THE USE OF iPTD ENGINEERED ANTIFREEZE PROTEINS FOR CRYOPRESERVATION OF CELLS

by

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Abstract

Antifreeze proteins from natural sources have been discovered to have cryoprotective function against freezing temperature, and have been tested for the application for cryopreservation of biological materials. However, none has been shown to match the effectiveness of current chemical cryoprotectants, such as dimethyl sulfoxide. One potential limitation with the application of antifreeze proteins is that they may only stay in the extracellular space around cells whereas chemical cryoprotectants can be penetrative. In this thesis project, we have designed, purified and explored the function of antifreeze proteins that were engineered with an intracellular delivery signal peptide, known as iPTD. We showed that iPTD-engineered antifreeze proteins had effective cell surface coverage within 30 minutes of incubation as shown by flow cytometry; however no intracellular protein delivery was observed under multiphoton microscopy. The plasma membrane was protected by iPTD-engineered antifreeze proteins during cryopreservation as seen in Calcein dye release assay, but cell recovery or proliferation was not observed after thawing. Given these properties of iPTD-engineered antifreeze proteins, we used them as red blood cell cryopreservation additives. By adding these modified antifreeze proteins, we were able to reduce the amount of glycerol (used for RBC cryopreservation) necessary to control freeze-induced hemolysis. Furthermore, the quality of thawed red blood cells is higher as protein addition resulted in high retention of intracellular ATP.

Preface

This thesis has been written in partial fulfillment of the requirement for the degree of Master of Science in Experimental Medicine. I have written this thesis under the supervision of Dr. Caigan Du, Dr. Horacio Bach, and Dr. Haishan Zeng from September 2014 to November 2016. All the experimental works and analysis of this research project were performed by Han Qi Zhao in the Immunity and Infection Research Centre, Vancouver Prostate Centre and iProgen Biotech Inc.

The iPTD engineered antifreeze protein plasmids used in this thesis project were designed and kindly provided by Leo Lin, head scientist at iProgen Biotech Inc.

Multiphoton microscopy (Chapter 2.3) was performed in Dr. Haishan Zeng's lab at the British Columbia Cancer Research Centre with the help of Graduate Student: Giselle Tian

The animal experiments in Chapter 5 were performed in accordance with the Canadian Council on Animal Care guideline under the protocol approved by the Animal Use Subcommittee of the University of British Columbia. The Ethics approval certificate number is A11-0409

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List of Abbreviations

AFP: Antifreeze protein

ATP: Adenosine Triphosphate

DGDG: Digalactosyldiacylglycerol

DMSO: Dimethyl sulfoxide

DNA: Deoxyribonucleic acid

2,3-DPG: 2,3-diphosphoglycerate

eGFP: Enhanced Green Fluorescent Protein

FACS: Fluorescence-activated cell sorting

IPTG: Isopropyl β-D-1-thiogalactopyranoside

KDa: Kilo Dalton

LB: Luria-Bertani broth

LDH: Lactate dehydrogenase

MTS: 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium

PAGE: polyacrylamide gel electrophoresis

PBS: Phosphate-buffered saline

PMSF: phenylmethane sulfonyl fluoride

RBC: Red blood cell

RES: Reticuloendothelial system

SDS: Sodium dodecyl sulfate

Acknowledgements

I would like to offer my most sincere gratitude to my mentor Dr. Caigan Du for helping me gain knowledge in academic research and learn the skills necessary to think independently and logically. His support to my thesis project has been unconditional and invaluable. I would also like to thank my colleagues at iProgen Biotech Inc. for training and supporting me during my time as a graduate student. I owe particular thanks to Ken Lee, CEO of iProgen Biotech Inc. who allow me the opportunity to experience the biotechnology industry.

I thank Dr. Horacio Bach for his constructive criticism, which directs me to think deeper into the goal of my research. I also want to extend my thanks to Dr. Haishan Zeng for introducing me the physics aspect of biomedical research and allowing me to conduct my experiment using his confocal microscope system.

I am also grateful to the Natural Sciences and Engineering Research Council of Canada who has supported me through the industrial post-graduate scholarship in order to cover my tuition and living fees for the past two years.

And finally, to my mother: Dong Wang, and my father: Dong Hui Zhao. I am truly fortunate to have them as my parents. Their reassurance restored my confidence when I swayed from my path. Their encouragement gave me hope when all seemed impossible. Without them, I would not be where I am today.

To all those that helped me throughout my time as a student, I cannot thank you enough for your kindness.

Dedication

To my parents

Chapter 1: Introduction and Background

1.1: AFP Origins and Properties

Antifreeze proteins (AFP) are a class of structural protein that can bind to ice crystal surfaces. AFPs were first discovered in the blood of Antarctic fish by DeVeries, *et al.* (1) where specific carbohydrate containing proteins (later known as antifreeze glycoproteins) were found to be responsible for up to 30% of the total freezing-point depression of fish blood serum. Later research into these AFPs has elucidated several important properties of these proteins. The first property, as discovered in DeVeries' research, is thermal hysteresis. Thermal hysteresis is the phenomenon where the freezing point of a solution separates from its melting point (2). AFPs purified from a variety of different sources have shown to exhibit this phenomenon. For example, AFP from Antarctic fish, *Pleuragramma antarcticum (Pa)* and *Notothenia coriiceps (Nc),* were shown to have thermal hysteresis of 0.08 ℃ at a concentration of 0.5 mg/mL (3). Addition of these fish AFPs lowered the freezing point of the solution, as observed by nanolitre osmometer. The second property of AFPs is their ability to bind and inhibit large ice crystal growth in a phenomenon known as ice recrystallization inhibition. In the research published by Jorov, A *et al.* (4), they showed that Winter Flounder (*Pseudopleuronectes americanus*) AFPs can bind to ice crystals. By binding to ice crystals in solution, these AFPs minimize the formation of large ice crystals at the expense of small ones. Furthermore, they reduce the complexity of ice morphology in the solution as well (4). The third AFP property elucidated to date is the ability to stabilize plasma membrane in cold conditions. Tomczak, *et al.* studied the effect of AFPs on membranes by employing artificial membrane model containing the plant lipid digalactosyldiacylglycerol (DGDG) obtained from chloroplast thylakoid. They found that the

addition of AFPs inhibits the leakage of artificial membranes and these AFPs exhibited dosage dependant activity (5).

1.2: Applications of AFP in Literature and Biotechnology

Given all the three special properties of AFP, these proteins have been widely applied in biomedical research and biotechnology. Their ability to lower the freezing point of a solution and inhibit large ice crystal growth has prompted researchers to use them as cryoprotectant of biological samples. Recently, researchers have used AFPs to preserve bovine embryos and mouse oocytes. Ideta, A *et al.* found that AFP from notched-fin eelpout (*Zoarce selongatus Kner*) was able to prolong the viability of bovine embryos stored at 4 ℃ from 4 days up to 10 days. Embryos treated with AFPs also had higher post-warm-up fertility compared to non-treated group (6). Furthermore, mouse oocytes supplemented with AFP purified from Arctic yeast (*Leucosporidium* sp.) had higher post-thaw viability after vitrification at -196 ℃ (7). Together, these results show that AFPs have the potential to be used as a biological cryoprotectant.
In addition to biomedical applications, AFPs have also found its target in the food industry. Recently, AFPs were used as a food additive to increase the creaminess of ice creams. Extract from cold-acclimated winter wheat grass containing AFPs were added to ice cream to enhance flavour by controlling the growth of large ice crystals. AFPs significantly reduced the growth of large ice crystals and enhanced the flavour and richness of the ice cream (8).

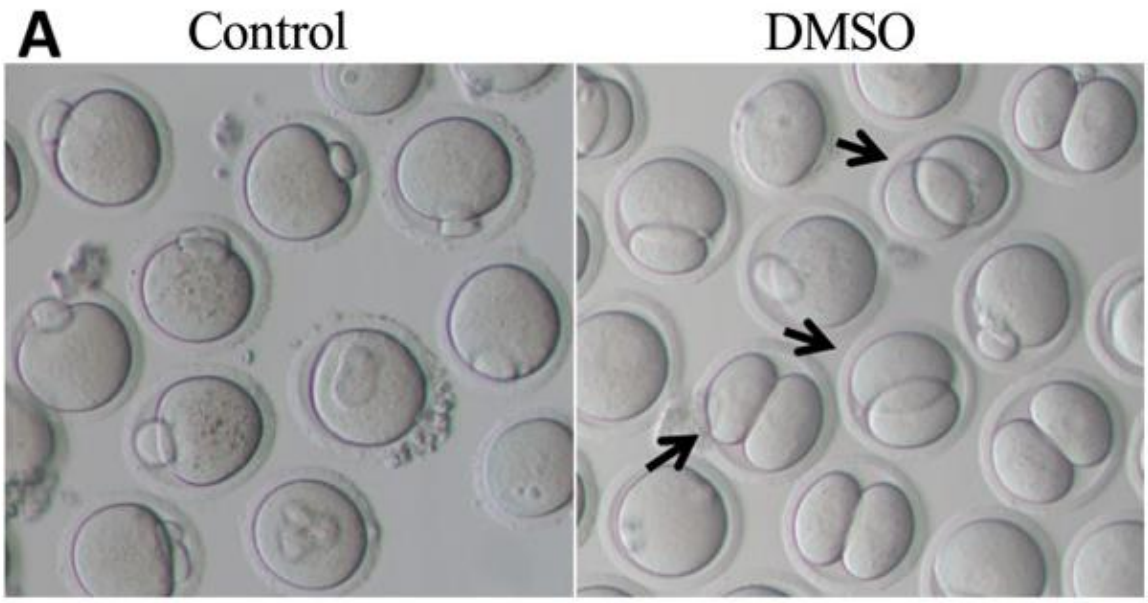
1.3: Cryobiology of Biological Samples

The purpose of cryopreservation is to lower temperature in order to limit the unregulated enzymatic activity and other chemical kinetics that damage the biological materials (9).

Cryopreservation is typically carried out in -80 ℃ using solid carbon dioxide or at -196 ℃ in liquid nitrogen (10). Several factors are important for successful cryopreservation. First, cooling rate and warming rate have a significant effect. Cooling rate determines the speed of water transportation across the membrane. If cooling rate is slow, water has time to flow outside of the cell to balance increased extracellular osmolarity (due to partial freezing of extracellular solution), and intracellular space will not be cooled below its freezing point (11). In this way, most ice will be extracellular, rendering the intracellular space undamaged. If cooling rate is too fast, then there is not enough time for water to flow out, and the intracellular space is super-cooled to a greater extend, exceeding the freezing point and increasing the possibility of internal ice formation (11). On the other hand, if the warming rate is slow, ice have to ability to recrystallize and thus forming large crystals instead of smaller ones (12). These large ice crystals damage the cells during the thawing process. Rapid warming rate prevents this by not giving enough time for the recrystallization to occur (12). However, more recent evidences have begun to show that cooling rate and warming rate are dependent on the biological sample to be cryopreserved (13,14,15)

Another important determinant in successful cryopreservation is the addition of cryoprotectants. Usually, cryopreserving biological samples without cryoprotectant is fatal. Some typical cryoprotectants in use today are glycerol, dimethyl sulfoxide (DMSO), and propylene glycol (16). At low cooling rate, cells without cryoprotectant will be subjected to exposure to high concentration of salt due to partial freezing of extracellular water (9). This high salt concentration damages the cell by extracting liquid out of the cell by the process of osmosis. Cryoprotectant, such as glycerol will modulate the rise in salt concentration outside of the cell as the water freezes (9). In addition, cryoprotectants eliminate ice formation during

cryopreservation and thus prevent ice damages to the cells (17). Cryoprotectants will also penetrate cells in order to prevent ice crystal formation internally. This means that the cryoprotectants are usually small enough to diffuse through the membrane by passive diffusion (61). Despite the success of ensuring high viability of biological samples post-thawing, standard cryoprotectants used today are shown to exhibit toxic effects on preserved biological samples. For example, a high concentration of glycerol used in cryopreservation induced acute kidney injury and subsequent kidney failure in rats through apoptosis and inflammation (18). Moreover, DMSO has been shown to alter the differentiation potential of human embryonic stem cells (19). DMSO has also been observed to induce early polar body formation of mouse moocyte during asymmetric differentiation (93). These evidences raise question about the risks associated with using chemicals as a standard cryoprotectant for biological samples.

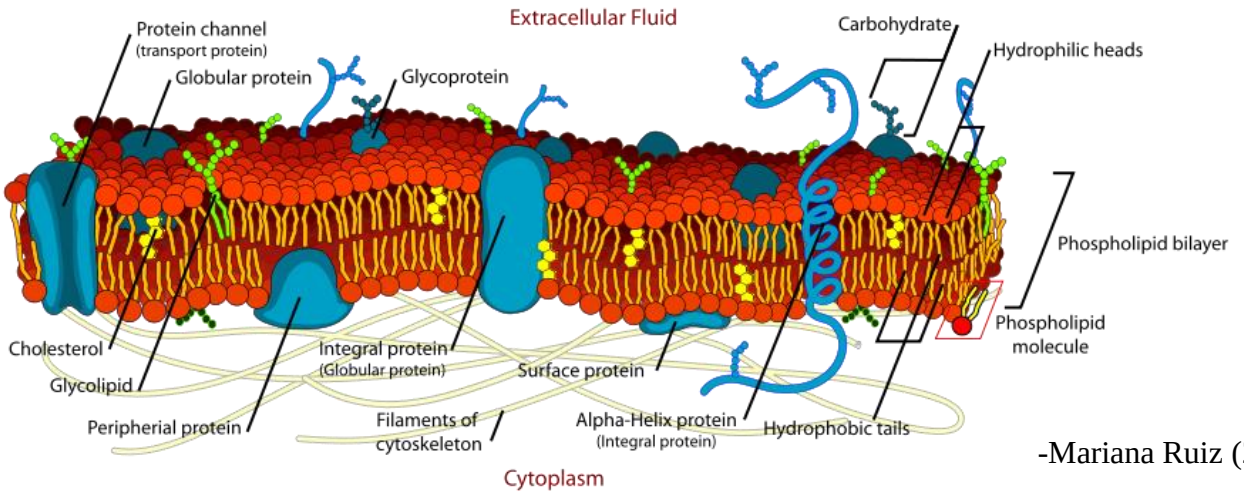


-Zhou, D *et al. 2014* (93)

Figure 1.1: Early polar body development of mouse moocyte during asymmetric differentiation after exposing to 3% DMSO

1.4: Plasma Membrane

Cell membrane, or plasma membrane, is a biological barrier that separates the interior environment of cell from the external environment (86). This biological cell membrane consists of phospholipids that assemble into bilayer with embedded proteins and other molecules (87). The cell membrane plays an important role in the life of the cell. Its functions include transportation of nutrients through passive diffusion or trans-membrane protein channel (88), and endocytosis of extracellular molecules (87). When temperature is lowered, the cell membrane fluidity decreases due to solidification of the phospholipids. Maintaining membrane fluidity is important for proper cell membrane function. For example, protein affinity to the plasma membrane decreases when the plasma membrane transition to a gel phase from a liquid phase with decreased temperature (89). Evidences have shown that there are numerous ways which organisms employ to maintain proper membrane fluidity. Cholesterol has been found to decrease lipid packing and increase membrane fluidity in the lipid bilayer at low temperature (90). Other organisms, such as bacteria, would change the lipid composition of the bilayer using an enzyme called desaturase to prevent solidification of membrane (91). AFPs have also been discovered to interact with cell membrane in low temperature conditions. By using circular dichroism, Kar *et al.* were able to find evidence of AFPs association with plasma membrane (92). Furthermore, Tomczak *et. al.* have suggested that AFPs bind to the lipid bilayer and alter the molecular packing of the lipid acyl chains (5). These evidences have led to the study of AFPs as a cell membrane stabilizer at low temperature.



-Mariana Ruiz (2007)

Figure 1.2: Eukaryotic cell membrane

1.5: iProgen Intracellular Delivery Technology

iProgen Biotech is a local biotech company (Richmond, BC) and has developed a novel technology that is capable of bringing cargo protein into the cell. In normal delivery platform, drugs have to be small enough to diffuse through the membrane, encapsulated in liposomes or enter the cell through electroporation for the delivery to occur (21,22,23), but these methods have certain limitations. For example, the first method of delivery relies on the size of the drug, the bigger the drug is, the more difficultly it is to diffuse across the membrane. Also, although this delivery is relatively efficient, there is a problem of off-target drug delivery. Moreover, many drugs, such as hormone-based drugs, are extremely difficult to produce in small sizes and therefore the variety of drugs that is capable of being delivered in this method is limited. Liposome suffers from quick clearance by the reticuloendothelial system (RES) (24). Therefore, drug-containing liposomes might be destroyed before they can reach the targeted cells. Moreover, liposomes can also be unstable *in vitro* and thus have a short shelf life (25). Electroporation of

the cell may induce cellular damage and cause the secretion of microparticles. These particles may illicit inflammatory responses, which may harm the patients (26). iProgen has formulated a special delivery tag that is produced with the cargo protein in a single polypeptide chain. The special tag contains signal peptide which cell recognizes for intracellular uptake (20). By recombinantly expressing a protein and iPTD tag as a single polypeptide, the protein can enter the cell through endocytosis pathway.

