 37°C and swirl until only a small amount of ice remains in the vial.
4. Pipet cells immediately into a petri dish with fresh pre-warmed media. If any ice remains, pipet a small amount of medium into the vial and pipet up and down until melted.
5. Exchange media 24 hours after thawing cells to remove residual DMSO.
6. Cells should be maintained in humidified incubators at 37°C and 5% CO2.
7. Label plates with the cell type, date, and your initials

Feeding Cells:

1. Once every 2 days.
2. Feeding cells refers to removing cell culture medium and replacing it with fresh cell culture medium.
3. Pre-warm the volume of medium needed to 37°C in a water.
4. When medium is warmed, remove cell culture medium by gently tilting the petri dish to pool the medium, and aspirating with a sterile Pasteur pipette attached to the vacuum trap.
5. Add fresh medium gently with a sterile serological pipette.

Passaging Cells:

1. When cells are 70-90% confluent (see below), aspirate medium and wash cells twice with 1X PBS (3-6 ml) by adding and then aspirating PBS.
2. Trypsinize cells by adding 0.25% trypsin EDTA (3 ml for 15 cm plate) to the plate, and move to the incubator for no more than 2-3 minutes.

3. Remove the plates and firmly tap them against your hand or the side of the table to detach cells (be careful not to splash medium). Check under the microscope that cells are floating and no longer attached (check the entire plate, not just one spot in the middle).
4. Add a volume of culture medium to the plate equal to the amount of trypsin added (move quickly, as over-trypsinizing can cause cells to clump). Tilt the plate so the suspension pools, and pipet up and down with a serological pipette to resuspend the cells and break up any clumps. Move the suspension into an appropriately sized conical tube.
   NOTE: Serum in culture medium inactivates trypsin.
5. Once the suspension is in the conical tube, resuspend with a serological pipette. Immediately take out 40ul cell suspension with a sterile pipette tip and add to 40ul trypan blue aliquot for cell counting (1:1 dilution; see Cell Counting section below).
   a. For plates of rat aortic SMCs with greater than 80% confluency, the suspension should be further diluted before removing the 40ul aliquot for counting. Otherwise, the cell density will likely be too high to easily count with a hemocytometer.
6. Centrifuge cell suspension into a pellet in the centrifuge tube for 5 minutes at 1000 rpm (200 rcf) in the Pins lab cell culture bucket centrifuge.
7. Aspirate supernatant, leaving cell pellet, and re-suspend cells in no more than 1ml media (less if your cell suspension needs to be more concentrated), pipet up and down with a 1000ul pipette to break up clumps.
8. After counting, add media to dilute the cell suspension to the desired concentration. Re-plate, freeze, or seed cells as rings. When re-plating for expansion, rat aortic SMCs should be seeded at a density of 5,600 to 11,000 cells/cm2.
9. Check growth each day, feeding every two days, and passage as necessary.
10. When passaging cells or changing medium, care should be taken to not let the cells dry out. When more than 4 plates need passaging, it is recommended to passage them in groups, so that plates do not dry out or become over-trypsinized.
    NOTE: Because rat aortic SMCs are immortalized, they will proliferate indefinitely, and are not affected by passage number. Thus, we do not record rat aortic SMC passage number.

Freezing Cells:

1. Trypsinize, count, and resuspend cells in growth medium as described above, at a density of approximately 1-2 x106 cells/ml
2. Add no more than 1 ml suspension (1-2 million cells) to each pre-labelled cryovial. Cryovials should be labelled with a sharpie, and state the cell type, cell count, your initials, and the date. Do not overfill cryovials!
3. Add 10% DMSO to each vial to prevent ice crystal formation during freezing. When freezing large volumes of cell suspension, 10% DMSO may be added to the suspension before distributing to the cryovials, and then the volume of mixture scaled up such that the correct number of cells is moved to each vial. Take care to move quickly during this process, as DMSO is harmful to cells at room temperature.

4. Make sure cryovial lids are tight, and place them in room temperature Mr. Frosty freezing container (shown to the right) and place in a -80°C freezer overnight then transfer vials to liquid nitrogen for storage immediately after 24 hours.

Appendix B.3 Tissue Ring Assembly Protocol

From Rolle Lab Protocol:
Strobel, H. A., Calamari, E. L., Alphonse, B., Hookway, T. A., Rolle, M. W. (2018). Fabrication of Custom Agarose Wells for Cell Seeding and Tissue Ring Self-assembly Using 3D-Printed Molds. *J. Vis. Exp.* (134), e56618, doi:10.3791/56618

Preparing PDMS Negative

1. Print plastic mold on a high resolution printer
2. Thoroughly wash the mold with detergent and water. Rinse with distilled water and let air dry.
3. Measure 25g PDMS base in a weight boat. Add curing agent in a 1:10 ratio by weight. Stir vigorously until thoroughly mixed.
4. FIx laboratory tape around the edges of the wall of the plastic mold, approximately 1 cm above the mold. Pour PDMS into the mold and de-gas until the bubbles are all gone.
5. Cure PDMS at 50C for 2-4 hours until solid. Extract the PDMS negative from the mold.
6. Incubate PDMS at 60C for 1 hour to fully cure.
7. Wash with detergent and water. Rinse with distilled water and let air dry.
8. Autoclave PDMS negative.

Making Agarose Wells (to be completed one day before seeding cells)

1. Make a 2% agarose solution (w/v) in DMEM. Autoclave.
2. Pipette 250 uL agarose into autoclaved PDMS negative. Make sure to pipette into the cavities for well posts.
3. Allow Agarose to cool for 2 minutes. Extract from PDMS mold and place into well.
4. Add enough culture media to submerge the molds and equilibrate in an incubator overnight.

Preparation of Cells

1. Culture rat smooth muscle cells until approximately 70% confluent.
2. Follow steps 1-7 of Passaging Cells section (see Appendix B.3)
3. Resuspend cells to achieve a concentration of 10 million cells/mL.

Fabrication of Rings

1. Aspirate media from equilibrated agarose molds.
2. Piped 50uL of cell suspension (10 million cells/mL) into each well.
3. Add 0.3 mL of fresh media outside agarose mold without letting medium overflow into agarose molds. Incubate overnight.
4. Gently aspirate medium from outside of molds. Add 4.5 mL fresh media to each well plate to completely submerge molds and rings.
5. Change media daily

Appendix B.4 Standard Freeze Protocol

Freezing Mammalian Cells

It is important to make sure that healthy, actively dividing cells are frozen for successful freezing and subsequent thaw and recovery. This can be achieved by freezing cells at about 70 to 80% confluency. Avoid freezing cells when their density is very low or very high. **A successful freezing technique should result in greater than 85% cell recovery after thawing.**

Procedure: (see diagram on next page)

1. Mark three 15 ml conical tubes as follows
   Freezing solution A **(one tube per team)**
   Freezing solution B (one tube per student)
   Freezing mixture (one tube per student)

2. Each team is provided with two 3T3 plates. Trypsinize the cells following the sub culturing protocol (3 ml trypsin + 2 ml CM). **Combine the cells from two plates** in to one 15 ml tube marked "freezing solution A". Centrifuge the cells at 200G. Determine cell count.

3. Prepare **freezing solution A**. The final cell number per vial will depend on the cell count you obtained in step 2 above. Once you get the total cell count, please discuss the procedure with the instructor, TA or PLA. Freezing solution A is prepared in complete media (10% FBS) at **double the cell density** of cells to be frozen in cryovials. That is, if you want to freeze cells at 1 x 10^6 cells/ml in the cryovial, freezing solution A should have double that density (2 x 10^6 cells/ml). **Team members will share cells from this tube.**

4. Prepare **freezing solution B** (with DMSO). This consists of complete medium supplemented with 20% DMSO. Each student will prepare this solution separately. In the tube marked Freezing Solution B, using a serological pipette, add 4 ml complete medium. To this, using a P-100 micropipette, and 1 ml of DMSO. Freezing solution B contains **twice the amount of DMSO (20%).** Once the final freezing mixtures are prepared, the final DMSO will be 10% in the cryovial.