1.6: Red Blood Cell Cryopreservation

According to report published by BCC Report Analysts, the global market for blood products was 23.5 billion dollars in 2013 (62). Red blood cells (RBCs) or erythrocytes are the most abundant type of blood cells. They are responsible for carrying oxygen to body tissues and pick up the toxic CO_2 for exchange and release to the atmosphere at the lung. RBCs are enucleated and lack many major organelles (e.g. mitochondria) common for other mammalian cells (27). The cytoplasm of RBCs is rich in hemoglobin, which is essential for their main function since this protein contains iron that binds to oxygen, facilitating its transportation in the body. The RBCs have an average life span of 100-120 days before they are recycled in the spleen (28). RBC cryopreservation is an important method for prolonging the shelf life of isolated blood product used mainly in blood transfusions.

There are several rationales for the research and development of RBC cryopreservation. First, the military is one of the biggest users of cryopreserved RBCs (63). During combat situation, injured soldiers require replenishment of RBCs. However, due to the extreme condition of the battlefield and the long transportation distances, blood products need to be cryopreserved with good post-thawing quality to fulfil combat requirement (29). Second, cryopreservation of RBCs allows for

stockpiling of RBCs in preparation for natural disaster situation when a sudden large number of blood units may be needed (30). Last, RBC cryopreservation can be used to prolong the life of rare blood type RBCs such as AB^- , which is present in approximately 1% of the population (31).

Currently, the standard protocol for cryopreserving RBCs involve perfusing the cells with 40% glycerol prior to slow freezing at 1°C/min up to -80°C (33). Glycerol is a penetrative cryoprotectant that enters the cell through plasma membrane and create hyperosmotic environment inside the cell. This prevents intracellular ice from damaging the RBCs (32). However, prior to transfusion to patient, the glycerol must be washed off to avoid osmotic lysis of patient cells. The glycerol washing step is strenuous, expensive and time consuming. Moreover, the washing procedure also induces RBC lysis and lowers the post-cryopreservation RBC quality (34).

Chapter 2: Protein Production and Delivery Kinetics Studies

2.1: Protein Production and Purification

Rationale: To recombinantly produce high quality, quantity and pure iPTD engineered AFPs

2.1.1: Materials and Methods

Plasmid Transformation and Protein Production

AFP and iPTD sequences were generated by iProgen Biotech and synthesized by Genscript (860 Centennial Ave. Piscataway, NJ 08854 USA). In total, three plasmids were synthesized. DNA 910 contained green fluorescent protein (GFP) linked to AFP from the insect *Rhagium inquisitor* (Coleoptera) was engineered with iPTD delivery tag with 9 histidine tags at the C-terminus. DNA 908 contained AFP from ocean pout *Zoarces americanus* engineered with iPTD delivery tag with a histidine tag at the C-terminus. DNA 1643 contained wild type AFP from ocean pout *Z. americanus* with a histidine tag at the C-terminus. The three plasmids were individually transformed into competent BL-21 *E. coli* according to protocols published by Chan, W *et al.* (35). Briefly, 2 μL of synthesized plasmid was added to 50 μL of competent BL-21 cells. The cell mixture was incubated on ice for 30 minutes, heat shocked using a table top heat plate set to 42 ℃ for 45 seconds, and incubated on ice again for 30 minutes before 200 μL of Luria-Bertani broth (LB) at room temperature was added to each cell mixtures. The cell mixtures were then left to recover for 1 hour in a 37 ℃ shaker. After recovery, 50 μL of each cell mixture was poured and spread onto an agar plate containing 50 μg/mL kanamycin. The plate was incubated at 37 ℃ overnight. Next day, an individual colony was inoculated into 5 mL of LB broth supplemented with the same concentration of kanamycin as stated earlier and shaken at 37 ℃ for 3 hours before

all of the volume was added to a 2 L flask containing 1 L of warmed LB broth supplemented with the same antibiotic and concentration. The flask was shaken at 37 ℃ until the OD_{600} reading of the culture was 0.6-0.7. Then, 1 mM of Isopropyl β-D-1-thiogalactopyranoside **(**IPTG) was added to each flask to induce protein production and the flasks were shaken overnight at room temperature. The next day, cell pellet was collected by centrifuging the cell culture from the flask at 2000 × g for 10 minutes. The bacterial pellet was stored at -80 ℃ or used immediately for protein purification.

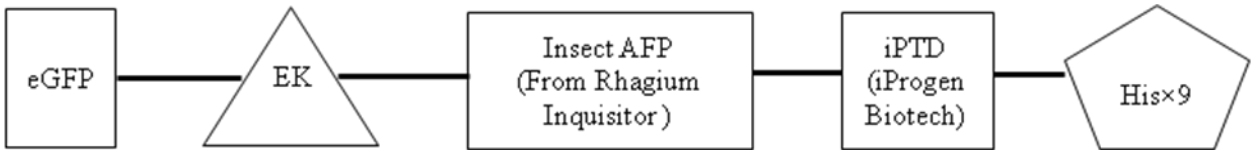
Protein purification:

Frozen bacterial pellet was completely thawed in 37 ℃ water bath and sonicated according to protocol published by Sellect, W *et al.* (36). Briefly, thawed cell pellet was suspended in lysis buffer (20 mM Tris pH 8, 400 mM NaCl, 20 mM Imidazole) with 10 mM of protease inhibitor phenylmethylsulfonyl fluoride (PMSF). The re-suspended cell pellet was sonicated using a VWR sonicator (50% duty cycle) for 9 cycles. After sonication, the cell suspension was centrifuged down at 9800 × g for 50 minutes and the supernatant was loaded onto a protein column pre-loaded with 4 mL of nickel resin (Bio-Rad 1329 Meyerside Drive Mississauga, Ontario, Canada). Immobilized metal affinity chromatography was performed according to published work by Yip, T *et al.* (37). Briefly, protein loaded column was washed with an increasing concentration of imidazole (20 to 500 mM). Eluted protein fractions were analyzed using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) following the work performed by Shapiro, A *et al.* (38). Briefly, 5 µL of eluted protein fractions was mixed with 5 µL of 4 × sample buffer (62.5 mM Tris-HCl pH 6.8, 2.5 % SDS, 0.002 % Bromophenol Blue, 0.7135 M (5%) β-mercaptoethanol, and 10 % glycerol in 1 × sample buffer) and 10 µL of de-ionized water. The

mixed protein was boiled for 5 minutes on a 100 ℃ heat plate. Sample was loaded onto a 10% SDS-PAGE and subjected to a constant voltage of 200 V for 40 minutes. After the gel finished running, fractions with purified target protein were pooled together and transferred to dialysis bag (Spectrumlabs Spectra/Por 3 cutoff 3.5 kDa and Spectra/Por 4 cutoff 12 kDa, 18617 S Broadwick St Rancho Dominguez, CA 90220, USA). Protein 910 and 908 were dialyzed in Spectra/Por 4 dialysis bag; whereas Spectra/Por 3 was used for protein 1643. The dialysis bag was placed overnight in 2 L of solution containing 20 mM Tris-HCl pH 8, 400 mM NaCl, 50 mM L-arginine, and 50 mM L-glutamate. Next morning, the dialyzed protein solution was concentrated using Amicon Ultra-15 Centrifugal Filter units (Millipore Sigma, 3050 Spruce St. St. Louis, MO 63103 USA) down to 1-1.5mL of volume. The concentration of the purified protein was determined using Bradford method (39).

2.1.2: Results

All of these three recombinant proteins were successfully produced by *E. coli* in our lab, and were purified by the nickel column. The purity of protein GFP-910-iPTD (71 kDa) is shown in Figure 2.1, protein 908-iPTD (39 kDa) in Figure 2.2 and protein 1643 (8 kDa, without iPTD) in Figure 2.3.



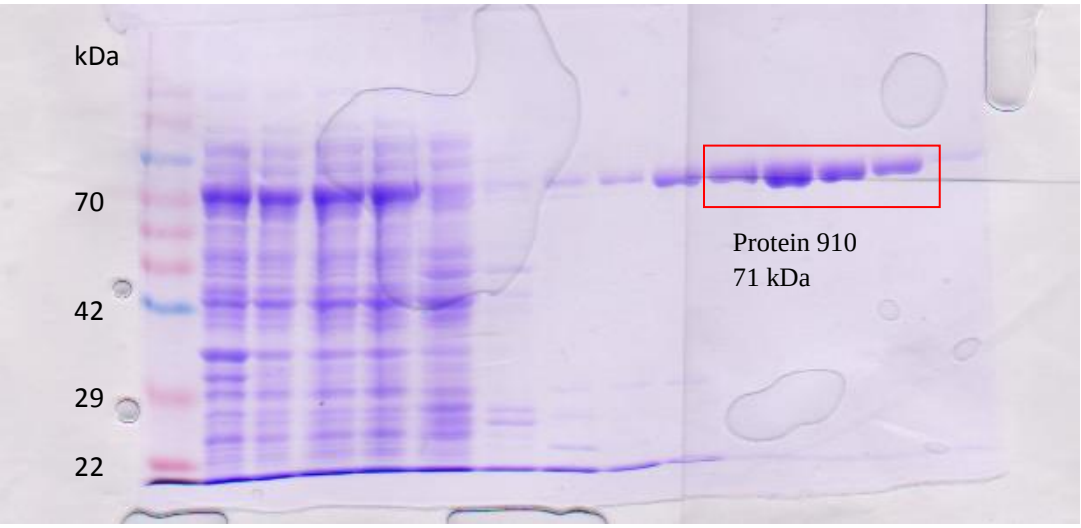


Figure 2.1: Purified protein 910 resolved by 10% SDS-PAGE analysis. Bands in the red box indicate purified proteins from the elution buffer. Protein molecular weight markers are indicated on the left. This gel illustrates a typical representation of 7 separated purification preparations.

The approximate molecular weight of protein 910 is 71 kDa. The protein was eluted with an increasing concentration of imidazole to compete the protein off stationary nickel. The protein started eluting at imidazole concentration of 100mM and finished at 450mM. The first few imidazole elutions (100mM to 200mM) contained an unidentified protein at 20 kDa. Other faint bands were also observed between 22-29kDa weight range. These initial fractions were therefore not included in the dialysis. Fractions without contaminating proteins were pooled together for dialysis and concentration (boxed in red, Figure 2.1). The final yield for this protein was 7mg per litre of culture.

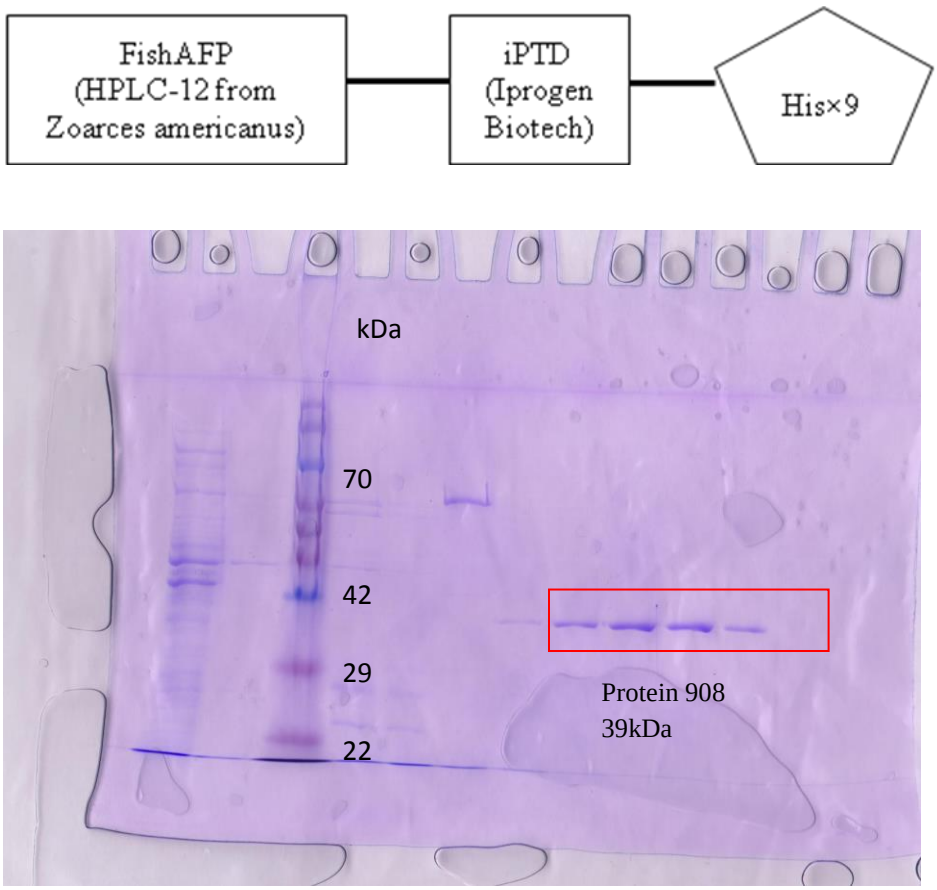


Figure 2.2: Purified protein 908 resolved by 10% SDS-PAGE analysis. Bands in the red box indicate purified proteins from the elution buffer. Protein molecular weight markers are indicated on the left. This gel illustrates a typical representation of 10 separated purification preparations.

The approximate molecular weight of protein 908 is 39 kDa. Protein 908 started eluting at imidazole concentration of 100mM and finished eluting at 300mM. Similar to protein 910 purification, the initial fractions contained contaminating proteins at around 20kDa. Furthermore, there was also an extra protein bands at 69kDa. Fractions without contaminating proteins were pooled together for dialysis and concentration (marked in red, Figure 1.2). The final yield for this protein was 3mg per litre of culture.

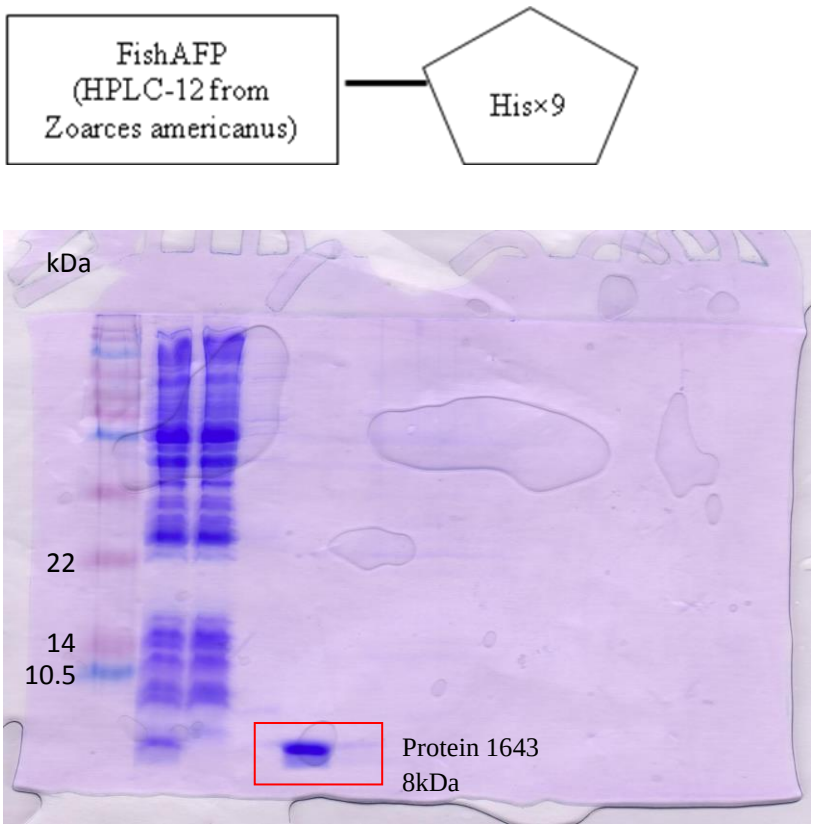


Figure 2.3: Purified protein 1643 resolved by 16% SDS-PAGE analysis. The band in the red box indicates the purified protein from the elution buffer. Protein molecular weight markers are indicated on the left. This gel illustrates a typical representation of 7 separated purification preparations.

The approximate molecular weight of protein 1643 is 8 kDa. This protein was eluted at imidazole concentration of 100mM. No further protein elution was observed in the subsequent imidazole concentrations. This fraction was dialyzed and concentrated. The final yield for protein 1643 was 1mg per litre of culture.

2.2: Flow Cytometry Analysis of iPTD Delivery Kinetics

Rationale: To determine the required time for cellular internalization of iPTD. Protein 910 is recombinantly expressed with Green Fluorescent Protein (GFP), which exhibit bright green fluorescence at 509 nm when exposed to light at a wavelength of 395 nm. By incubating protein 910 with Jurkat cells at different time points, we can determine the time kinetics of iPTD interaction with target cells using flow cytometry.

2.2.1: Materials and Methods

Flow cytometry was used to analyze the cell GFP fluorescence at various time points following the addition of protein 910. The protocol of detecting GFP fluorescence using flow cytometry was adopted from work by Chu, Y, *et al.* (40). Briefly, 2.5×10^5 Jurkat cells (Clone E6-1, ATCC® TIB-152™, 10801 University Boulevard Manassas, VA 20110 USA) were seeded in 500 μL of RPMI-1640 (GE Healthcare Life Sciences, HyClone Cat No.: SH30027.01, 100 Results Way Marlborough, MA, USA) supplemented with 10% fetal bovine serum (GE Healthcare Life Sciences, HyClone Cat No.: SH30071.01) and 1% Penicillin-Streptomycin (GE Healthcare Life Sciences, HyClone Cat No.: SV30010). Protein 910 was added at 50 μg/mL. The cells were then incubated in an incubator supplemented with 5% CO_2 at 37 ℃. Cell samples were taken out at 10, 30, 60, 120, 180 and 300 minutes after protein addition. Cells were washed twice with 10 mL of phosphate buffered saline (PBS) (Thermo Fisher Scientific Cat No: 10010023, 168 Third Avenue Waltham, MA USA 02451). Final volume was adjusted to 500 μL and was analyzed by BD FACSCanto II Flow Cytometer. Percentage of GFP^+ cells at each time point was recorded, as well as the mean fluorescence intensity for each sample.

2.2.2: Results

We used protein 910 (GFP-AFP-iPTD) to investigate the kinetics of AFP-iPTD binding to the target cells. As shown in Figure 2.4, after 10 min of incubation, the staining of Jurkat cells with protein 910 reached a maximum of 70%, which remained unchanged for the next 300 minutes. However, the fluorescence intensity of GFP in these stained cells started declining after 30 min of incubation (Figures 2.5).

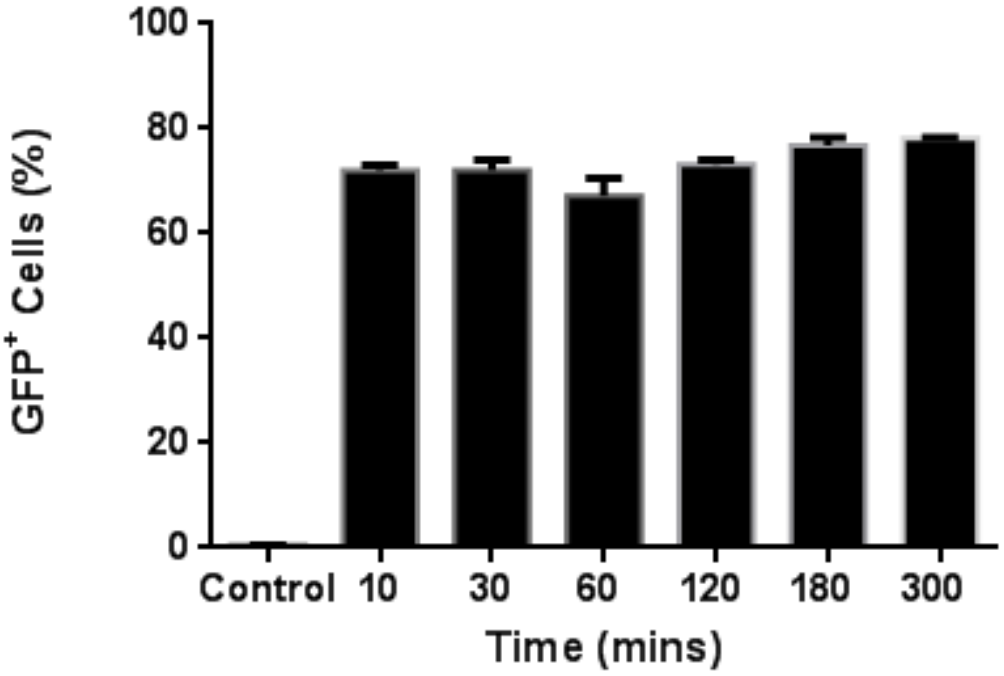


Figure 2.4: Percentage of GFP positive cells at various time-points after cell incubation with the protein. PBS was added to "Control" sample in place of protein 910. A total of 10,000 events were counted in each sample. The error bar represents the standard deviation (SD) from mean of three independent samples in one experiment.

Figure 2.4 shows the percentage of cells that is detected with protein 910 (GFP-AFP-iPTD) at different time-points. There is close to 0% of GFP^+ cells in "control" sample. This was expected as protein 910 was not added. Within 10 minutes of protein 910 and cell incubation, up to 70% of the Jurkat cells could be detected with proteins on their cell membrane. Longer incubation time did not significantly increase or decrease the percentage of GFP^+ Jurkat cells.

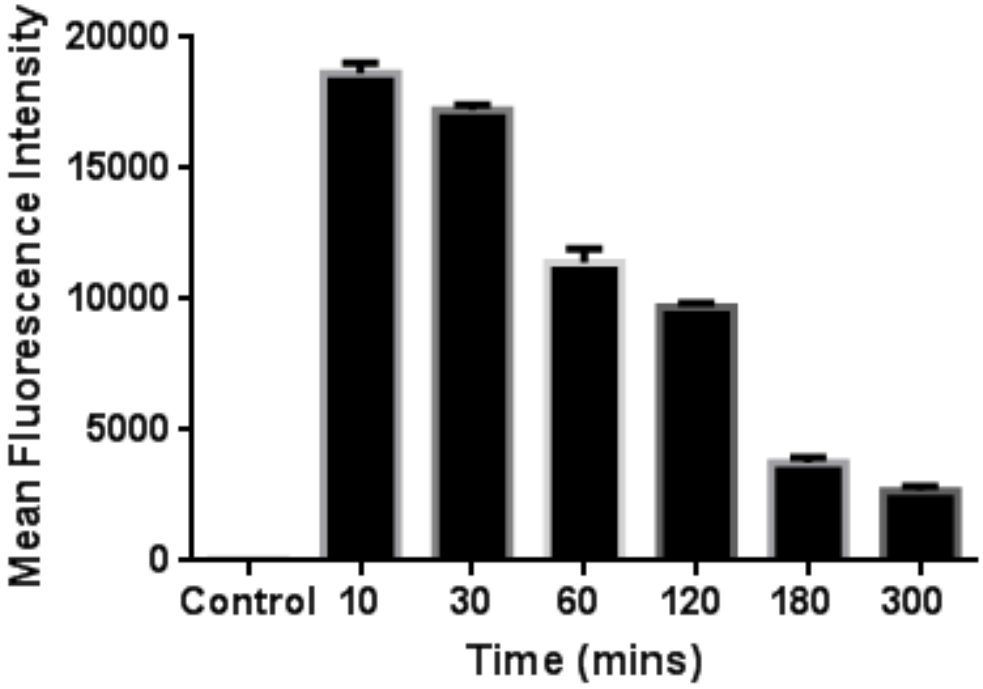


Figure 2.5: Mean GFP fluorescence intensity of cells incubated with protein 910 for various duration of time. PBS was added to "Control" sample in place of protein 910. The error bar represents the standard deviation (SD) from mean of three independent samples in one experiment.

Although the percentage of GFP^+ cells remains relatively unchanged over 300 minutes of protein and cell incubation as shown in Figure 2.4, the intensity of GFP fluorescence declined significantly. As seen in Figure 2.5, the fluorescence intensity of cells decreases with longer incubation time. The decrease in intensity was more significant past 30 minutes of incubation time. By 60 minutes, the fluorescence intensity has decreased to almost half of the original intensity shown at 10 minutes. This means that although protein 910 was still detectable on 70% of the cells after 300 minutes, the concentration of 910 on cell surface decreased significantly overtime. This disappearance of signal could mean that protein 910 has begun entry into the cell intracellular space, which is the hypothesized function of iPTD. In order to answer these questions, we need to observe the intracellular space of the cell.

2.3: Multiphoton Microscopic Analysis of Cells Treated with Protein 910

Rationale: To confirm whether protein 910 was intracellularly delivered at 30 minutes incubation with the target cells, using multiphoton microscopy.

2.3.1: Materials and Methods

Jurkat cells were cultured as previously described (see section 2.2.1). Protein 910 was added to Jurkat cells at 50 μg/mL and incubated for different periods of time and analyzed by a multi-photon microscope constructed in-house at Dr.Zeng's lab (BC Cancer Research Centre). This microscope utilizes a tunable fs Ti:Sapphire laser. It is scanned over the back aperture of a 60 × water-immersion objective lens. The Field of View is 20 μm × 20 μm. The signal collected is epi-two photon fluorescence.

2.3.2: Results

To further verify whether protein 910 is internalized by the cell, the cellular location of GFP-labelled 910 was examined in a Jurkat cell using a real-time multiphoton microscope after 10 and 30 minutes of incubation. As shown in Figure 2.6, at both time points, GFP was localized on the cell surface, but no sign of fluorescence was observed inside the cell.

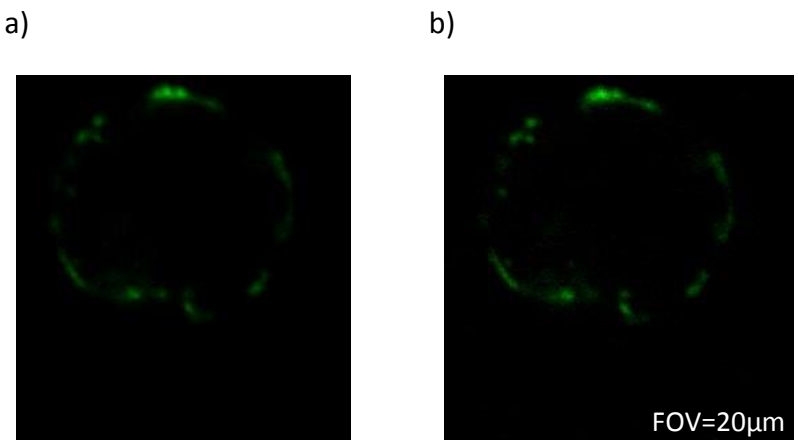


Figure 2.6: Multi-photon microscopy analysis of iPTD protein 910 cell delivery kinetics. Protein 910 and cells were incubated for (a) 10 minutes and (b) 30 minutes before microscopic analysis. Both images were generated by averaging the fluorescence intensity of 60 frames.

Figure 2.6 shows result of protein 910 attachment to Jurkat cell. After 10 and 30 minutes of protein and cell incubation, there was membrane coverage as shown by the GFP fluorescence around the cell plasma membrane. In both images, around 70% of the cell membrane was covered by GFP fluorescence. The intracellular space of the cell appears to be dark, suggesting very little or no GFP protein was detectable inside the cell.

Summary

In this chapter, we have purified iPTD engineered antifreeze proteins and investigated their delivery kinetics. The iPTD engineered antifreeze proteins were produced with relatively high purity. In order to find out how iPTD delivers protein to cells, we conducted some kinetics studies. By using flow cytometry analysis, we found that the majority of cells surface can be detected with iPTD-engineered protein after only 10 minutes of incubation. However, the concentration of the protein on the surface decreases dramatically if the incubation time past 30 minutes. Furthermore, Multi-photon microscopy confirmed that iPTD-engineered protein had good plasma membrane coverage of cell; but little to no intracellular delivery was observed.

Chapter 3: AFP-iPTD Functional Analysis

3.1: Membrane Integrity Assessed by Lactate Dehydrogenase (LDH) Release Assay

Rationale: After elucidating the iPTD kinetics, we begin to understand the functions of iPTD conjugated AFPs. Since the effective range of the temperature of our proteins is unknown, we decided to try a variety of conditions. From the last chapter, we observed good cell membrane coverage of iPTD-AFPs. Therefore, we first wanted to see whether our iPTD-AFPs have any preventive activity against ice induced cell membrane damage. Lactate dehydrogenase (LDH) is an intracellular enzyme that is released into extracellular space upon plasma membrane damage. We use the ratio of extracellular LDH compared to total cell LDH as a measurement for membrane integrity.

3.1.1: Materials and Methods

Jurkat cells were cultured at 37 ℃ supplemented with 5% CO_2 in RPMI-1640 supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin according to protocol by Abraham, R *et al.* (41). Briefly, frozen Jurkat cells (2×10^6 cells/mL) were thawed quickly for 2 minutes in 37 ℃ water bath. Cell tubes are swirled around in water bath until only a little bit of ice remained in the cell solution. Then 10 mL of pre-warmed (37 ℃) supplemented RPMI-1640 media was added to the thawed Jurkat cells and centrifuged at 2000 × rpm for 5 minutes. Supernatant was aspirated and cell pellet was re-suspended in 1 mL of fresh media before adding to a Corning T25 cell culture flask containing 10 mL of fresh, pre-warmed RPMI media. The Jurkat cells were sub-cultured when the cell density reached 8×10^6 cells/mL to maintain an optimal cell density of 1-

2×10^6 cells/mL. Jurkat cells were sub-cultured for at least 5 times before they were used for the experiments (42).

LDH release-based Cytotoxicity Detection Assay

Cytotoxicity Detection kit of LDH release assay was purchased from Roche Applied Science (Cat.No. 11644793001 9115 Hague Road P.O. Box 50414 Indianapolis, IN 46250-0414, USA). General procedures were adopted from Smith, *et al.* (43). Briefly, 2.5 $\times 10^4$ cells in 500 µL of RPMI-1640/ well were dispensed in a flat bottom 24-well Corning® Costar® culture plate (2149 Winston Park Dr. Oakville, Ontario L6H 6J8). Protein 910 and protein 908 were added at 50µg/mL. DMSO at final concentration 10% was used as control, whereas culture media without protein was used as a negative control. The culture plates were incubated for 30 minutes. Then, cells in each individual well were transferred to 2 mL cryotubes (Greiner Bio-One International; Item No.: 126279, Bad Haller Str. 32 4550 Kremsmunster, Austria) and placed at the following temperatures: 4, 0, -6, -20, or -80 ℃. Cells were incubated in cold or freezing temperature for 24 hours and were then thawed in 37 ℃ water bath quickly for 2 minutes. Thawed cells were centrifuged at 1000 × g for 5 minutes, and 10 µL of supernatant was carefully removed without extracting any cell. The supernatant was mixed with 90 µL of double distilled H_2O in a flat bottom 96-well Corning® Costar® culture plate. LDH assay working solution was pre-mixed according to the instructions of the manufacturer (https://pim-eservices.roche.com/LifeScience/Document/dc220baa-d3ed-e311-98a1-00215a9b0ba8). 100 µL of LDH working solution was added to each well containing 100 µL diluted cell supernatant. Cells were incubated at room temperature in dark for 30 minutes before measuring the optical density at 490 nm using Epoch microplate spectrophotometer (BioTek, 100 Tigan Street

Winooski, VT 05404 United States). Percentage of LDH released is calculated by dividing the measured absorbance of extracellular LDH by the total LDH of the sample released by 2% Triton X-100 treatment.

Statistical Analysis:

All data has been analyzed using Graphpad Prism 6. The asterisk (*) on the graphs denote the p value of significant differences between indicated samples. One-way ANOVA and Tukey's Comparison Test was used for comparison. Samples that did not have significant differences ($p>0.05$) were not labelled. $*$ = $p\leq0.05$, $**$ = $p\leq0.01$, $***$ = $p\leq0.001$, $****$ = $p\leq0.0001$.

3.1.2: Results

In this chapter, we first used LDH release assay to determine the function of modified AFP at different temperatures. After 24 hours of incubation at 4^{o}C, 0 ^{o}C and -6^{o}C (Figure 3.1-3.3, respectively), no significant difference of LDH release between groups was measured. At -20 ^{o}C and -80 ^{o}C, treating cells with DMSO 10% prior to freezing significantly reduced the amount of cell LDH release while cells treated with protein 1643 and 908 did not have the same effect.

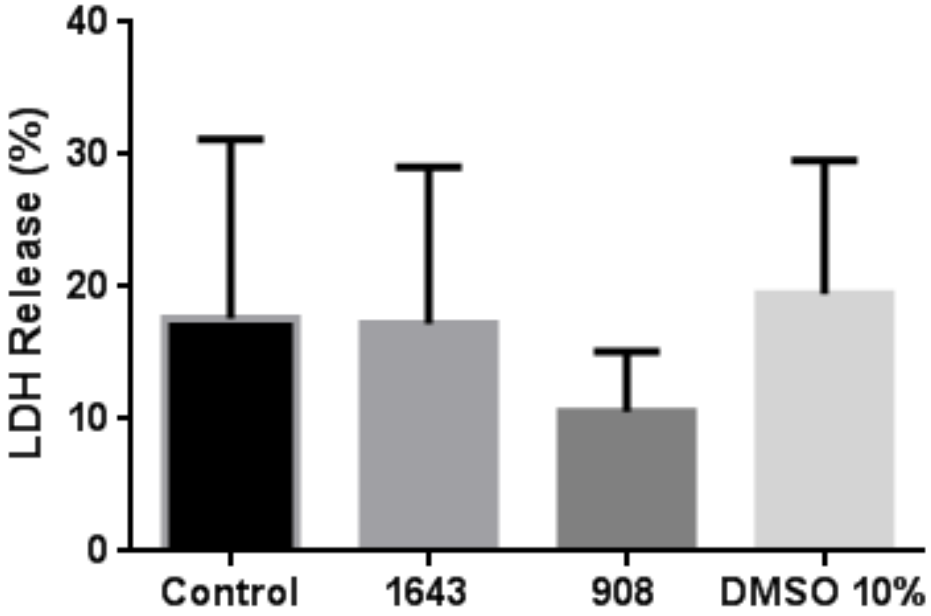


Figure 3.1: Percentage of LDH release in supernatants after 24 hours of incubation at 4°C. The error bars represent the SD from mean of data pooled from three separated experiments. "Control" sample was incubated with PBS instead of proteins or DMSO. No significant differences were observed between samples using one-way ANOVA test on Graphpad Prism (p=0.7523).