5. For "**No DMSO**" freezing, mix the cells in "Freezing solution A" tube by closing the cap and inverting the tube 3-4 times.

6. Using a P-1000 micropipette, quickly transfer 500 µl ml of cell suspension from "Freezing solution A" tube to the cryovial marked "**No DMSO**". To this cryovial, add 500 µl regular complete medium (without DMSO) and pipette up and down 3-4 times to mix the solutions. Set the cryovial aside. This will result in 1 ml of cell mixture in the cryovial at half the cell number, but without DMSO.

7. For the regular cell freezing "**With DMSO**", mix the cells in "freezing solution A" by closing the cap and inverting the tube 3-4 times to ensure that the cells are resuspended uniformly.

8. Using a P-1000 micropipette, quickly transfer 1 ml of cell suspension from "freezing solution A" tube to the "freezing mixture" tube (see the flowchart below).

9. In this step, you will **gradually** add freezing solution B to the cell suspension in the "freezing mixture" tube. This is called progressive mixing where DMSO is introduced to the cells progressively. Using a P-1000 micropipette, measure 1 ml of freezing solution B. Introduce the solution slowly to the cells in the "freezing mixture" tube, while mixing the solutions gently.

10. Transfer 1 ml of the mixture from the "Freezing mixture" tube to the cryovial marked "With DMSO". Tighten the cap and transfer the tube to "CoolCell" freezing container in the -20 freezer.

11. The tubes will be later transferred to -80 freezer. Next week, you will thaw and plate the cells from today's freezing to determine the success of the two freezing methods.

Appendix B.5 Vitrification Protocol

1. Prepare ring samples to be moved into cryovial under biological hood
2. Aspirate the media where the rings were being incubated and move ring into labeled cryovial
3. Add 1ml of the specified media to the cryovials
4. Depending on time required for permeation, let samples soak or move to step 5.
5. Move cryovial to liquid nitrogen cryotank using the required safety protocols and protections, including eye protection wear and appropriate gloves to prevent frostbite.

Appendix C: Trial List

Trial Identifier	Sample Type	CPA	Freeze Method	Time Frozen	Thaw Method
C1	Tissue Rings	10% DMSO	Slow Freeze	24 Hours	Standard
C2	Tissue Rings	10% DMSO	Slow Freeze	48 Hours	Standard
C3	Tissue Rings	10% DMSO	Vitrification	24 Hours	Standard
C4	Tissue Rings	10% DMSO	Vitrification	48 Hours	Standard
T1	Tissue Rings	10% DMSO	Vitrification	24 Hours	Standard
T2	Tissue Rings	6% 1-3 CHD + 15% Trehalose	Vitrification	24 Hours	Standard
T3	Tissue Rings	6% 1-3 CHD + 6% HES + 15% Trehalose	Vitrification	24 Hours	Standard

Appendix D: Experimental Data

Appendix D.1 Viability Data

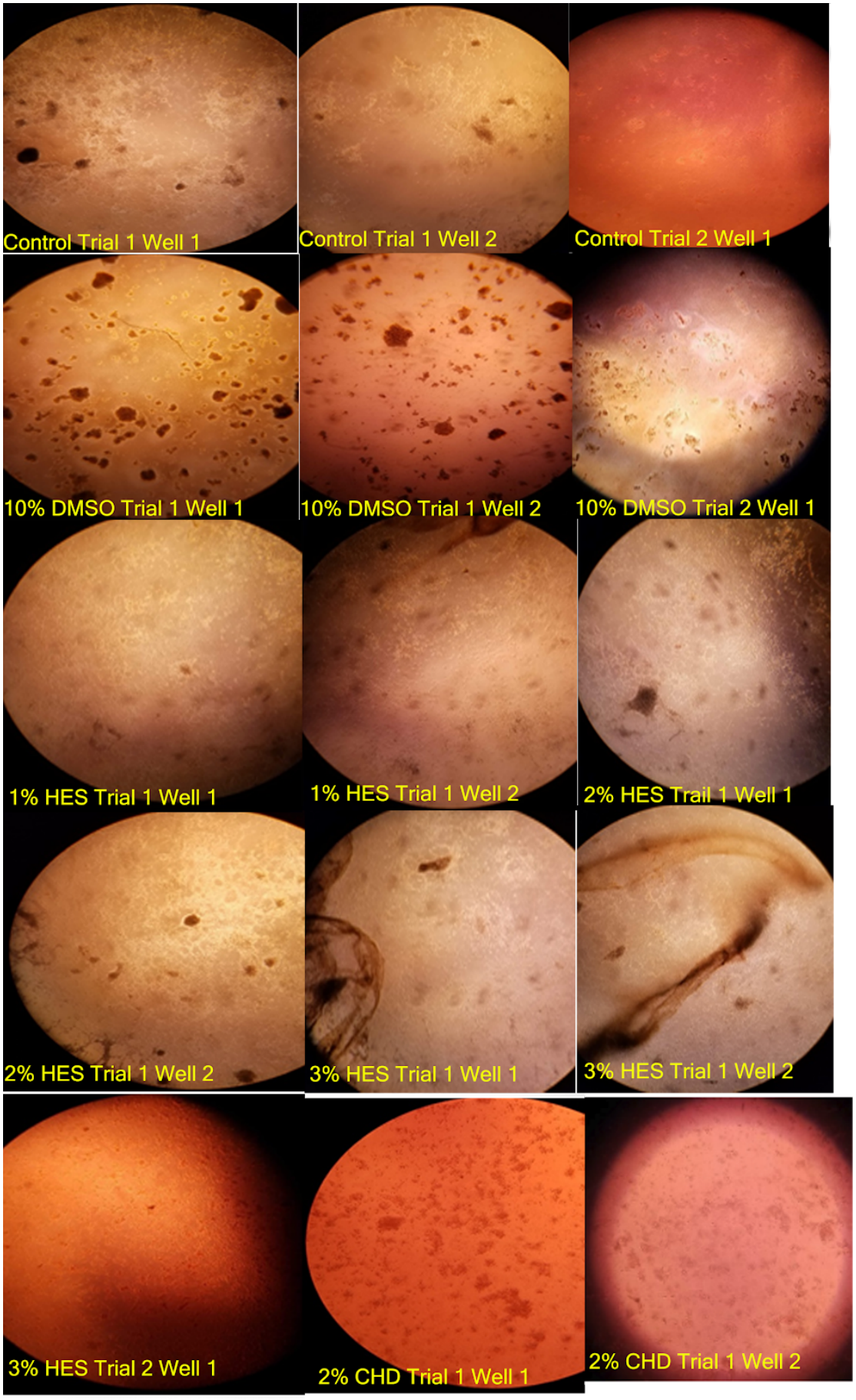
The alamarBlue assay results for the control groups using 10%DMSO are included in the following table:

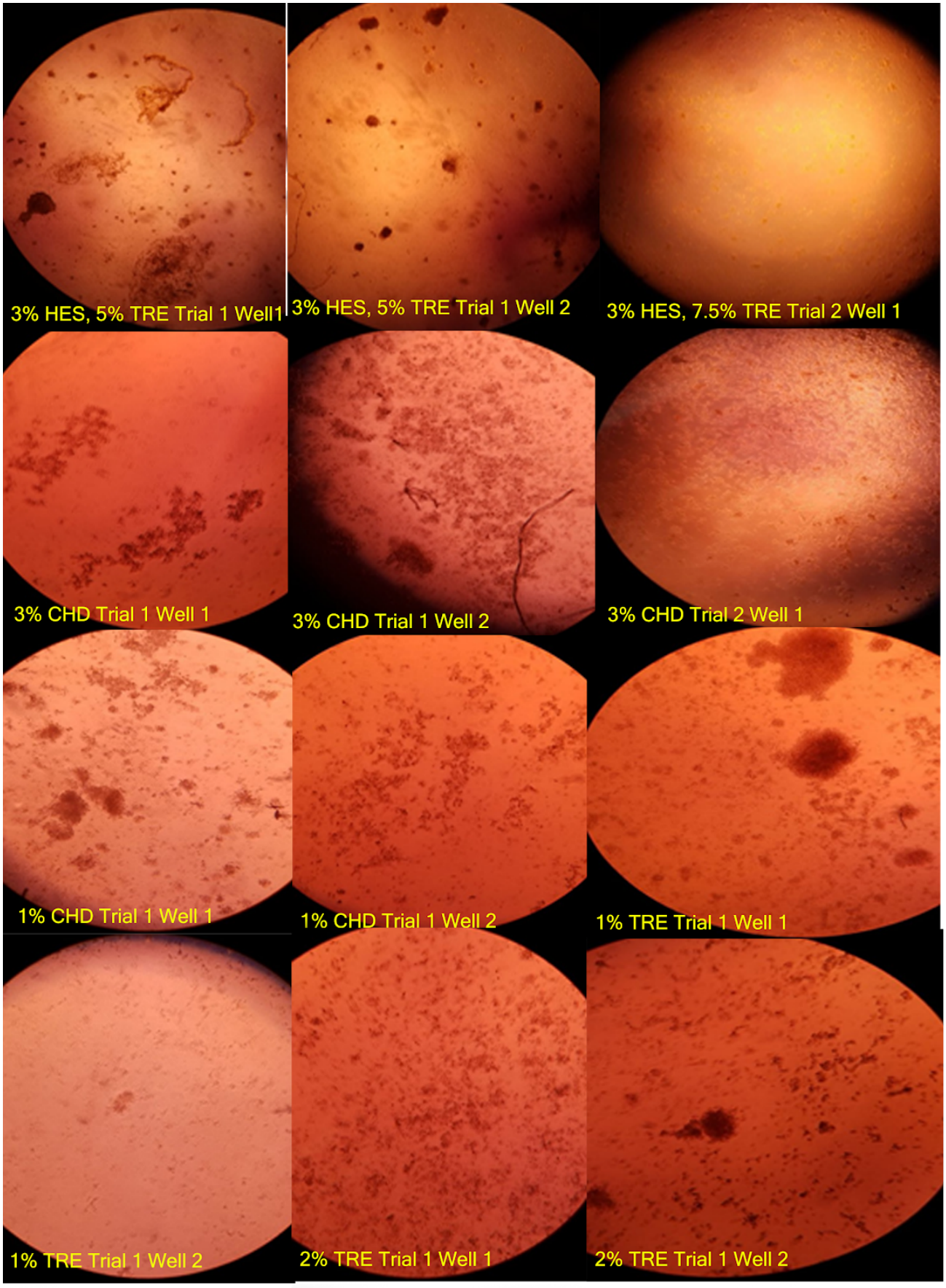
Experiment	Pre_FreezeAbsorbanceValue	DateFrozen	DateThawed	Post_ThawAbsorbanceValue	Difference	Viability	Viability_avg
C1.1	1148090	2/13/2019	2/16/2019	1138365	9725	0.9915294097	0.916
C1.2	1189094	2/13/2019	2/16/2019	991475	197619	0.8338070834	
C1.3	1012437	2/13/2019	2/16/2019	940976	71461	0.9294168427	
C 2.1	1175217	2/13/2019	2/17/2019	687907	487310	0.5853446644	0.70816
C 2.2	1000605	2/13/2019	2/17/2019	408998	591607	0.4087507058	
C 2.3	1311427	2/13/2019	2/17/2019	1483015	-171588	1.13084068	
C 3.1	1358387	2/13/2019	2/15/2019	537447	820940	0.3956508712	0.5298
C 3.2	1071547	2/13/2019	2/15/2019	1300494	-228947	1.21366025	
C 3.3	1043505	2/13/2019	2/15/2019	734003	309502	0.703401517	
C 4.1	1011874	2/13/2019	2/16/2019	708440	303434	0.7001266956	0.7876
C 4.2	988944	2/13/2019	2/16/2019	933132	55812	0.9435640441	
C 4.3	1139907	2/13/2019	2/16/2019	820127	319780	0.7194683426	
C 1.4	1720778	2/18/2019	2/20/2019	1534297	186481	0.8916298326	0.894
C 1.5	1748918	2/18/2019	2/20/2019	1731109	17809	0.9898171327	
C 1.6	1687361	2/18/2019	2/20/2019	1352746	334615	0.801693295	

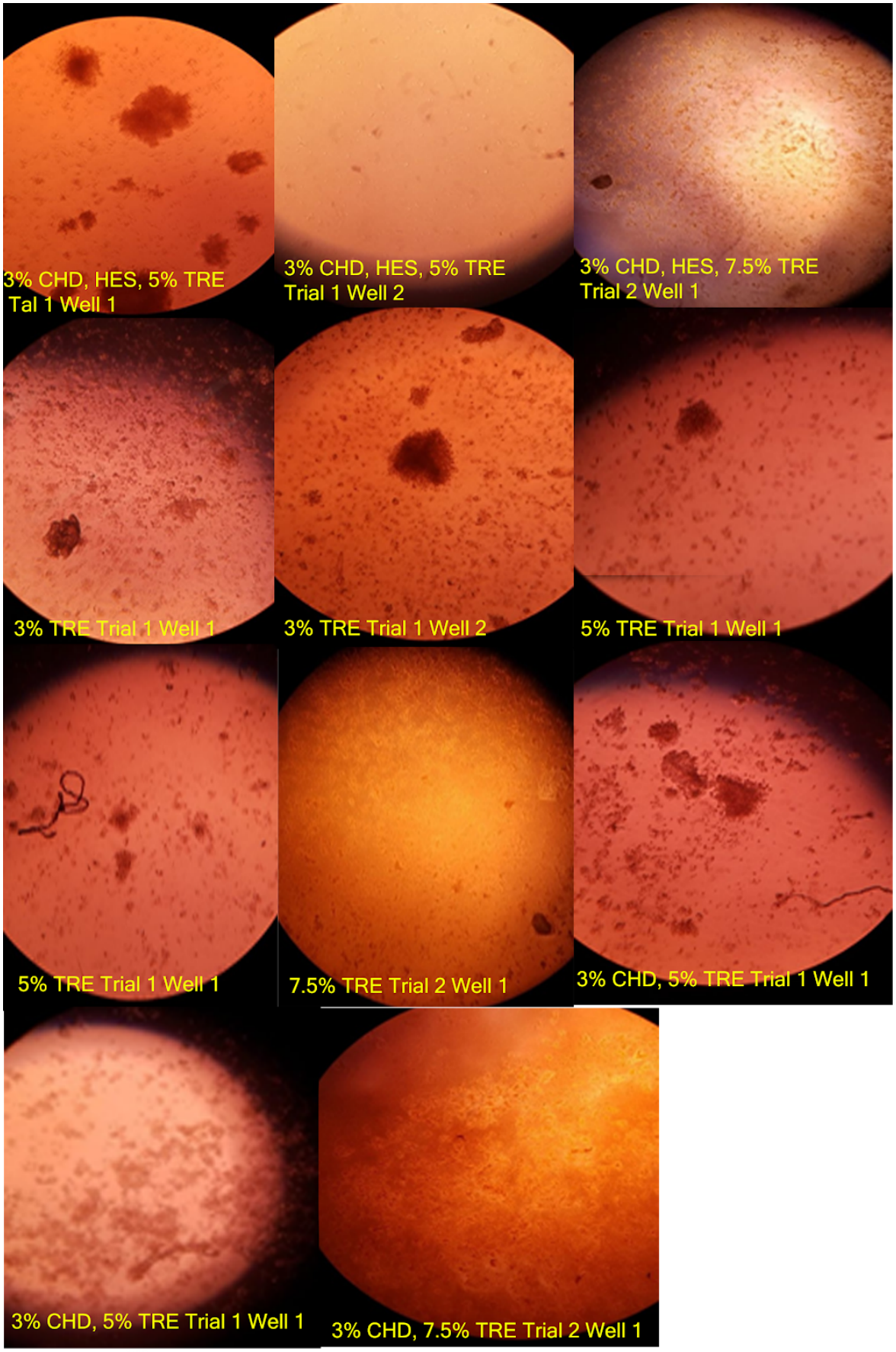
C 2.4	1450670	2/18/2019	2/21/2019	1551740	-101070	1.069671255	1.0319
C 2.5	1431310	2/18/2019	2/21/2019	1374926	56384	0.9606067169	
C 2.6	1366443	2/18/2019	2/21/2019	1456008	-89565	1.065546093	
C 3.4	1428807	2/18/2019	2/19/2019	1039020	389787	0.7271940857	0.85509
C 3.5	1277818	2/18/2019	2/19/2019	1095140	182678	0.8570391089	
C 3.6	1332070	2/18/2019	2/19/2019	1306989	25081	0.9811714099	
C 4.4	1315905	2/18/2019	2/20/2019	1413996	-98091	1.074542615	0.95062
C 4.5	1264343	2/18/2019	2/20/2019	1134517	129826	0.897317421	
C 4.6	1239134	2/18/2019	2/20/2019	1090507	148627	0.8800557486	
C 3.7	1352266	2/25/2019	2/26/2019	1001287	350979	0.740451213	0.7023
C 3.8	1486943	2/25/2019	2/26/2019	998473	488470	0.6714937963	
C 3.9	1442268	2/25/2019	2/26/2019	1002476	439792	0.695069155	