During cold incubation at 4°C (Figure 3.1), cells with PBS (Control) had less than 20% of LDH release after 24 hours. This was similarly observed in protein 1643 and 10% DMSO treated cell samples. Protein 908 treated samples appear to have lower percentage of LDH released into the extracellular space, but this difference was not statistically significant.

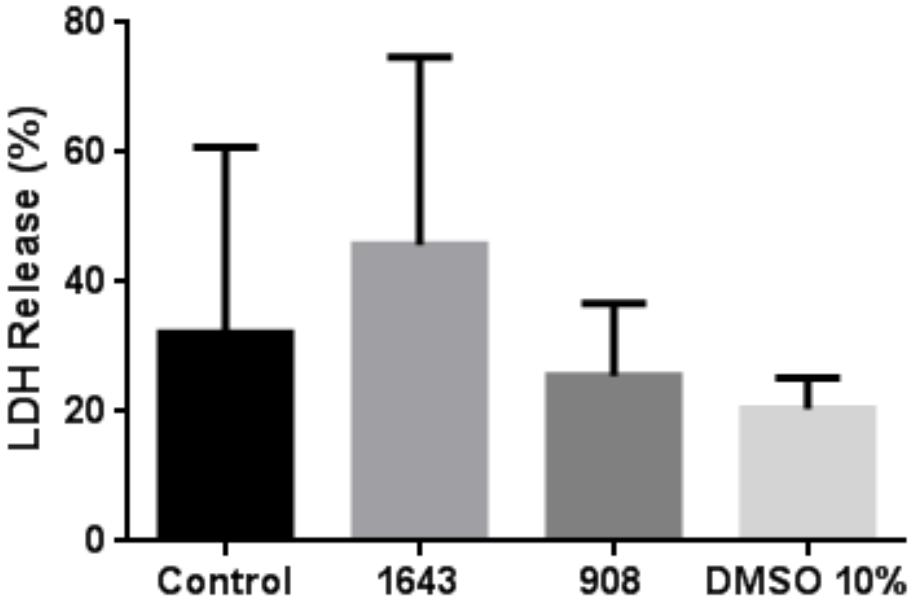


Figure 3.2: Percentage of LDH release in supernatants after 24 hours of incubation at 0°C. The error bars represent the SD from mean of data pooled from three separated experiments. "Control" sample was incubated with PBS instead of proteins or DMSO. No significant differences were observed between samples using one-way ANOVA test on Graphpad Prism (p=0.5283).

During cold incubation at 0°C (Figure 3.2), cells with PBS (Control) now has almost 40% LDH release into the supernatant which is an increase from the result at 4°C. Lowering the temperature appears to induce more membrane damage on the cells. Similar to the previous condition, AFP addition did not significantly reduce the LDH release from cells.

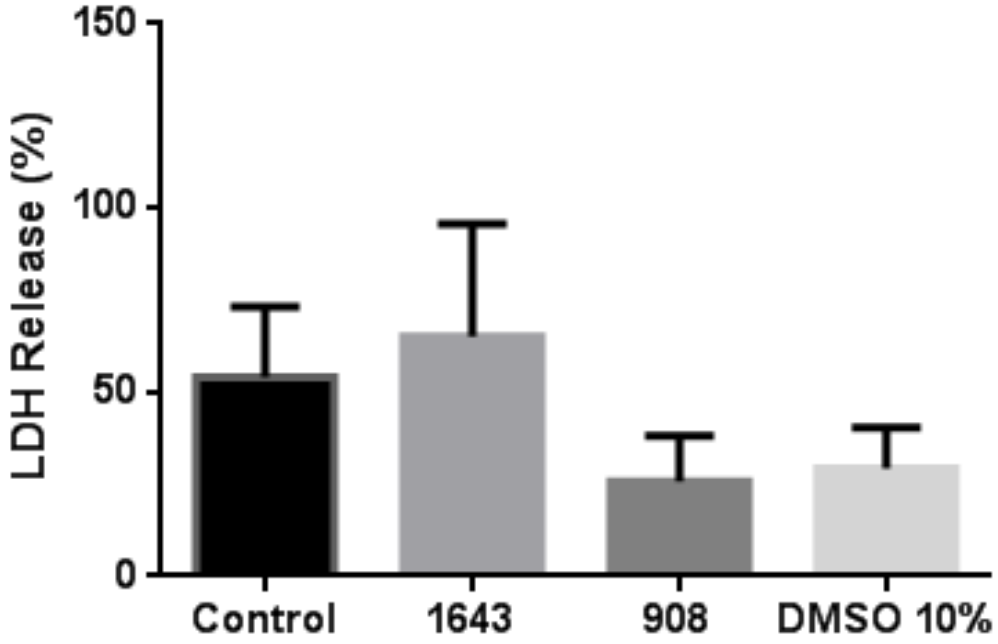


Figure 3.3: Percentage of LDH release in supernatants after 24 hours of cryopreservation at -6°C. The error bars represent the SD from mean of data pooled from three separated experiments. "Control" sample was incubated with PBS instead of proteins or DMSO. No significant differences were observed between samples using one-way ANOVA test on Graphpad Prism (p=0.1123).

Lowering the incubation temperature to -6°C further increased cell membrane damage as observable in cells with PBS (Control), where over 50% of total LDH has been released into the extracellular space. Protein 1643 addition did not significantly reduce the percentage of LDH release when compared to the Control. Protein 908, which is engineered with iPTD, appears to have an effect on reducing the LDH release when added to the cells; however, this apparent effect was not statistically significant. Standard cryoprotectant DMSO also appeared to have a similar effect on the cell as protein 908, but its effect was also not statistically significant. Our

AFPs did not seem to have any positive effect on reducing the amount of LDH release at these temperatures so far.

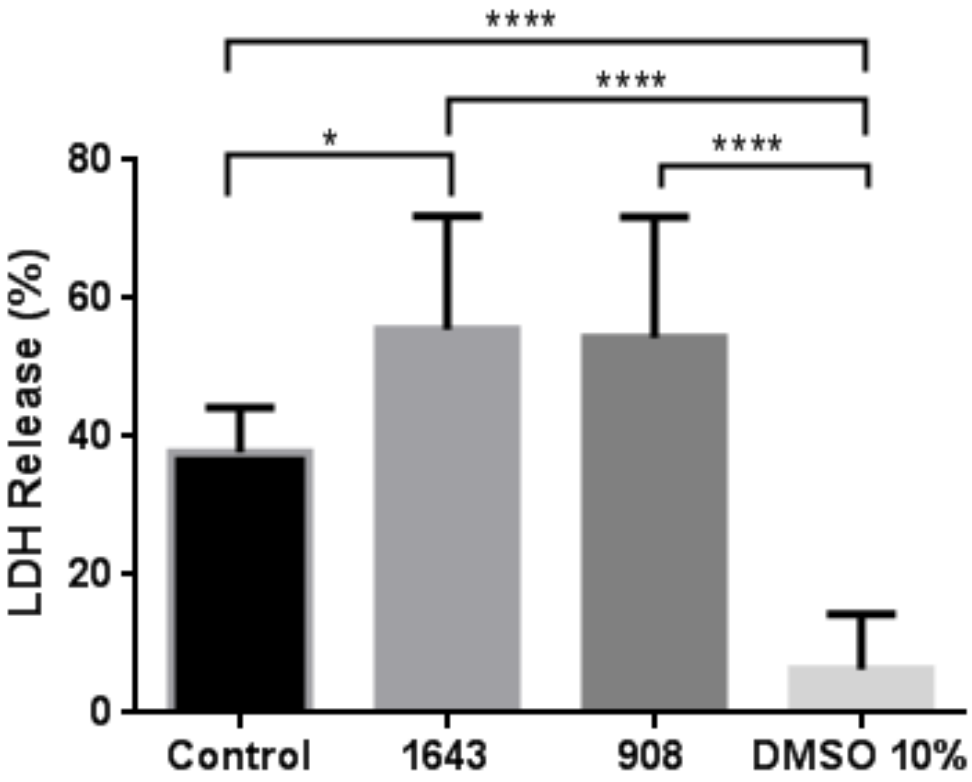


Figure 3.4: Percentage of LDH release in supernatants after 24 hours of cryopreservation at -20°C. The error bars represent the SD from mean of data pooled from three separated experiments. "Control" sample was incubated with PBS instead of proteins or DMSO. Significant differences were observed between samples using one-way ANOVA test on Graphpad Prism ($p<0.0001$). Tukey's multiple comparison test was used to compare the mean of each sample with every other sample.

When the temperature has been further lowered to freezing condition of -20°C, 10% DMSO treated cells appear to have significantly reduced LDH release compared to the control, protein 1643 and protein 908 treated cells. (Tukey's multiple comparison test, $p<0.0001$). Interestingly, protein 1643 addition to cells appear to have significantly increased the LDH release from the intracellular space (Tukey's multiple comparison test, $p\leq0.05$). Even though the temperature has been decreased further, the control sample only had around 40% of LDH release. This was unexpected as freezing temperature should have caused extensive ice formation and thus punctures the cell membrane, resulting in more LDH release.

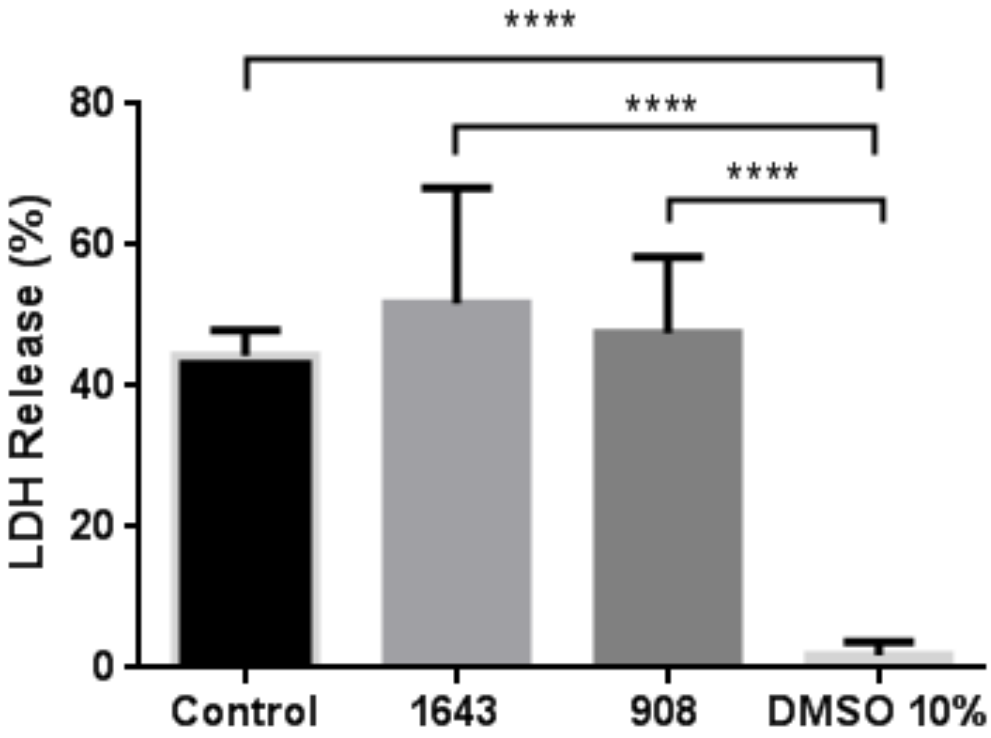


Figure 3.5: Percentage of LDH release in supernatants after 24 hours of cryopreservation at -80°C. The error bars represent the SD from mean of data pooled from three separated experiments. "Control" sample was incubated with PBS instead of proteins or DMSO.

Significant differences were observed between samples using one-way ANOVA test on Graphpad Prism ($p<0.0001$). Tukey's multiple comparison test was used to compare the mean of each sample with every other sample.

In cryopreservation temperature of -80°C, the standard cryoprotectant DMSO has significantly reduced the LDH release from cells compared to all other samples (Tukey's multiple comparison test, $p<0.0001$). However, the LDH assay appears to be unreliable at these freezing temperatures. Cells in control sample had less than 50% LDH release. This infers that over 50% of cells were alive after 24 hours of freezing at -80°C and similarly at -20°C (Figure 3.4). Maybe the LDH assay itself is unsuitable to measure membrane integrity at freezing temperatures. LDH assay relies on measuring the release of the intracellular enzyme lactate dehydrogenase into extracellular solution upon plasma membrane damage. Freezing temperature conditions could destabilize this enzyme, thus giving false positive results. Starnes, J *et al.* reported that cold storage could significantly affect LDH activity. As much as 79% and 40% reduction in LDH activity was measured after 24 hours of incubation at -20°C, and at -80 ℃, respectively (44). Thus, in this case, LDH assay is an inadequate assay to test AFP function in cells at low temperatures. Even though data from Figure 3.1, 3.2 and 3.3 did not show the false positive trend observed in Figure 3.4 and 3.5, the cold incubation temperature could still adversely affect the accuracy of the LDH assay.

3.2: Development of Calcein Dye Release Assay

Rationale: We chose calcein-AM as an alternative to measure membrane integrity. This compound is an inactive fluorescent dye that readily crosses the cell membrane of viable cells. Once inside the cell, calcein-AM is metabolized by a cytosolic esterase to become calcein, which

is fluorescently active (45). The active dye is trapped inside the cell unless membrane damage or leakage events lead to their escape from the cell. We took advantage of this property and used this assay to test cell membrane damage.

3.2.1: Materials and Methods

Jurkat cells were passaged for at least 5 times to dilute the concentration of DMSO before they were used for the experiment. Live Jurkat cells were counted by trypan blue staining on a haemocytometer. The final concentration of cells was adjusted to 1×10^6 cells/mL of media. The total number of cells needed for the experiment was 0.5×10^6 cells/500µL of RPMI media per well/per treatment). Before treatment, cells were centrifuged at 500 × g for 5 minutes. Supernatant was aspirated and cell pellet was resuspended in 1 mL of RMPI media. Calcein-AM dye was purchased from *e*biosciences (cat.No. 65-0853-39, 10255 Science Center Drive San Diego, CA 92121 USA). The dye was prepared by dissolving 1 mg in 1mL of DMSO to make a stock solution of 1mg/mL. 15 µL of the stock Calcein-AM dye solution was added to 1 mL of media containing cells. Cells were incubated in darkness at 37°C supplemented with 5% CO_2 for 30 minutes. Then, cells were dispensed in a Corning Costar 24 well tissue culture plate and treated with AFPs (50 µg/mL), DMSO 10%, or PBS. Cells were incubated for 30 minutes before transferring to 2 mL cryotubes (Greiner Bio-One International; Item No.: 126279, Bad Haller Str. 32 4550 Kremsmunster, Austria) and placed at 0, -6, -20 and -80°C for 24 hours.

After 24 hours, cryotubes were thawed in 37°C water bath for 2 minutes by constant swirling. Triplicates were seeded from each sample in Corning Costar flat bottom 96-well tissue culture plate at 140 µL per well. The culture plate was centrifuged at 300 × g for 5 minutes. Then, 100 µL of supernatant was removed from each well and transferred into a Falcon™Efluorec Black-

walled 96 well plate. Fluorescence was measured using Gemini Spectromax XPS using an excitation of 495 nm, and an emission of 515 nm. Each sample was completely lysed by 2% Triton X-100 to release total Calcein dye fluorescence as a control.

Statistical Analysis:

All data has been analyzed using Graphpad Prism 6. The asterisk (*) on the graphs denote the p value of significant differences between indicated samples. One-way ANOVA and Tukey's Comparison Test was used for comparison. Samples that did not have significant differences ($p>0.05$) were not labelled. $*$ = $p\leq0.05$, $**$ = $p\leq0.01$, $***$ = $p\leq0.001$, $****$ = $p\leq0.0001$.