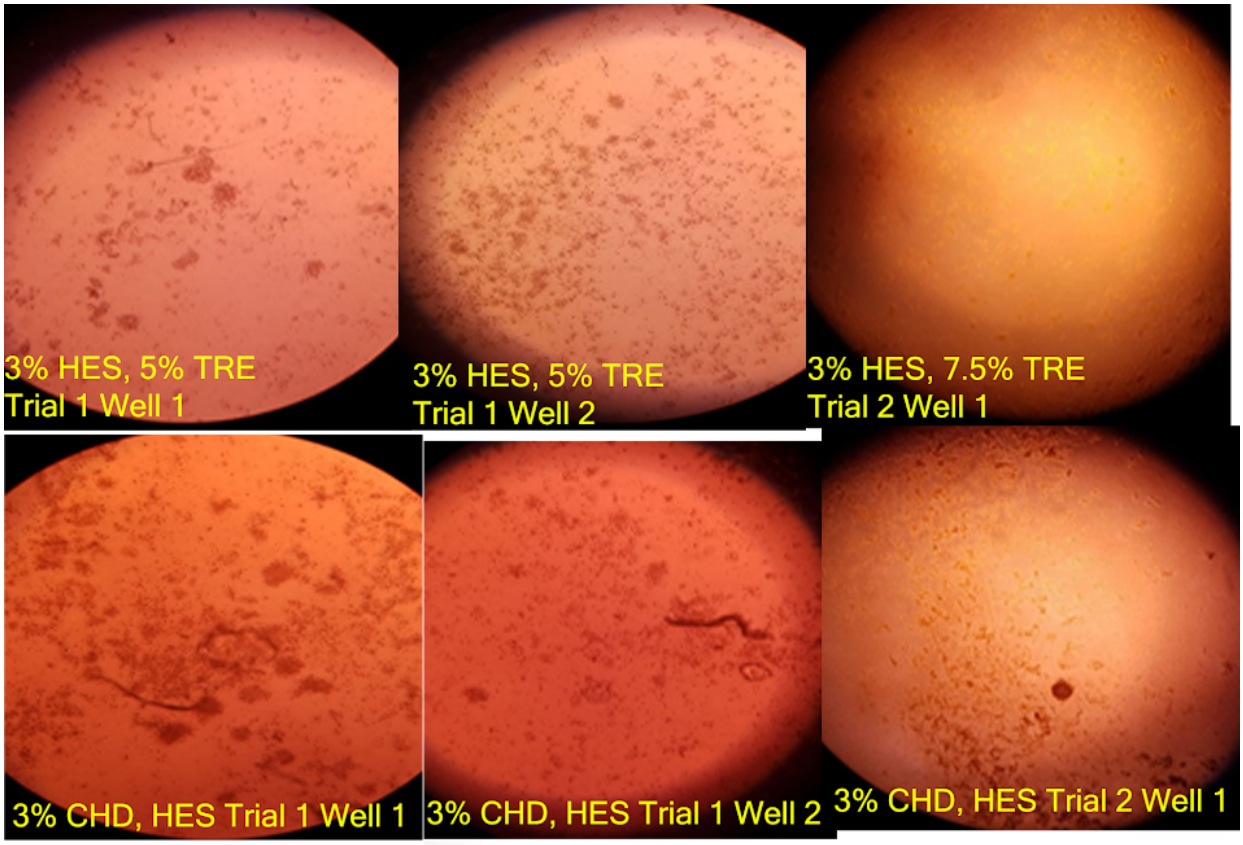
Appendix D.2 Cytotoxicity Data:

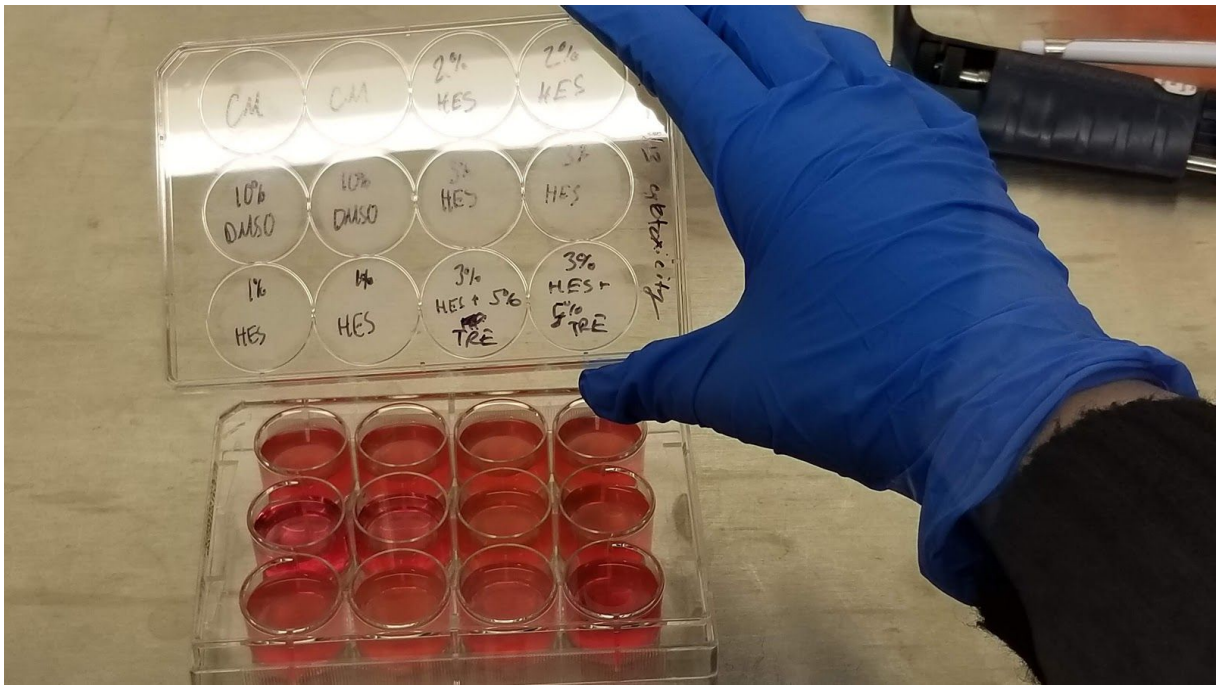
Cytotoxicity results after approximately 45 hours post plating cells in media solutions for trial 1 images in the following wells: control wells 1 and 2, 10% DMSO wells 1 and 2, 1% HES wells 1 and 2, 2% HES wells 1 and 2, 3% HES wells 1 and 2, 3% HES and 5% TRE wells 1 and 2. Photographed on 1/15/19 at 1:20pm. Second part of trial 1 included the remaining wells in trial 1. These pictures were taken with same camera on 1/17/19 at 10:46am. Pictures taken with a Samsung Galaxy S8 camera, as the lab's microscope camera was broken for all of January and February 2019. It is likely that the media used for trial 2 was contaminated prior to pipetting into the cells and was not caught until the following week. Trial 2 photos were taken on 2/13/19 at 12:10pm.


Control Trial 1 Well 1
Control Trial 1 Well 2
Control Trial 2 Well 1
10% DMSO Trial 1 Well 1
10% DMSO Trial 1 Well 2
10% DMSO Trial 2 Well 1
1% HES Trial 1 Well 1
1% HES Trial 1 Well 2
2% HES Trail 1 Well 1
2% HES Trial 1 Well 2
3% HES Trial 1 Well 1
3% HES Trial 1 Well 2
3% HES Trial 2 Well 1
2% CHD Trial 1 Well 1
2% CHD Trial 1 Well 2


3% HES, 5% TRE Trial 1 Well1
3% HES, 5% TRE Trial 1 Well 2
3% HES, 7.5% TRE Trial 2 Well 1
3% CHD Trial 1 Well 1
3% CHD Trial 1 Well 2
3% CHD Trial 2 Well 1
1% CHD Trial 1 Well 1
1% CHD Trial 1 Well 2
1% TRE Trial 1 Well 1
1% TRE Trial 1 Well 2
2% TRE Trial 1 Well 1
2% TRE Trial 1 Well 2


3% CHD, HES, 5% TRE
Tal 1 Well 1
3% CHD, HES, 5% TRE
Trial 1 Well 2
3% CHD, HES, 7.5% TRE
Trial 2 Well 1
3% TRE Trial 1 Well 1
3% TRE Trial 1 Well 2
5% TRE Trial 1 Well 1
5% TRE Trial 1 Well 1
7.5% TRE Trial 2 Well 1
3% CHD, 5% TRE Trial 1 Well 1
3% CHD, 5% TRE Trial 1 Well 1
3% CHD, 7.5% TRE Trial 2 Well 1


3% HES, 5% TRE
Trial 1 Well 1
3% HES, 5% TRE
Trial 1 Well 2
3% HES, 7.5% TRE
Trial 2 Well 1
3% CHD, HES Trial 1 Well 1
3% CHD, HES Trial 1 Well 2
3% CHD, HES Trial 2 Well 1


CM
CM
2% HES
2% HES
10% DMSO
10% DMSO
HES
HES
1% HES
1% HES
3% HES + 5% TRE
3% HES + 5% TRE

The following table contains the alamarBlue assay results of the fourth and last cytotoxicity experiment:

Sample	#1	#2	#3	**Average**
Cell Media	1448457	1504030	1448226	1466904
10% DMSO	1495714	1481456	1699518	1558896
Big Boy	1645428	1524576	1617717	1595907
3% HES	1481305	1662736	1518419	1554153
3% CHD	1267404	1634204	1404006	1435205
7.5% Trehalose	1579036	1527292	1734843	1613724
3% HES + 3% CHD	1558307	1591380	1587278	1578988
3% HES + 7.5%Trehalose	1416120	1544162	1800127	1586803
3% CHD + 7.5% Trehalose	1579151	1471823	1491205	1514060

Appendix D.3 Infrared Spectra

The following spectra correspond to the solid state of the different CPAs employed in this study.

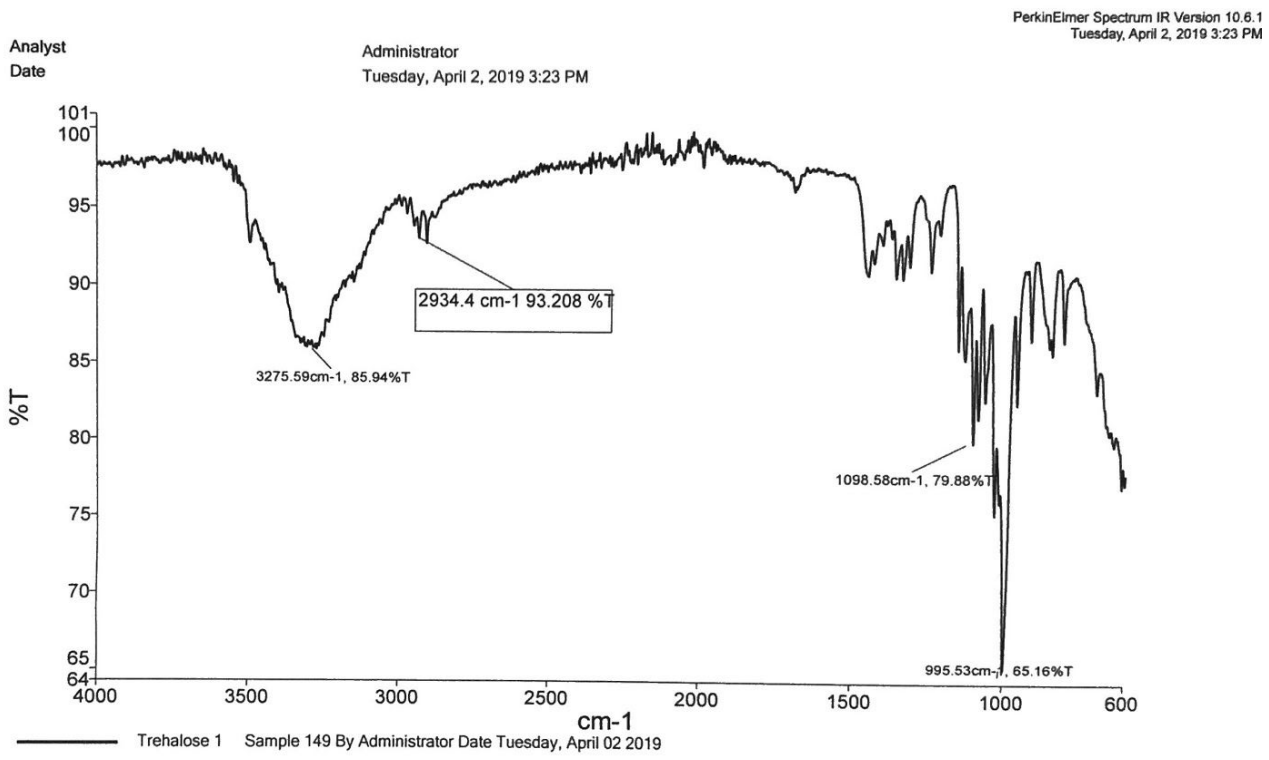


Fig. IR spectra for Trehalose.