3.2.2: Results

Since LDH release assay was affected by freezing temperature, we used temperature-insensitive calcein dye release assay to measure the integrity of cell membrane after preservation with AFPs. As shown in Figure 3.6, there was no significant dye release from cells treated with protein 908 compared with medium control at 0^{o}C, while both DMSO and protein 1643 caused significant dye release. Similar results were seen under all of the different freezing temperatures (-6 to -80^{o}C) (Figure 3.7 – 3.9)

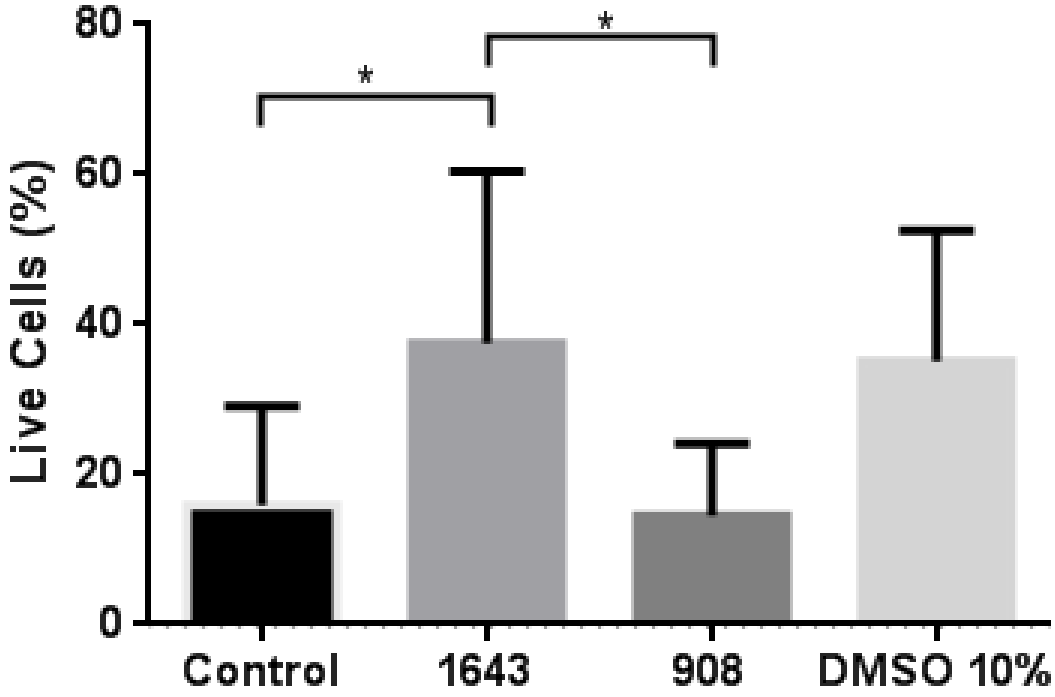


Figure 3.6: Intracellular fluorescence after 24 hours of incubation at 0°C. The error bars represent the SD from mean of data pooled from three independent experiments. "Control" sample was incubated with PBS instead of proteins or DMSO. Significant differences were observed between samples using one-way ANOVA test on Graphpad Prism (p=0.0066). Tukey's multiple comparison test was used to compare the mean of each sample with every other sample.

The result from this assay shows that after 24 hours of cold treatment at 0°C, cells without any cryoprotectant (Control) retained less than 20% of active calcein dye inside. This suggests that the cell membrane integrity is severely affected by the cold temperature resulting in dye release. This effect was similarly observed in cells incubated with protein 908. The addition of engineered AFP 908 did not reduce calcein dye release significantly when compared to Control

sample. However, when wildtype AFP (protein 1643) was added to cells prior to cold treatment, calcein dye release has been significantly reduced ($p \leq 0.05$). This could be because wildtype AFP addition has controlled ice formation and thus reducing ice damage to the cell membrane. Overall, this suggests that the wildtype AFP is effective at helping to maintain cell membrane integrity during cold treatment. Although 10% DMSO treated cells appear to have reduced calcein dye release as well, the results were not statistically significant.

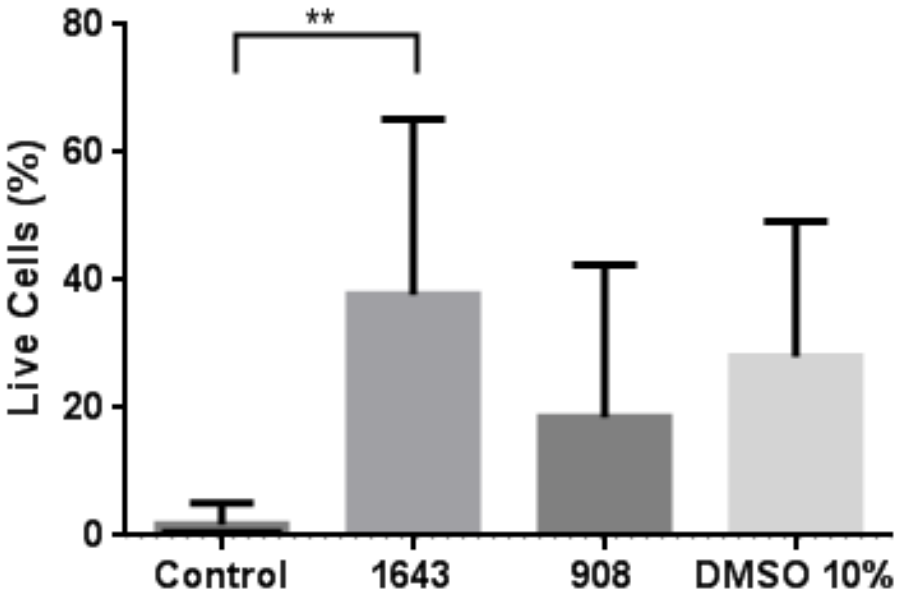


Figure 3.7: Intracellular fluorescence after 24 hours of incubation at -6°C. The error bars represent the SD from mean of data pooled from three individual experiments. "Control" sample was incubated with PBS instead of proteins or DMSO. Significant differences were observed between samples using one-way ANOVA test on Graphpad Prism (p=0.0079). Tukey's multiple comparison test was used to compare the mean of each sample with every other sample.

Lowering the temperature further to -6°C has resulted in almost complete dye release in the control sample. While protein 908 and DMSO 10% addition to cells appear to have reduced the calcein dye release from cells, the results were not significant compared to control. Protein 1643 addition has retained around 40% intracellular calcein dye. This result was similarly observed in Figure 3.6. When compared to cells in control sample, protein 1643 addition significantly reduced the amount of calcein dye release ($p \leq 0.01$). Lowering the temperature to -6°C did not seem to change the dye retaining capability of protein 1643.

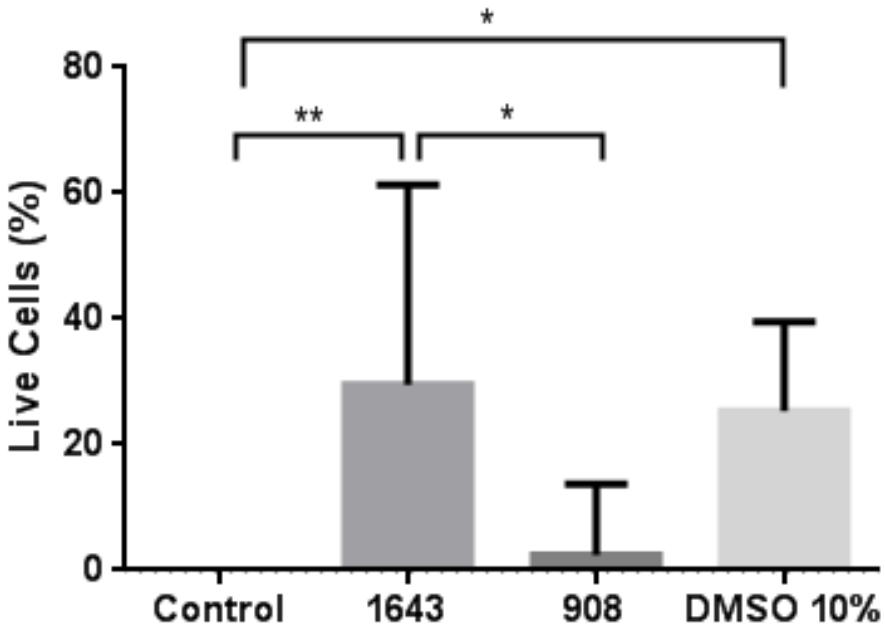


Figure 3.8: Intracellular fluorescence after 24 hours of cold incubation at -20°C. The error bars represent the SD from mean of data pooled from three independent experiments. "Control" sample was incubated with PBS instead of proteins or DMSO. Significant differences were observed between samples using one-way ANOVA test on Graphpad

Prism (p=0.0018). Tukey's multiple comparison test was used to compare the mean of each sample with every other sample.

Freezing for 24 hours at -20°C has resulted in complete calcein dye release of cells in the control sample. Formation of ice crystals probably has completely punctured the cell membrane of cells without any cryoprotectant. Protein 908 addition did not seem to significantly reduce the ice damage. The effect of 10% DMSO, however, is now significant ($p \leq 0.05$) when compared to control. Around 20% of the intracellular calcein dye has been retained when DMSO was added. In the case of protein 1643, the amount of dye retention has been reduced compared to Figure 3.6 and 3.7. However, protein 1643 addition still resulted in around 30% of calcein dye retention when compared to control ($p \leq 0.01$). There is no significant difference between the effect of protein 1643 and DMSO 10% at reducing calcein dye relese. At this temperature, both protein 1643 and 10% DMSO are effective at reducing calcein dye release.

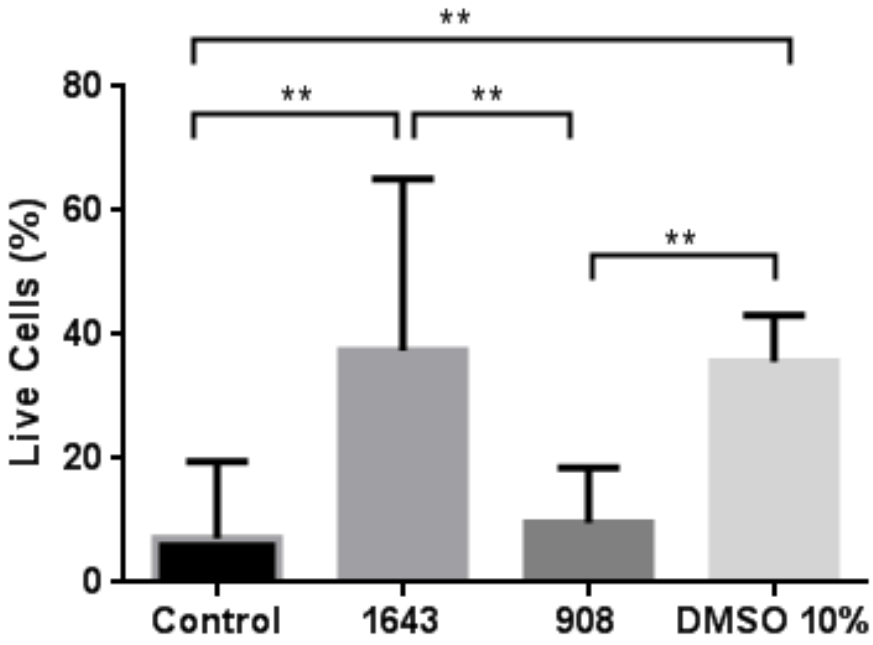


Figure 3.9: Intracellular fluorescence after 24 hours of incubation at -80°C. The error bars represent the SD from mean of data pooled from three independent experiments. "Control" sample was incubated with PBS instead of proteins or DMSO. Significant differences were observed between samples using one-way ANOVA test on Graphpad Prism (p=0.0002). Tukey's multiple comparison test was used to compare the mean of each sample with every other sample.

At extremely low temperature of -80°C, protein 1643 addition significantly improved calcein dye retention compared to the control ($p \leq 0.01$). DMSO 10% treated cells also retained around 40% of their intracellular calcein dye. The effect between protein 1643 and DMSO 10% was not significantly different. Both of these two treatments were also significantly more effective at retaining intracellular calcein dye when compared to protein 908 treated sample ($p \leq 0.01$).

Summary

From the data in this chapter, we have elucidated that the standard LDH assay is not a suitable model to test the function of AFPs due to the cold temperature factor that affect LDH enzyme activity. In order to properly assess protein function, we developed the calcein dye release assay. Since calcein is trapped in membrane intact cells and is only released into the extracellular space upon membrane damage or leakage, it is reasonable to suggest that AFP may have had a role in protecting the cellular membrane during cold/freezing storage of cells. Despite the positive results, calcein assay only suggests membrane integrity and thus may not be directly related to cell survival post cryopreservation. To test for cell viability after cryopreservation, cell recovery and growth must be measured after freezing and thawing of the cells.

Chapter 4: AFP's Effect on Cell Recovery

Rationale: To determine cell viability after cryopreservation. In spite of a significant membrane protection shown by the addition of AFP, the viability of the cells post-thawing should be investigated.

4.1: Trypan Blue Exclusion Assay

4.1.1: Materials and Methods

Jurkat cells were passaged for at least 5 times before they were used for the experiment. Live Jurkat cells were counted by trypan blue staining on a haemocytometer. 1×10^6 cells were seeded in 500 μL of RPMI medium/well in a Corning Costar 24-well tissue culture plate. Protein 908 and 1643 were added to the wells at 50 μg/mL. DMSO was added at 10% (*v/v*) as a control. Cells were incubated for 30 minutes. Then, each sample was transferred to 2 mL cryovials and placed at 0, -6, -20 or -80°C for 24 hours. The next day, cryovials were thawed in a 37°C water bath for 2 minutes. Cells from cryovials were transferred to individual 15 mL Falcon tubes containing 10 mL of pre-warmed RPMI medium. Tubes were centrifuged at 300 × g for 5 minutes and the supernatants were aspirated. Cells were re-suspended in 1 mL of warmed RPMI medium. Then cells from each Falcon tube were seeded in triplicate in a Corning Costar 96 well tissue culture plate (100 μL of cells/well). This procedure was repeated 3 times to generate three 96-well plate containing post-thawed cells. One of the three plates was assessed at 24, 48, and 72 hours post-thawing using trypan blue staining assay. The percentage of live cells was calculated by dividing the trypan blue cell count by the total number of cells prior to the freezing and thawing process.

Trypan blue exclusion assay was performed according to work published by Strober, W (46). Briefly, each well of cells were mixed with 100 μL of 0.4% trypan blue solution (Thermo Fisher Scientific Cat.No.:15250061, 168 Third Avenue Waltham, MA USA 02451) making it 2 × dilution (46). Cells and trypan blue dye were mixed thoroughly by constant pipetting up and down, while avoiding the formation of air bubbles. Then, 10 μL of each sample was transferred to a Hausser Scientific hematocytometer purchased from New York Microscope Co. (product code HS3110, 100 lauman lane, suite A 11801 Hicksville, New York, USA). Cells were counted using a Nikon TS100 inverted light microscope. Bright, unstained cells in four large 4 by 4 squares were counted and the average of the four squares was recorded. This average live cell number is multiplied by dilution factor (2×) and then multiplied by 10^4. This gives the number of live cells per mL. This number is then multiplied by 0.1 mL to get the total number of live cells/well.

Statistical Analysis:

All data has been analyzed using Graphpad Prism 6. The asterisk (*) on the graphs denote the p value of significant differences between indicated samples. Two-way ANOVA and Tukey's Comparison Test was used for comparison. Samples that did not have significant differences ($p>0.05$) were not labelled. $*$ = $p\leq0.05$, $**$ = $p\leq0.01$, $***$ = $p\leq0.001$, $****$ = $p\leq0.0001$.

4.1.2: Results

To verify if cells with less dye release were viable, trypan blue exclusion assay was used to determine their recovery. As shown in Figures 4.1 – 4.4, except for cells preserved with 10% DMSO at -80^{o}C, cells from other treatment groups, including protein 1643 treated ones, were not

viable, indicated by the absence of cell growth after incubation in culture medium at 37°C supplemented with 5% CO_2 for up to 72 hours.

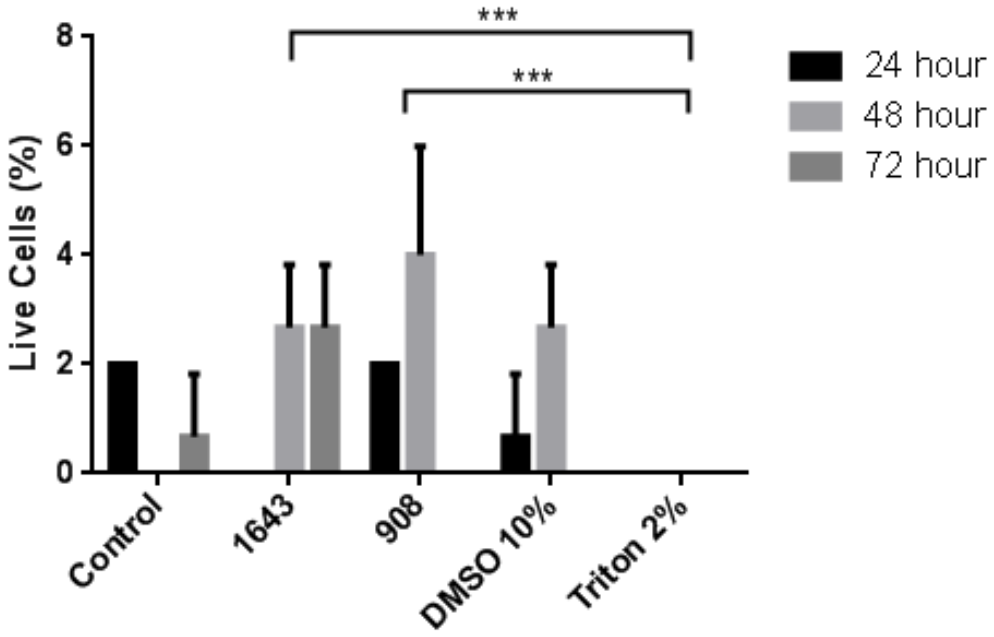


Figure 4.1: Cell number proliferation measured by trypan blue exclusion after 24 hours of treatment at 0 ℃. The error bars represent the SD from mean of data pooled from three independent experiments. "Control" sample was incubated with PBS instead of proteins or DMSO. Significant differences were observed between treatment using two-way ANOVA test on Graphpad Prism (p=0.0002). Tukey's multiple comparison test was used to compare the mean of each sample with every other sample.