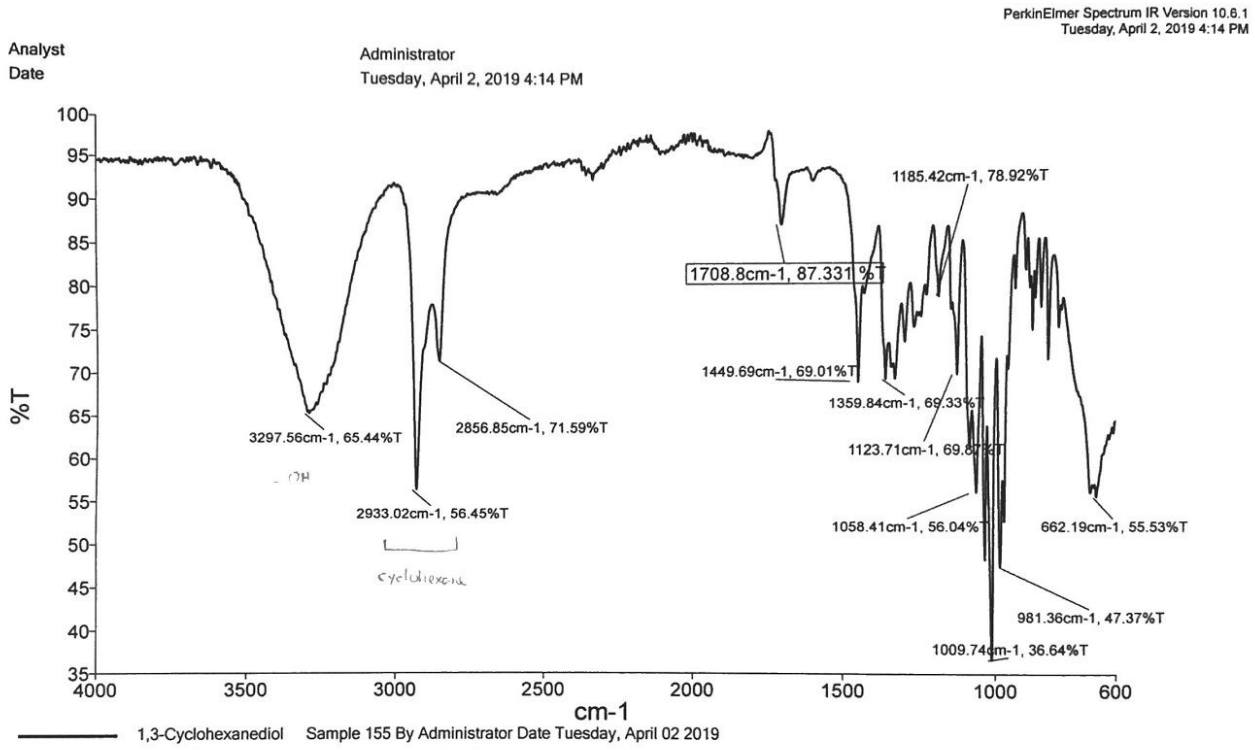


Fig. IR Spectra corresponding to 1,3-cyclohexanediol

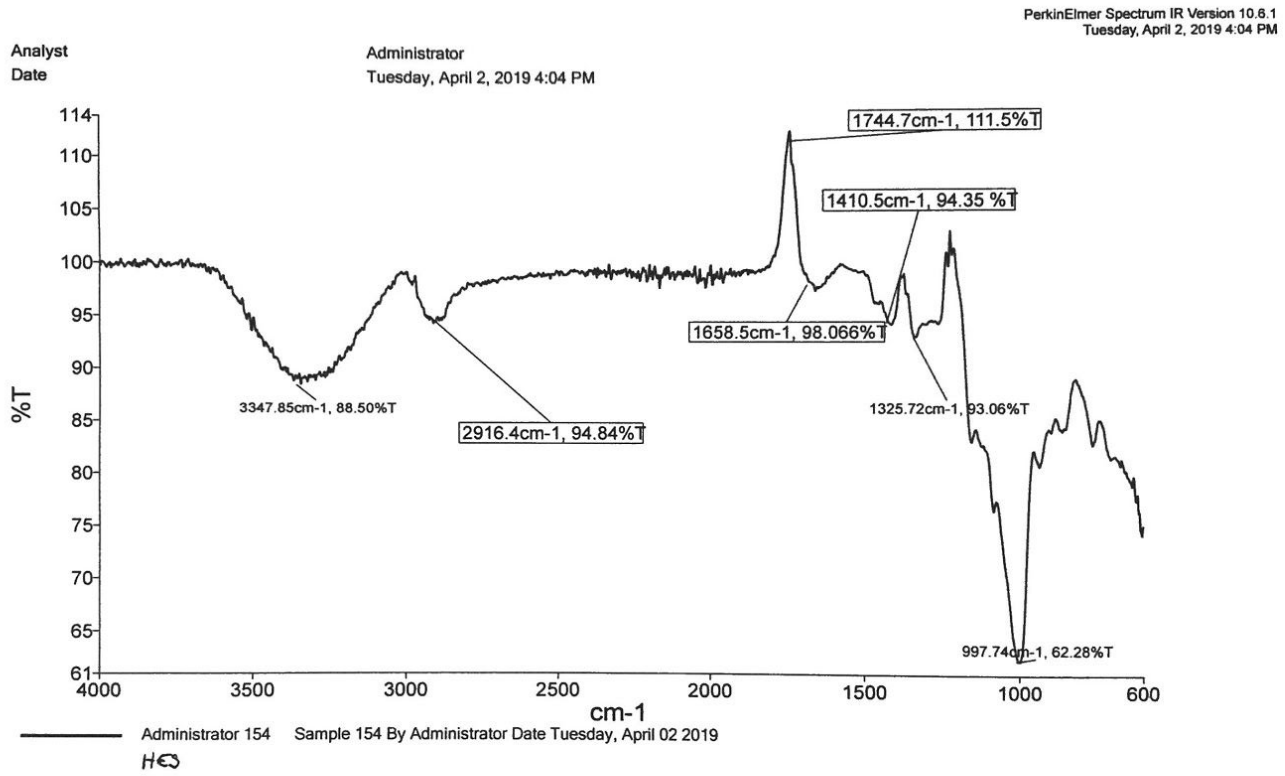


Fig. IR spectra corresponding to 2-hydroxyethyl starch

Appendix E: Workflows

Appendix E.1: Tissue Ring Workflow

1. Begin Culture (*derived from Rolle Lab Rat Aortic Smooth Muscle Cell Culture Manual*)

    a. Creating Media **[10 min]**
        i. DMEM (435 mL)
        ii. 10% FBS (50 mL)
        iii. 1% Penn/Strep (5 mL)
        iv. 1% GlutaMAX (5 mL)
        v. 1% NEAA (5 mL)

    b. Feeding Cells **[10 min, once every two days]**
        i. Pre-Warm Media to 37°C
        ii. Aspirate out old media
        iii. Add pre-warmed media (10 mL)

2. Splitting Cells **[30 min]**
    a. Once cells have reached 70% - 90% confluency, they are ready to be split.
    b. Aspirate Media
    c. Wash (2x) with DPBS- (5 mL)
    d. Trypsinize with .05% Trypsin (3mL)
    e. Place in Hood for 5 min
    f. Remove from hood and check under microscope that cells are detached.
    g. Add Culture Media (3mL)
    h. Spin cells at 200 rcf for 5 min
    i. Aspirate media and resuspend pellet in fresh Media (5 mL)
    j. Count Cells
        i. Pipette up and down in resuspended cells to ensure even distribution
        ii. Pipette 10 uL of suspension onto hemocytometer
        iii. Record cell count in top outside corners (squares with 4x4 squares in them)
        iv. Calculate average number of cells

3. Creating Molds *(from JOVE protocol)*
    a. Prepare PDMS negative by following Rolle JOVE Protocol
    b. Agarose Mold Production **[2.5 hrs]**
        i. Prepare 2% agarose solution
            1. 2% agarose

2. 98% DMEM

ii. Autoclave 2% agarose Solution & PDMS negative **[1.5 hrs]**
iii. Pipette 250 uL agarose into PDMS negative
iv. Allow to cool **[2 min]**
v. Gently 'pop' out agarose well using fingers/forceps
vi. Place molds in a plate **[48 molds takes approximately 2.75 hours]**
vii. Submerge molds in media (~ .3 mL)
viii. Let equilibrate overnight

4. Seeding Rings **[1 hr]** *(from JOVE protocol)*
    a. Prepare a solution of 10 million cells/ mL **[30 min]**
    b. Pipette 50 uL of cell suspension into each well. **[30 min]**
    c. Add (2 mL) fresh medium around outside of wells without letting spill into wells
    d. Incubate overnight
    e. Aspirate media outside mold
    f. Add (.45 mL) fresh media into plate to cover entire mold
    g. Replace media daily

5. Preparing CPA's **[1 hr]**
    a. Prepare stock CPA solutions
        i. Measure the corresponding amounts for each mixture and concentration (50ml)
        ii. Mix with media, store in cool area away from possible contaminants

6. Freezing
    a. Standard Freeze **[1 hr]**
        i. Retrieve CPA stock solution
        ii. Using forceps, place agarose mold containing tissue ring into cryovial
        iii. Quickly fill cryovial with CPA solution (.5 mL)
        iv. Depending on CPA solution, let sit
            1. Control **[0 min]**
            2. 10% DMSO **[0 min]**
            3. 6% 1-3 CHD + 15% Trehalose **[20 min]**
            4. 6% 1-3 CHD + 6% HES +15 % Trehalose **[20 min]**
        v. Place cryovial into Mr. Frosty, and place Mr. Frosty into -80 Freezer
    b. Vitrification **[1 hr]**
        i. Retrieve CPA stock solution
        ii. Using forceps, place agarose mold containing tissue ring into cryovial
        iii. Quickly fill cryovial with CPA solution.
        iv. Depending on CPA solution, let sit. See above
        v. Move cryovial from bath into cryotank rod/box
        vi. Place cryotank rod/box into cryotank

7. Thawing
    a. Standard Thaw **[30 min]**
        i. Take a fresh 15 mL conical tube and add in 9 mL of pre-warmed media
        ii. Take a small beaker with lukewarm tapwater
        iii. Take the cryovial out of the liquid N2/freezer and place in warm water
        iv. Let the solution completely thaw. **[1-2 min]**
        v. Using a p-1000 pipette, move the thawed solution into the conical tube.
        vi. Spin the tube at 200G **[5 min]**
        vii. Aspirate the supernatant
        viii. Resuspend cells/ rings
        ix. Store cells in incubator
    b. Checkpoint Thaw **[1 hr]**
        i. Follow Standard thaw procedure. At step iii, deviate to the following do not put in warm water.
        ii. Prepare a cooled bath at -4°C.
        iii. Take cryovial from freezer/ liquid N2, and place in bath **[5 min]**
        iv. Continue from step iii from standard thaw procedure by placing cryovial in prewarmed water.