Triton X-100 is a known detergent that disrupts cellular membrane, leading to a complete trypan blue dye staining of cells (49). After cold storage at 0°C (Figure 4.1), none of the treatment has shown significant cell proliferation post-thawing. Overall, the cell viability after cold storage in all samples was less than 4%. Some live cells were observed at 24 hours post-thawing; however,

these cells gradually die with longer incubation time. This shows that cells were not able to recover and proliferate after cold storage. Protein or chemical treatment did not result in cell proliferation at this temperature.

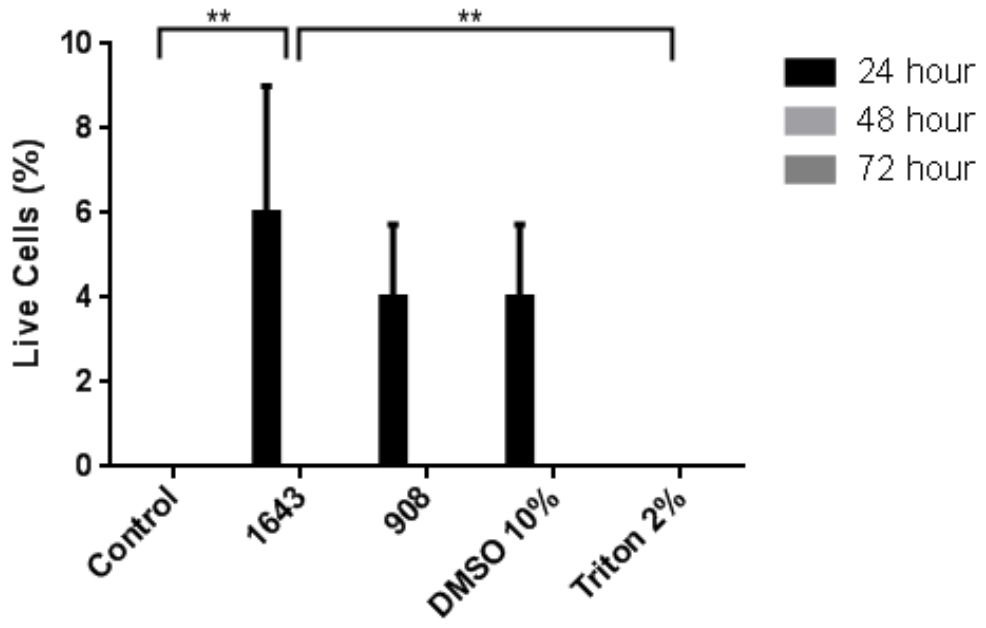


Figure 4.2: Cell number measured by trypan blue exclusion after 24 hours of treatment at -6℃. The error bars represent the SD from mean of data pooled from three independent experiments. "Control" sample was incubated with PBS instead of proteins or DMSO. Significant differences were observed between treatment using two-way ANOVA test on Graphpad Prism (p=0.0003). Tukey's multiple comparison test was used to compare the mean of each sample with every other sample.

After cold storage at -6°C (Figure 4.2), all the cells have died by the 48 hour time point. Similar to the result in Figure 4.1, there were some live cells at 24 hours post-thawing. However, these cells gradually die off. In this case, no live cells were observed after 48 hours of post-thaw

incubation. 24 hours after the cells were thawed and placed in 37°C 5% CO_2 incubator, there were more live cells in sample treated with protein 1643 compared to cells treated with just PBS (Tukey's multiple comparison test, $p \leq 0.01$). The same significant difference was also observed between protein 1643 treated and Triton 2% treated cells. However, with longer incubation time, these live cells eventually die as well. While protein 908 and DMSO 10% treated cells appear to have some live cells at the 24 hour time-point, these data were not statistically significant when analyzed by two-way ANOVA on Graphpad Prism.

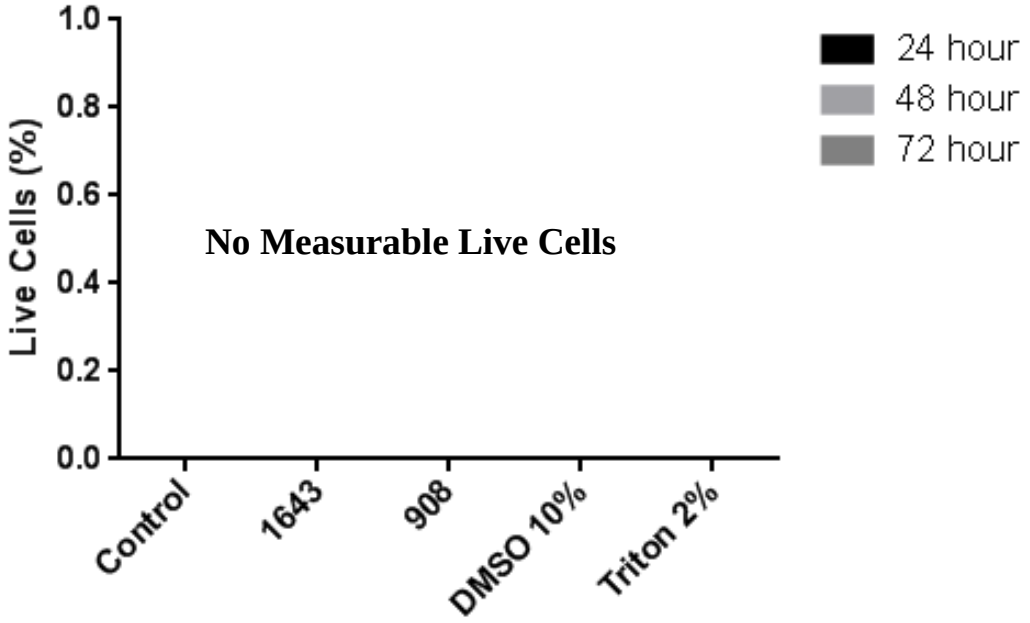


Figure 4.3: Cell number measured by trypan blue exclusion after 24 hours at -20 ℃. Data was pooled from three independent experiments. "Control" sample was incubated with PBS instead of proteins or DMSO. No significant differences were observed between treatment using two-way ANOVA test on Graphpad Prism (p>0.9999).

Freezing cells at -20°C for 24 hours and then thawing them have resulted in complete trypan blue staining of cells in all the tested samples. Unlike at 0°C or -6°C where some live cells could be observed at 24 hours post-thaw, no live cells could be observed in any of the sample at this temperature.

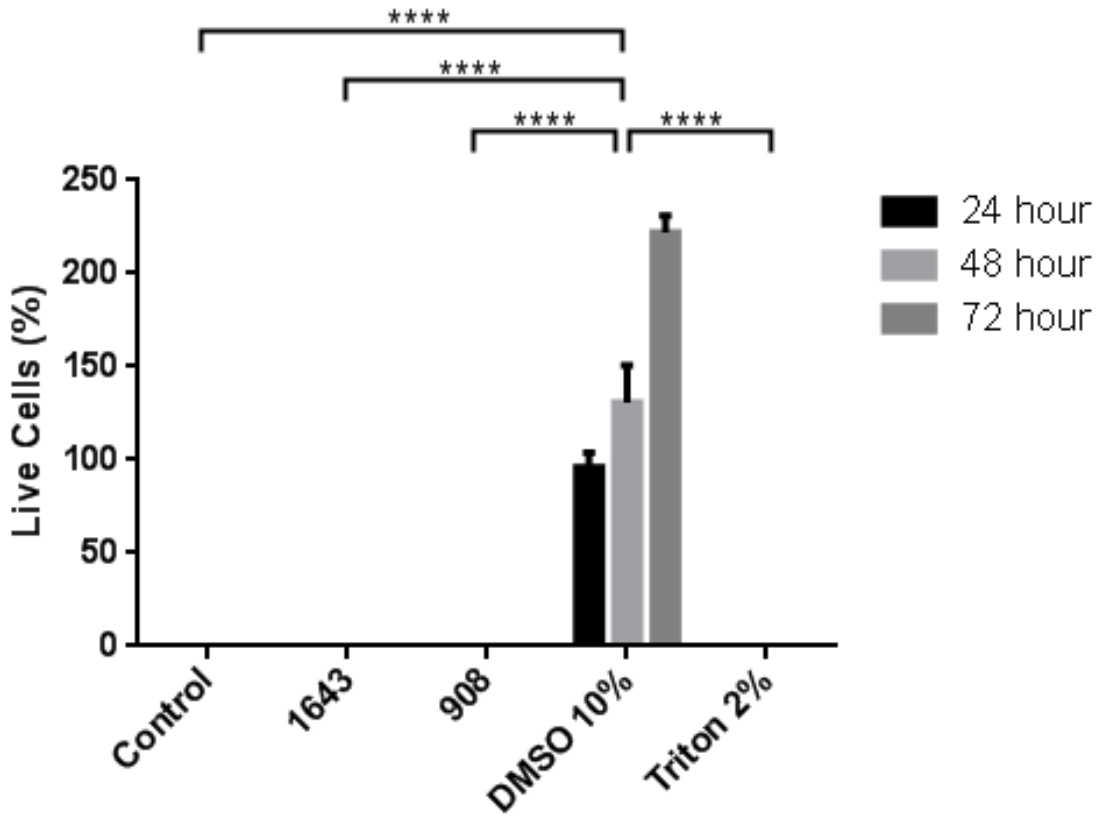


Figure 4.4: Cell number measured by Trypan Blue Exclusion after 24 hours of at -80°C. The error bars represent the SD from mean of data pooled from three independent experiments. "Control" sample was incubated with PBS instead of proteins or DMSO. Significant differences were observed between treatment using two-way ANOVA test on

Graphpad Prism (p<0.0001). Tukey's multiple comparison test was used to compare the mean of each sample with every other sample.

After freezing at -80°C for 24 hours, only DMSO 10% treated cells appear to have successfully recovered. After just 24 hours of incubation at 37°C supplemented with 5% CO_2 , DMSO 10% treated cells were able to recover to almost 100%, which is the number before cells were frozen down. At the subsequent recovery time-points, DMSO 10% treated cells kept on proliferating. At the end of the experiment (72 hour time-point), there were twice as many Jurkat cells as there were prior to freezing. No cell proliferation was observed in all other samples similar to Figure 4.3. Despite the possibility of having better membrane protection immediately after thawing, 908 and 1643 treated cells gradually die off and no signs of significant recovery were observed.

4.2: MTS Assay

4.2.1: Materials and Methods

Cell culture and cell seeding were performed exactly as described previously for trypan blue exclusion assay (See 4.1.1)

MTS Assay:

CellTiter 96® AQueous One Solution Cell Proliferation Assay (MTS) was purchased from Promega Corporation (cat. No: G3582, 2800 Woods Hollow Road Madison, WI 53711 USA).

The MTS ((3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium)) solution was taken out and thawed completely at room temperature for 2 hours

prior to the experiment. When MTS is fully thawed, 10 μL of MTS working solution was added to each well containing 100 μL of cell suspension. The tissue culture plate containing cells and MTS was incubated for 60 minutes. The absorbance of the plate was then measured at 490 nm using a Epoch microplate spectrophotometer (BioTek, 100 Tigan Street Winooski, VT, United States). MTS readings of cells, which were not frozen and thawed, were used as the 100% live cell control. MTS reading from 2% Triton X-100 treated cells were used as background absorbance. This background absorbance was then subtracted from both the 100% live cell control and experimental sample MTS readings. Percentage of live cells is then calculated by dividing the sample MTS reading by the 100% live cell control and multiplying by 100.

Statistical Analysis:

All data has been analyzed using Graphpad Prism 6. The asterisk (*) on the graphs denote the p value of significant differences between indicated samples. Two-way ANOVA and Tukey's

Comparison Test was used for comparison. Samples that did not have significant differences ($p>0.05$) were not labelled. $*$ = $p\leq0.05$, $**$ = $p\leq0.01$, $***$ = $p\leq0.001$, $****$ = $p\leq0.0001$.

4.2.2: Results:

To further confirm the experimental result from trypan blue exclusion assay, we performed MTS assay that was designed to measure cellular metabolism. As shown in Figures 4.5 – 4.8, the MTS assay results were similar to the trypan blue exclusion assay result (Figures 4.1 – 4.4). There was no significant number of cells recovering from preservation with AFPs despite their protected

plasma membrane.

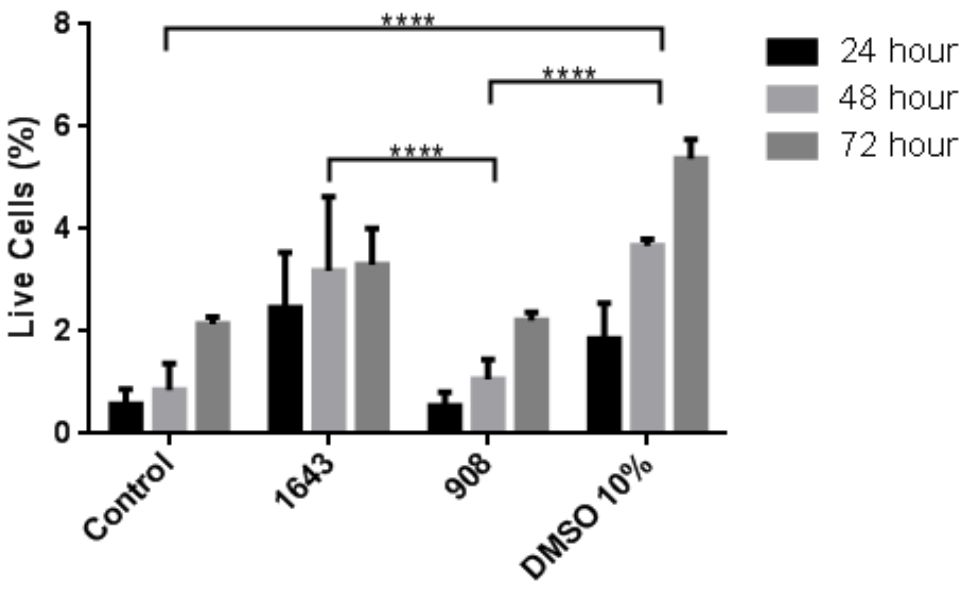


Figure 4.5: Cell metabolic activity assessed by MTS after 24 hours of storage at 0 ℃. The error bars represent the SD from mean of data pooled from three independent experiments. "Control" sample was incubated with PBS instead of proteins or DMSO. Significant differences were observed between treatment using two-way ANOVA test on Graphpad Prism ($p<0.0001$). Tukey's multiple comparison test was used to compare the mean of each sample with every other sample.

MTS is a compound that is metabolized by NADPH and NADH produced by dehydrogenase enzymes in metabolically active cells (50). In metabolically active cells, MTS can be metabolized to form formazan, which is a dye that has a maximum absorbance peak at 490 nm (51). Therefore, a high MTS absorbance suggests high cellular mitochondrium activity. After cold treatment at 0°C (Figure 4.5), only around 4% of live cells have been observed after 72 hours of incubation under 37 ℃ 5% CO_2 condition. However, the cell metabolic rate in each

sample appears to be increasing with longer incubation time. Furthermore, cells treated with DMSO 10% appear to be recovering better compared to control and protein 908 treated cells (Tukey's multiple comparison test, $p \leq 0.001$). Although protein 1643 treatment appear to be enhancing cell metabolic activity recovery compared to control, this difference was not statistically significant. Furthermore, the addition of protein 908 did not significantly increase cell post-thaw metabolic rate compared to control.

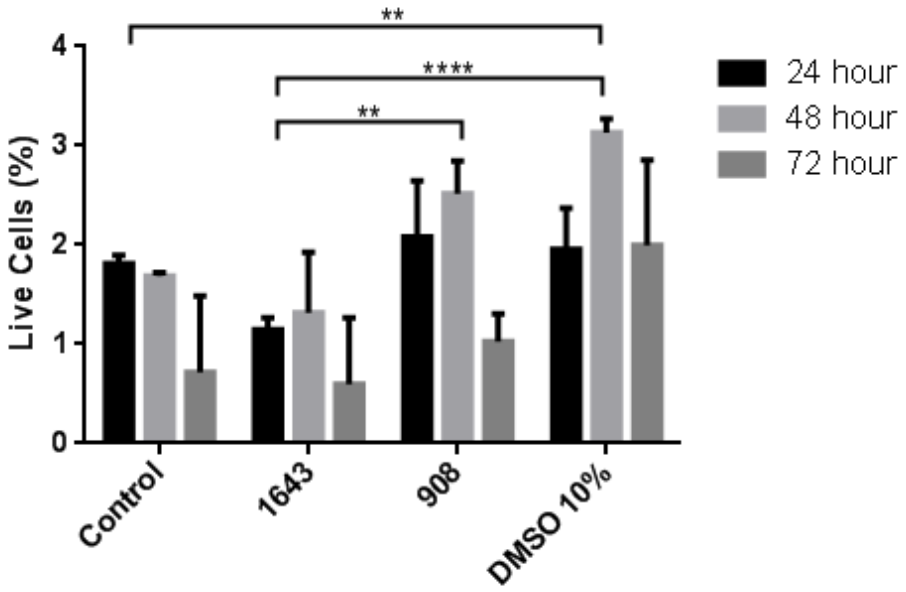


Figure 4.6: Cell metabolic activity assessed by MTS after 24 hours at -6°C. The error bars represent the SD from mean of data pooled from three independent experiments. "Control" sample was incubated with PBS instead of proteins or DMSO. Significant differences were observed between treatment using two-way ANOVA test on Graphpad Prism ($p<0.0001$). Tukey's multiple comparison test was used to compare the mean of each sample with every other sample.