8. Viability Testing
    a. AlamarBlue Protocol from ThermoFisher

9. Mechanical Testing
    a. Test method

Appendix E.2: Cytotoxicity Workflow

Plating Cells in 12 well Plate

1. Plate cells at 5,600 to 11,000 cells/cm^2 (wells are 3.8 cm^2)
2. Incubate overnight
3. Once the cells have reached 70-80% confluency, the media they are in is aspirated and the media with chemicals can be added
4. Incubate overnight
5. Add 1/10th alamarBlue reagent to the wells and let incubate 3-4 hours
6. Measure absorbance with spectrophotometer

Chemical Preparation

1. Determine the CPA concentrations needed to prove cytotoxicity
    a. Concentrations of CPA's used on this experiment
        i. 3% CHD + Media
        ii. 3% HES + Media
        iii. 7.5% Trehalose + Media
        iv. 3% CHD + 3% HES + Media
        v. 3% HES + 7.5% Trehalose + Media
        vi. 3% CHD + 7.5% Trehalose + Media
        vii. 7.5% Trehalose + 3% CHD + 3% HES + Media
    b. Controls created:
        i. Media
        ii. Media (no cells)
        iii. 10% DMSO
2. Stock solutions of each concentration to be tested were made in 15ml conical tubes. (Alternatively 50ml solutions can be prepared if more is needed)
    a. Chemicals measured in the following amounts
        i. 0.448g of CHD
        ii. 0.675g of HES
        iii. 1.778g Trehalose
        iv. 0.448g of CHD, 0.675g of HES
        v. 0.675g HES, 1.778g Trehalose
        vi. 0.448g CHD, 1.778g Trehalose
        vii. 1.778g Trehalose, 0.448g CHD, 0.675g HES
    b. Media added using sterilized pipettes, mix the solution until dissolved.
    c. Keep away from sources of contamination

Appendix F: Issues and troubleshooting

F.1 The design and production of tissue rings

Creating the rings and their molds took approximately six weeks before the rings were reliably made and the procedure was consistent. Some complications encountered during this time were the PDMS mold being broken, incorrectly sized plates (Figures 9 and 10), and rings tipping in the media solutions. Figures 9 and 10 shows plates that are too large or too small. The suggested plate size to grow and store tissue rings in their agar molds is a 28 well plate. A 96 well plate is too small to fit a ring in, and a 24 well plate is too large, so the rings are unstable and quick movements of the plate or pipetting in media can cause the agar molds to tip over in the well. If the mold tips over, the tissue rings will not form properly. The use of 48 well plates allowed for an appropriate fitting of the agarose molds into the wells without the complications of the larger wells (Figure 11).

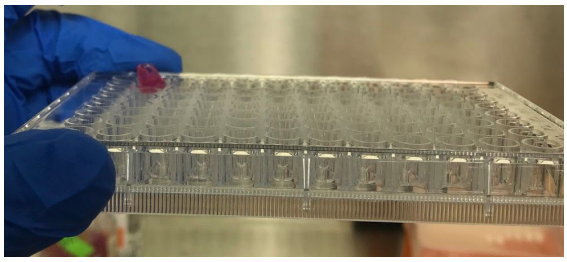


Figure 9. **Plate-to-mold sizing difficulty.** A tissue ring protrudes from the opening of a well in a 96 well plate, showing that the diameter of the ring is too large to fit into the well.

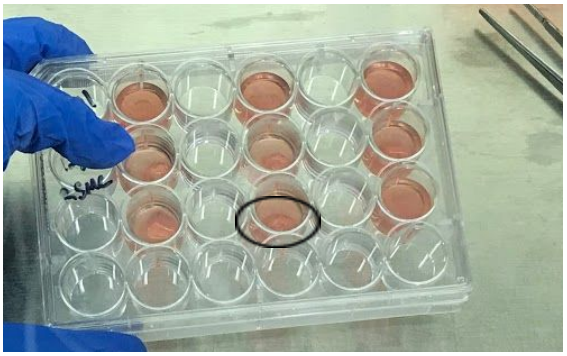


Figure 10. Tipped over mold in plate. 24 well plate, too large for rings. Circled in black is the ring placement in the well, and how they tip toward the bottom when the plate is slightly slanted.

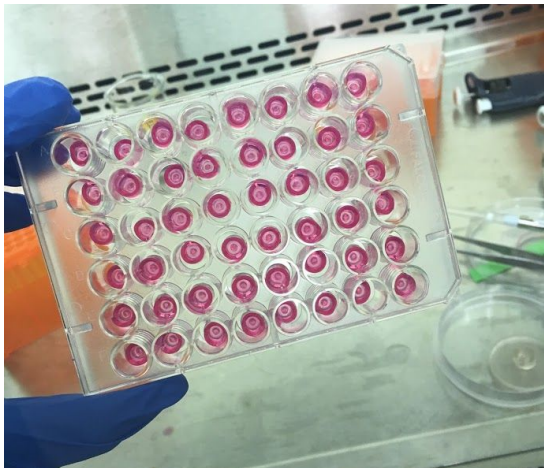


Figure 11. **Molds in a 48 well plate.** 48 well plate, with agarose molds before cells are seeded.

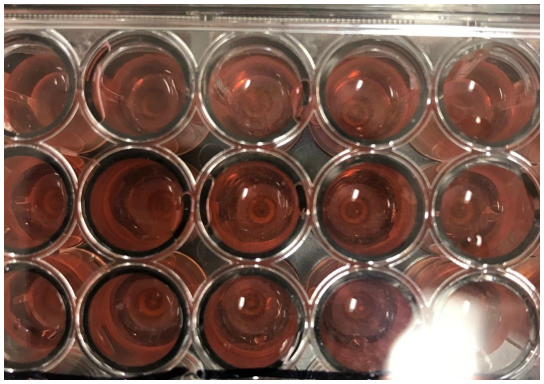


Figure 12. **Molds with tissue ring inside.** Rings growing in agarose molds in 48-well plate.

F2 Issues with cytotoxicity trials

Trials 1 and 2 were ran using 12 well plates, and trials 3 and 4 were ran with 96 well plates. The first trial was performed by adding in the cells and stock solutions of chemicals at the same time, then allowed to incubate. This produced over-confluency, along with additional stress in the cells, as they had to attempt to adhere to the wells while also undergoing stress from the chemical solutions. Images for the wells from this test can be found in Appendix D.2 under Trial 1. Trial 2 still had over confluency as they were left too long before analysing, however the difference was that the cells were given 24 hours to adhere to the wells before the stock solution of chemicals were added. Another fallback to this trial was that the alamarBlue was not yet available to use to test for viability after letting the cells incubate in the stock solutions for 24 hours. These images are also located in Appendix D.2 under Trial 2.

Trial 3 had the correct calculations to insure that the cells were not over confluent, the cells were added 24 hours prior to the stock solutions, and the alamarBlue had arrived to do viability tests. However, it was during this trial that we discovered the stock solutions used for trial 2 and 3 had become contaminated by bacteria, as discussed in sections 5.2.2. Therefore, none of the data from these two tests was usable. Figure 20 shows the contamination in the 12 well plates used in trial 2.