Lowering the storage temperature to -6 ℃ has further decreased the percentage of live cells in all samples compared to the previous condition (Figure 4.5). Interestingly, after 24 hours of freezing at -6 ℃, cell metabolic rate appear to be declining at the end of 72 hour incubation. Cell metabolic rate has increased at 48 hour post-thawing for AFPs and DMSO 10% treated cells but declined at 72 hour. The effect of DMSO 10% on cell metabolic recovery is still significant compare to control ($p \leq 0.01$) and protein 1643 treated cells ($p \leq 0.0001$). The addition of either AFPs did not significantly increase the post-thaw cell metabolic activity.

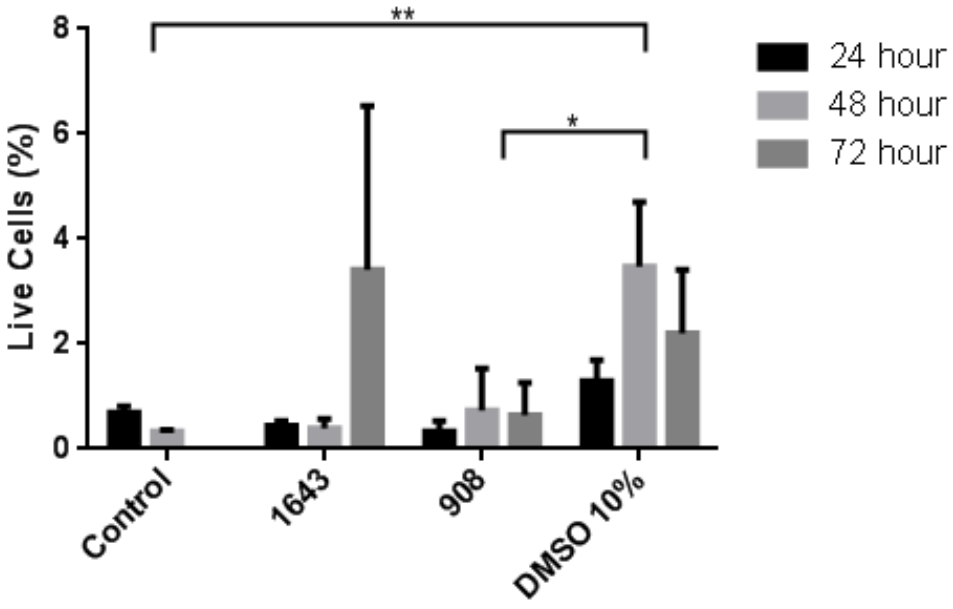


Figure 4.7: Cell metabolic activity assessed by MTS after 24 hours at -20°C. The error bars represent the SD from mean of data pooled from three independent experiments. "Control" sample was incubated with PBS instead of proteins or DMSO. Significant differences were observed between treatment using two-way ANOVA test on Graphpad Prism (p=0.0027). Tukey's multiple comparison test was used to compare the mean of each sample with every other sample.

After 24 hour of freezing at -20°C, the cells without any cryoprotectant (Control) has minimal cell metabolic activity post-thawing and no significant activity could be detected at 72 hour post-thaw. This suggests that these cells did not recover after freezing. Adding protein 908 or protein 1643 did not significantly improve the metabolic recovery of cells compared to control. Although recovery is low, at around 4% of live cells 48 hours post-thaw, DMSO 10% treated cells did recovery significantly better compared to control cells ($p \leq 0.01$). However, DMSO 10% treated cell had decreased metabolic activity at 72 hour post-thaw compared to the previous time-point. This might be due to the toxicity of DMSO.

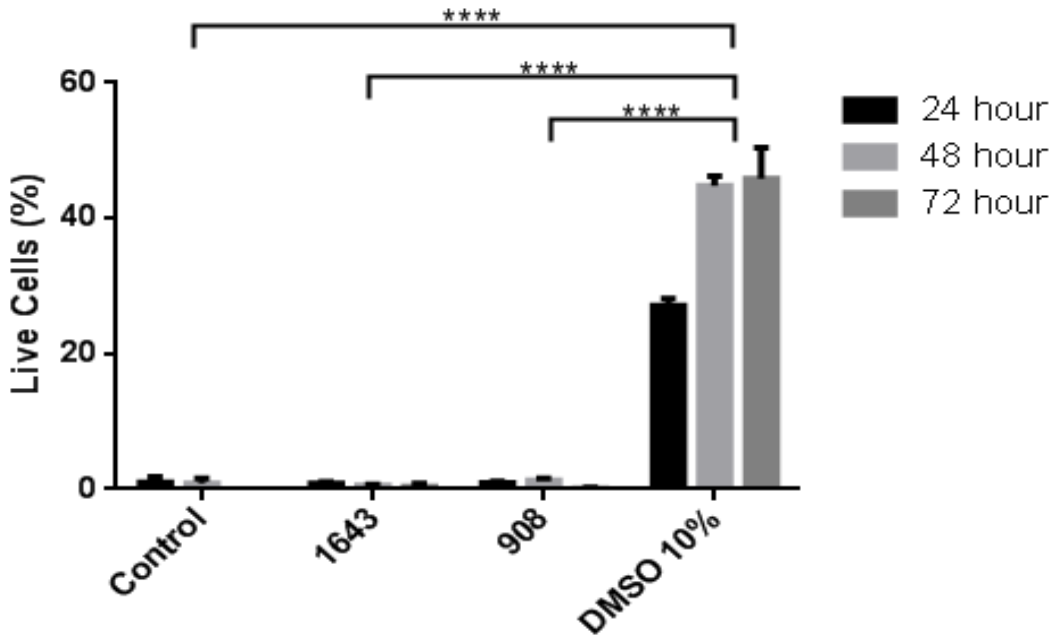


Figure 4.8: Cell metabolic activity assessed by MTS after 24 hours at -80°C. The error bars represent the SD from mean of data pooled from three independent experiments. "Control" sample was incubated with PBS instead of proteins or DMSO. Significant differences were observed between treatment using two-way ANOVA test on Graphpad Prism ($p<0.0001$). Tukey's multiple comparison test was used to compare the mean of each sample with every other sample.

Freezing at ultra-low temperature of -80°C has generated similar result observed in Figure 4.4, where DMSO 10% treated cells have successfully recovered and proliferated. In this assay, these cells also have significant metabolic activity recovery. This effect is significant compared to all other samples ($p \leq 0.0001$). AFPs effect on cell metabolic rate recovery was similar to data in Figure 4.5 through 4.7 where their effects were minimal.

Summary

From the results in this chapter, we conclude that the membrane protective effect of AFPs did not translate to cell proliferation or metabolic activity recovery after freezing and thawing. The next step would be to find a suitable model for our AFP's function.

Chapter 5: AFP in Red Blood Cell Cryopreservation

Rationale: Red blood cells (RBCs) do not have the ability to self-renew and lack many membrane bound intracellular organelles. Furthermore, plasma membrane integrity is crucial in RBC cryopreservation. We hypothesize that our AFPs may protect RBC membrane in cryopreservation and increase their post-thaw viability.

5.1: RBC Hemolysis and ATP Retention

5.1.1: Materials and Methods

Adult Balb/c mice (both male and female) were sacrificed by CO_2 suffocation following the protocol published by Pritchett, K *et al.* (54). Briefly, mice were transferred to lockable cage that is connected to CO_2 tank (54). CO_2 was administered for 10 minutes or until the mouse is completely euthanized. The mouse was taken out from the cage and 1-2 mL of blood was taken out and put into a 10 mL glass plasma tube containing sodium heparin (BD Biosciences, Cat No. 366480, 2350 Qume Drive San Jose, CA, USA 95131). The extracted whole blood was then transferred to a 15 mL Falcon tube and centrifuged at 2000 × g for 10 minutes. After centrifugation, the plasma and buffy coat were aspirated. 12 mL of sterile cold PBS (4 ℃) was added to the RBC pellet. A 10 mL sterile pipette was used to fully re-suspend the RBC pellet. Then the suspension was centrifuged again at 2000 ×g for 10 minutes. The supernatant was aspirated out and 12 mL of sterile cold PBS was used to wash the cells again. This process was repeated two more times. After the supernatant was aspirated in the last spin, 60 µL of the remaining RBC pellet was taken into a Corning Costar 96 well plate and mixed with 40 µL of pre-diluted AFPs or BSA used as a control. The RBC mixtures were incubated at room

temperature for 30 minutes. Each sample was then transferred to 2 mL sterile cryotubes (Greiner Bio-One International; Item No.: 126279). Glycerol was added very slowly to make either 25% or 40% final concentration *(v/v).* The final concentrations of AFPs used were: protein 908 at 30 µM, protein 1643 at 30 µM, and BSA at 30 µM. 40 µL of sterile cold PBS was added to glycerol as a negative control. RBCs in cryotubes were thoroughly mixed with glycerol. The cryotubes were then transferred to Mr. Frosty Freezing Container (ThermoFisher Scientific Cat No.: 5100-0001, Thermo Fisher Scientific 168 Third Avenue Waltham, MA USA 02451) containing 250 mL of isopropyl alcohol. The Mr. Frosty container was placed at -80 ℃. After 24 hours, cryotubes were thawed quickly in 37 ℃ water bath for 2 minutes. The thawed RBCs were transferred to 1.5 mL microfuge tubes and were centrifuged on a tabletop centrifuge at 12,900×g for 1 minute. Supernatant and pellet were both collected for further experiment.

Drabkins Hemolysis Assay:

Drabkin's reagent was purchased from Sigma-Aldrich (Cat No.: D5941-6VL, 3050 Spruce St. St. Louis, MO 63103 USA). 1 litre of double distilled water was added to 1 vial of Drabkin's reagent powder to make the working solution. The assay was performed according to the protocol used by Lee, S. G. *et.al* (55). Briefly, 5 µL of RBC post-thawed supernatant was added to 195 µL of Drabkin's reagent working solution in a Corning Costar 96 well plate (55). The solution was incubated at room temperature covered by aluminum foil in darkness for 15 minutes. The cyanomethaemoglobin absorbance was read at 540 nm using the Epoch microplate spectrophotometer as previously described. This represents the absorbance of hemoglobin that was released into the supernatant and termed supernatant hemoglobin. Total hemoglobin of a given RBC sample was also measured. Briefly, the thawed RBCs were re-suspended thoroughly

with equal volume of 1 × somatic cell ATP releasing reagent purchased from Sigma-Aldrich (cat. No: FLSAR-1VL, 3050 Spruce St. St. Louis, MO 63103 USA). 5 µL of the lysate was added to 195 µL of Drabkin's reagent in a 96 well plate. The plate was incubated in darkness for 15 minutes. Absorbance was read at 540 nm using the Epoch microplate spectrophotometer as explained previously. The obtained value was termed total hemoglobin.

Hemolysis Percentage Calculation:

Supernatant hemoglobin ÷ (total hemoglobin × 2) × 100% = Percentage of hemoglobin released into the supernatant = Percent Hemolysis

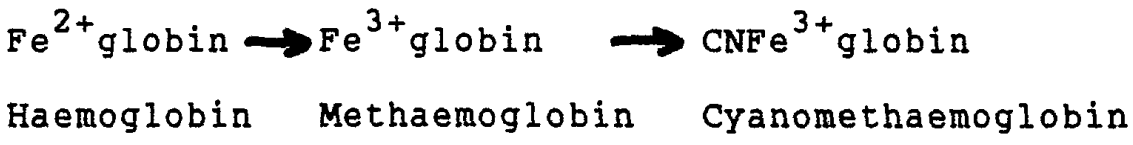


Figure 5.1: Drabkin's reagent reaction formula

ATP Determination Assay

ATP Determination kit was purchased from ThermoFisher Scientific (Cat No.: A22066). The working solution was made according to protocol published on ThermoFisher's website (https://tools.thermofisher.com/content/sfs/manuals/mp22066.pdf). The ATP determination assay was performed according to work published by Miloš, B *et al.* (57). Briefly, 10 µL of post-thawed RBC supernatant was mixed with 90 µL of deionized water, pH 8. 10 µL of this mixture was added to wells of an opaque white bottom 96 well plate (Corning 96 Well Cat.No.: CLS3362). This is defined as the supernatant ATP. Total ATP was also measured by re-suspending thawed RBCs (combination of pellet and supernatant) with equal volume of 1 ×

somatic ATP release reagent (cat. No: FLSAR-1VL). 10 µL of the lysate was added to 90 µL of deionized water, pH 8. 10 µL from this mixture was added to the opaque white bottom 96 well plate. The amount of ATP was measured using pre-installed bioluminescence luciferase assay software on Tecan Infinite m200 luminometer. The ratio of pellet to supernatant ATP was calculated by the following formula:

(Total ATP -Supernatant ATP) ÷ Supernatant ATP = Pellet ATP: Supernatant ATP ratio

Statistical Analysis:

All data has been analyzed using Graphpad Prism 6. The asterisk (*) on the graphs denote the p value of significant differences between indicated samples. One-way ANOVA and Tukey's Comparison Test was used for comparison. Samples that did not have significant differences ($p>0.05$) were not labelled. $*$ = $p\leq0.05$, $**$ = $p\leq0.01$, $***$ = $p\leq0.001$, $****$ = $p\leq0.0001$.

5.1.2: Results

To test if the engineered AFPs could be used for RBC preservation, we pre-treated mouse RBCs with AFPs before they were cryopreserved with glycerol. As shown in Figure 5.2, protein 908 might not significantly prevent hemolysis in RBCs preserved with 40% glycerol at -80^{o}C, but it significantly reduced the leakage of intracellular ATP from these cells after cryopreservation (Figure 5.3). To further examine the potential of protein 908 for RBC cryopreservation, the protective activity of this protein was tested in cryopreserving RBCs with a reduced amount of glycerol (25%). As shown in Figure 5.4 and 5.5, pre-treatment with protein 908 significantly reduced both hemolysis and intracellular ATP loss in RBCs cryopreserved with 25% glycerol.

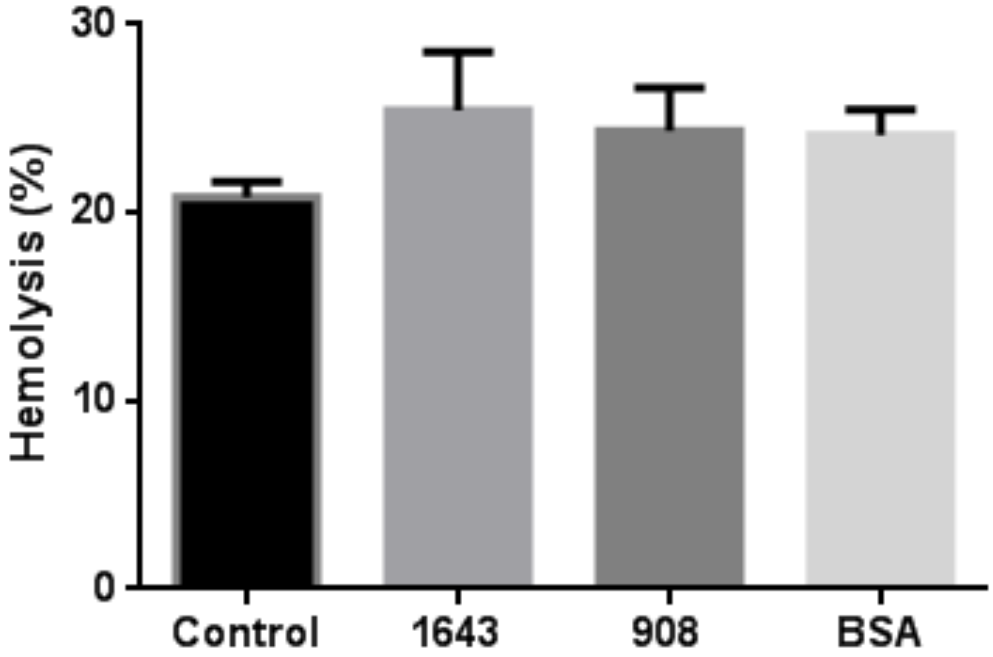


Figure 5.2: Hemolysis of 40% glycerol perfused RBCs after 24 hours at -80 ℃. All samples contained 40% glycerol. Hemolysis was measured by Drabkin's assay. Error bars represent the SD from mean of data pooled from three independent experiments. "Control" sample was incubated with PBS instead of AFPs or BSA. No significant differences were observed between treatment using one-way ANOVA test on Graphpad Prism (p=0.1092).

After 24 hours of freezing at -80 ℃ supplemented with 40% glycerol in accordance with North American RBC cryopreservation standards, AFPs and BSA (protein control) were added to determine whether there is any enhancement of RBC viability and quality post-cryopreservation. Figure 5.2 shows the result of hemolysis of RBCs. 40% glycerol perfused cells had approximately 20% of hemolysis after cryopreservation. This result was not further improved even by adding either the wild type AFP (1643) or iProgen engineered protein 908 as shown by statistical analysis.