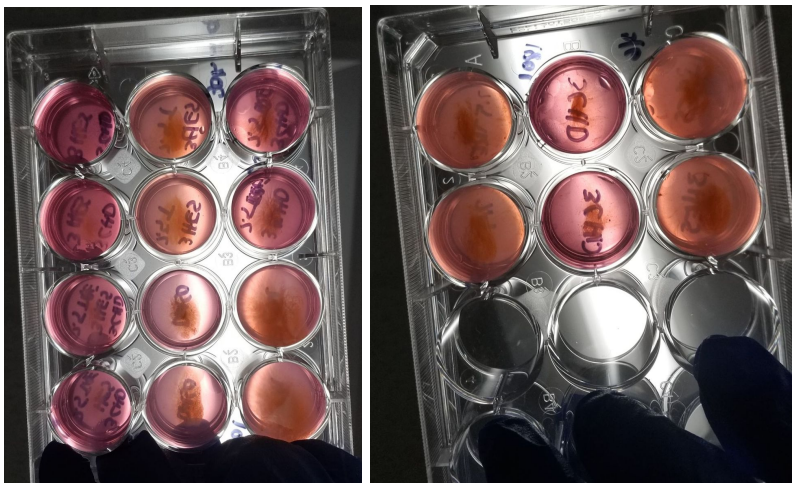


Figure 20. **Contaminated wells for the cytotoxicity test.** The color difference between the wells shows the contamination present in Trial 2 of the cytotoxicity tests. The oranger wells are the ones that contaminated media was used in and the pinker wells are the ones that contain CHD and did not get contaminated.

After the contamination of the initial set of stock solutions, a new set of stock solutions was made by taking into consideration the issues that occured with the first set. We reduced the amount of solution made in the conical tubes, used warm media and the centrifuge to dissolve the chemicals, and made sure to store them in the fridge with the rest of the media.

The solutions were checked after 9 days (Fig. 25); no signs of precipitation, color changes or other signs of contamination were present.

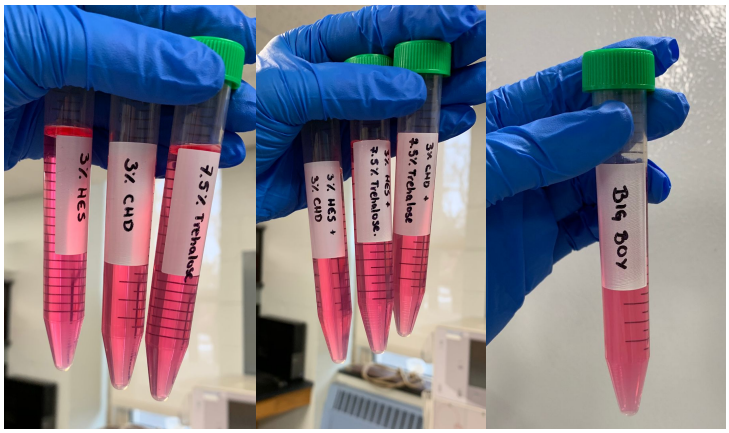


Fig 21. New stock solutions remain uncontaminated when not stored in incubator. Second set of stock solutions made for the cytotoxicity study.

6.3 1,3-Cyclohexanediol has antibiotic properties

Additionally, during cytotoxicity testing, we discovered that CHD can also work as an antibiotic. Each conical tube without CHD became contaminated, shown in Figure 18, where the contaminated solutions' media was orange and the tubes with CHD was still clear and bright pink, as sterile, unused media is. To prove the cause of this was from bacterial contamination, we performed a Trypan Blue test on the 3% HES stock solution tube, as shown in Figure 19. We did not have access to a microscope with a high enough magnification to examine the bacteria at the cellular level, so instead the Trypan Blue stained the outside of the cells to show their location.

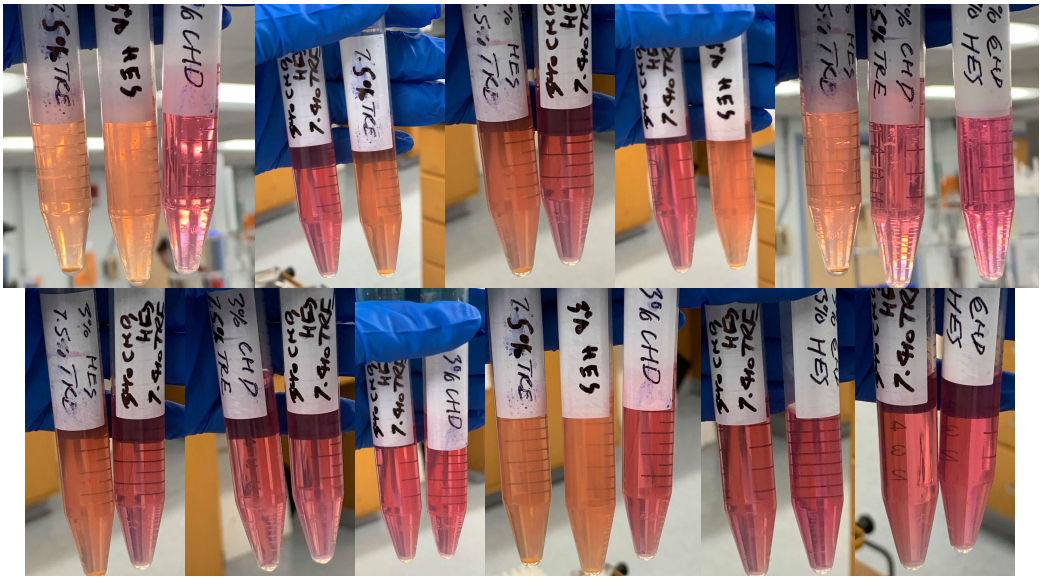


Figure 18. Media color comparisons show contamination in stock solutions. Combinations of stock solutions compared together to show the color difference of contaminated solutions containing HES and TRE, and non-contaminated solutions containing CHD.

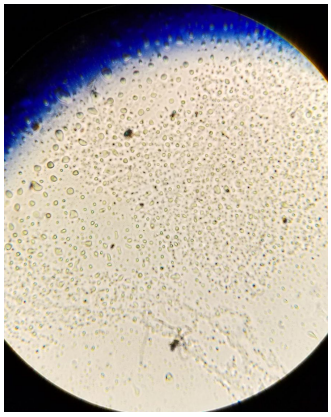


Figure 19. Bacterial contamination test was positive in stock solutions. Trypan Blue assay on contamination in HES stock solution, tested on 2/18/19, magnification of 40x.

F.3 Tensile testing issues

The rings used for these tests were grown for 6 days, whereas a healthy, fully mature ring would normally do so for 12. This meant that while the rings still aggregated, they were smaller than what was planned to be tested. This resulted in some samples having very thin sections that would break immediately upon removal of the agarose mold. Due to the fact the load cells for the Instron in the project lab did not have the precision to test the small tissue rings, a custom fixture was created to mechanically test the samples. This fixture can be seen in Figure 16.The rings that maintained integrity by themselves were placed on the top “grip” of the fixture while the bottom “grip” was placed to hang from the ring. This bottom “grip” consisted of a small hook that weights would be incrementally placed onto. Due to the immaturity, all rings failed upon releasing the bottom “hook”. This fixture can be seen in Figure . The rings tested for the custom CPAs were only able to grow for 6 days instead of the 12-14 days that previous rings used in viability tests had been growing.

Figure . Custom Fixture for Mechanical Testing. Custom load cell mechanism created by John Schwamb

Likely due to the immaturity of these rings, all samples (10% DMSO, 6% CHD and 15% Trehalose, and 6% HES, 6% CHD and 15% Trehalose) mechanically failed with just the weight of the bottom hook. This failure was under a load of 0.04 N. Given that healthy rings

normally have a UTS of 105 kPa, this shows that the mechanical testing was inconclusive and no relevant data should be drawn from it (Gwyther).