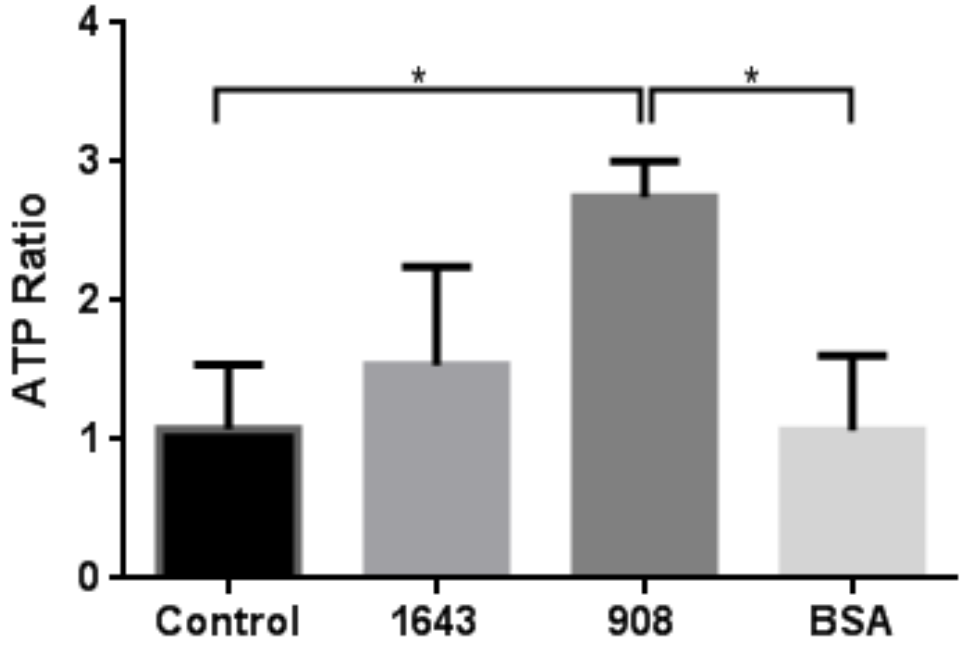


Figure 5.3: Intracellular to extracellular ATP ratio of 40% glycerol perfused RBCs after 24 hour cryopreservation at -80 ℃. All samples contained 40% glycerol. Error bars represent the SD from mean of data pooled from three independent experiments. "Control" sample was incubated with PBS instead of AFPs or BSA. Significant differences were observed between samples using one-way ANOVA test on Graphpad Prism (p=0.0124). Tukey's multiple comparison test was used to compare the mean of each sample with every other sample.

In Figure 5.3, the ratio between intracellular ATP and extracellular ATP was measured. A higher ratio indicates that there is more ATP remaining in the intracellular space compare to extracellular environment. This suggests less ATP leakage has occurred. RBCs that were frozen with only 40% glycerol had ATP ratio around 1. This suggests that these RBCs' intracellular ATP level was roughly equal to their extracellular ATP concentration. In the case of RBCs

treated with protein 908, there was almost three times as much ATP inside the cell as there are outside ($p \leq 0.05$). This suggests that with protein 908 addition, RBC membrane was less leaky to ATP during cryopreservation. The membrane covering ability of protein 908 might have had a contribution to this effect. Neither BSA nor protein 1643 was able to achieve the same reduction of ATP leakage effect as 908.

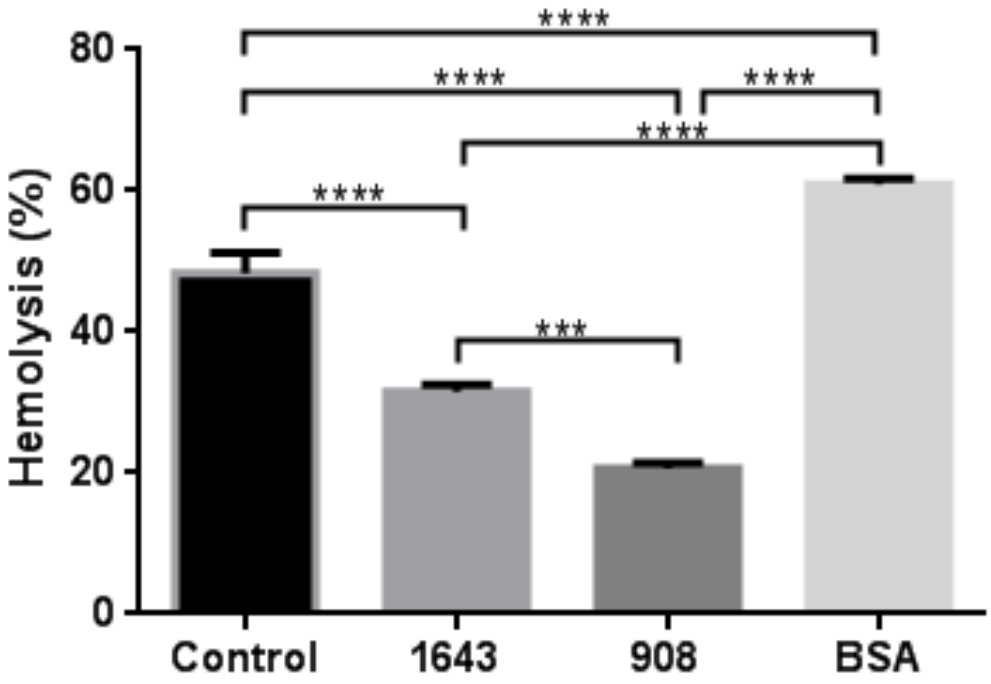


Figure 5.4: Hemolysis of 25% glycerol perfused RBCs after 24 hours at -80 ℃. All samples contained 25% glycerol. Hemolysis was measured by Drabkin's assay. Error bars represent the SD from mean of data pooled from three independent experiments. "Control" sample was incubated with PBS instead of AFPs or BSA. Significant differences were observed between samples using one-way ANOVA test on Graphpad Prism ($p<0.0001$). Tukey's multiple comparison test was used to compare the mean of each sample with every other sample.

From Figure 5.4, it is evident that the reduced glycerol concentration resulted in an increase of hemolysis in the control sample. RBCs frozen with only 25% glycerol had around 50% hemolysis compared to 20% hemolysis when 40% glycerol was used (Figure 5.2). However, when the wild type AFPs (1643) was added to RBCs, the hemolysis was reduced to approximately 30%. This difference was statistically significant when analyzed by Tukey's multiple comparison test ($p \leq 0.0001$). Similar hemolysis reduction effect was not observed in BSA treated samples. The difference between protein 1643 and BSA was also statistically significant ($p \leq 0.0001$) suggesting the effect of AFP was functional rather than simple protein crowding effect. iProgen engineered antifreeze protein 908 had superior effect on hemolysis reduction. 30 μM of protein 908 has reduced the hemolysis to around 20%. This is a significant reduction of hemolysis compare to the control ($p \leq 0.0001$). When compared to protein 1643 treated RBCs, protein 908 treated RBCs also appears to be experiencing less hemolysis. This difference is statistically significant ($p \leq 0.001$). Interestingly, the addition of 30μM of BSA has resulted in significant more hemolysis ($p \leq 0.0001$). High concentration of BSA might have altered the osmotic environment during cryopreservation thus leading to more severe hemolysis.

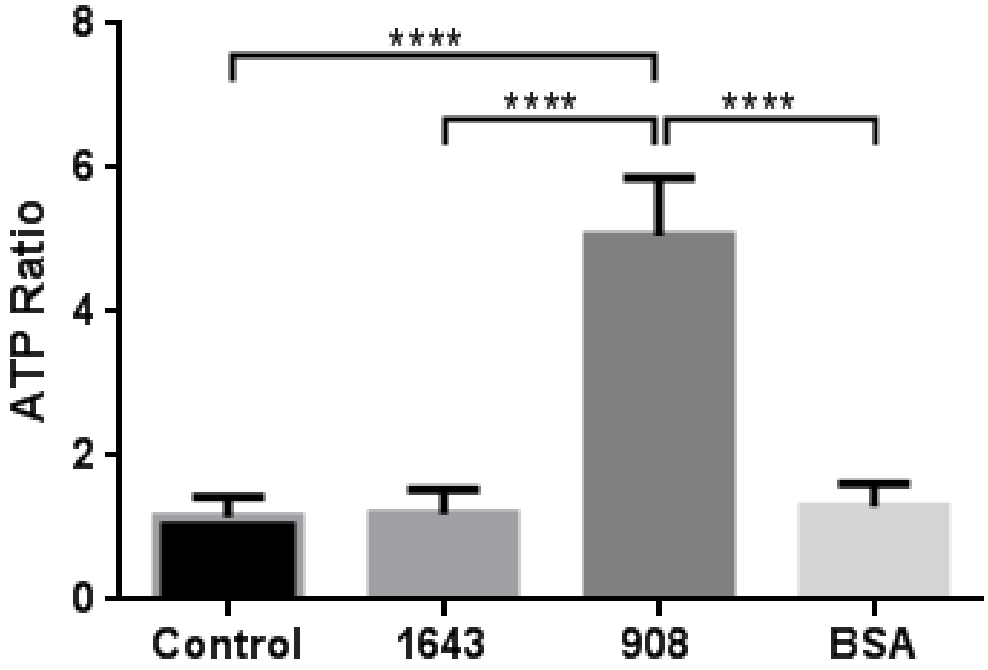


Figure 5.5: Intracellular to extracellular ATP ratio of 25% glycerol perfused RBCs after 24 hour cryopreservation at -80°C. All samples contained 25% glycerol. Error bars represent the SD from mean of data pooled from three independent experiments. "Control" sample was incubated with PBS instead of AFPs or BSA. Significant differences were observed between samples using one-way ANOVA test on Graphpad Prism ($p<0.0001$). Tukey's multiple comparison test was used to compare the mean of each sample with every other sample.

Figure 5.5 shows the intracellular ATP retention ratio of 25% glycerol perfused RBCs after 24 hours of cryopreservation at -80°C. Similar to 40% perfused RBCs, the 25% glycerol perfused RBCs (control) had roughly a 1:1 ratio of intracellular ATP versus the extracellular ATP. Wild type AFP (1643) addition did not significantly enhance the ATP retention ($p>0.05$). When protein 908 was added at 30μM, the ATP retention ratio was significantly increased compared to control sample ($p\leq0.0001$). Protein 908 treated RBCs' intracellular ATP concentration was around 5 times higher than the extracellular ATP. The addition of protein 1643 or BSA did not lead to significant ATP leakage reduction. With protein 1643, although hemolysis was reduced as observed in Figure 5.4, this protein did not reduce ATP leakage.

Summary

Engineered protein 908 can achieve similar percentage of hemolysis with lower glycerol concentration (25%) compared to the standard 40% glycerol used in clinics today. Protein 908-treated RBCs have higher intracellular ATP concentration and therefore may have better activity post-thawing.

Chapter 6: Discussion and Conclusion

In this thesis project, I have optimized the purification protocol for proper production of iPTD engineered AFPs. I then studied the intracellular delivery kinetics of recombinantly produced iPTD-AFP in cell culture models. Finally, after understanding the kinetics, I moved on to test these engineered AFPs' function in various *ex vivo* models.

AFPs were discovered as early as 1969 in the blood of Antarctic fish (1). Since then, scientists have studied extensively their functions, morphologies and mechanism of their action (67, 68, 69). Published work in the literature that was able to successfully utilize AFPs in order to enhance biological sample post-cryopreservation viability mostly has two properties in common. The AFPs were used in large quantities (in scale of more than 10 mg/mL) and functioned in the extracellular space (70, 71). Although limited success has been achieved, AFPs have never been shown to completely replace the standard cryoprotectant, such as DMSO. One possibility is that as described before, potent cryoprotectants must be able to penetrate the cell membrane and enter the intracellular space. By preventing ice formation and recrystallization both intracellularly and extracellularly, cryoprotectants ensure optimal survival for biological samples after freezing and thawing. Since standard cryoprotectants are usually toxic at high concentrations (72), they must be immediately removed, through extensive washing and sub-culturing after thawing the biological samples. This process is not only time-consuming, but DMSO and its associative cryoprotectant might have already caused damage to the sample. As an example, DMSO was shown to alter gene expression in certain *in vitro* models (73). Therefore, we initially thought to combine the idea of active AFP and the need for intracellular delivery together to develop a novel concept and eventually a new class of artificial biological cryoprotectant.

We selected a few wild type AFPs that have shown protective effects in the literature (74,75) and contacted a local Biotech company, iProgen. This biotech company has a proprietary patented technology that can bring cargo proteins into cells. This technology was termed iPTD and has been shown in the literature to delivery recombinant produced proteins into the intracellular space with high efficiency (76). Therefore, collaboration was formed to combine AFPs with iPTD technology.

Through optimization, the iPTD engineered AFPs have been successfully produced and are high in purity. We also purified the wild type version of the AFP to be used as a comparison.

Since iPTD tag is capable of protein delivery into the cell, we first have to understand the kinetics of this process. Using flow cytometry, we established that the iPTD delivery system is time dependent, with 30 minutes of iPTD protein incubation with cells demonstrating the greatest fluorescence intensity signal. After 30 minutes, the amount of iPTD protein signal is gradually lost. In this experiment, we only detected the cell surface GFP fluorescence (no cell permeabilization) over a period of 300 minutes. iPTD could have begun to deliver the cargo protein into the intracellular space via endocytosis pathways already. This would explain the disappearance of GFP fluorescence over time. In order to find evidence of intracellular delivery, multiphoton microscopy was performed. From the microscopy images, it was clear that iPTD engineered protein 910 was localized on the cell membrane within 30 minutes of incubation. However, no GFP fluorescence was detected in the cell. iPTD was modified from a mammalian secretion signal peptide and is hydrophobic (20). This particular version of the iPTD may have a quick and efficient plasma membrane coverage, but with little intracellular delivery capability. Since no intracellular delivery was observed, intracellular ice formation may still damage the

cells during cryopreservation. However, we believe that the iPTD engineered AFPs can still improve the cryoprotective effect of wild type AFPs. Plasma membrane coverage may reduce the AFP concentration needed to achieve cryoprotective effect. Also, an intact cell membrane post-cryopreservation could also lead to better cell recovery and proliferation.

To determine the membrane protective effect of engineered AFPs, we decided to perform LDH release assay. LDH is an intracellular enzyme that is released into the extracellular space upon cell membrane damage. Previous studies have widely used LDH assay as an important assessment of cell viability post-cryopreservation (77, 78, 79). Our result of this assay was rather interesting in that we found LDH assay is not an adequate assay to test cell viability at low temperature. We hypothesized that LDH enzyme, which the assay relies on, is temperature sensitive. We could not adequately assess the membrane protective effect of our AFP by using the LDH assay.

Calcein dye release assay was developed to replace the LDH assay in assessment of membrane integrity. Traditionally, Calcein-Am dye has been used to stain live cells and analyzed by fluorescent microscopy and flow cytometry (45, 77). However, we took advantage of the fact that fluorescent calcein is trapped within intracellular space of live cells and is released upon membrane damage to develop a new membrane integrity test. Our data demonstrated that calcein release assay is more reliable compared to LDH assay in all tested temperatures.

When AFPs were added, our data from the Calcein assay suggests that the wild type AFP 1643 have reduced dye leakage. This result confirms the membrane leakage protection theory proposed by Tomczak, M *et al* (5). However, whereas Tomczak, M *et al.* demonstrated the membrane protective effect at 0°C to 25°C, we found that our AFP had membrane protective

effect at freezing temperatures up to -80°C. Unfortunately, the iPTD-engineered AFP 908 did not show similar protective effect as wild type AFP shown by the assay. One possibility could be the size difference between the two proteins. Since wild type AFP was only around 8.5 kDa with the engineered protein being 39.4 kDa, the amount of protein molecules added to the cells differ in each case. Each protein was added at 50 μg/mL for the Calcein experiment, and thus there will be more molecules of wild type fish AFP (1643) (5.88 μM) in the culture media compared to engineered fish AFP (908) (1.27 μM). There were almost 5 times as many molecules of protein 1643 than protein 908. Since AFPs are structural proteins that bind ice crystals (1), more molecules of AFPs may result in better cryoprotective effect. Despite this difference of two AFPs, the natural form of AFP has shown to be actively protecting the plasma membrane during cryopreservation. This is a surprising discovery as previous work in the literature has used much higher concentration to achieve similar protective effects.

In order to investigate whether AFP protective function could translate to cell recovery, cryopreserved cells were thawed and incubated. In the subsequent time points, trypan blue exclusion and MTS proliferation assay were performed. Trypan blue exclusion assay would elucidate the number of cells in each sample, and thus reflect cell proliferation; while the MTS assay test for metabolic activity of the cell, specifically the mitochondrial activity. Cell mitochondrial activity is critically important for the function of cells. Cellular processes such as citric acid cycle and electron transport chain to generate ATP would not be possible without active mitochondria (80). Damaged mitochondria may also initiate cell apoptosis, resulting in cell death (81). From the results of trypan blue exclusion assay, it can be deduced that AFP addition did not enhance proliferation of post-thawed Jurkat cells. If AFP had an effect on cell recovery, then we would expect to see an increase in the number of live cells. Live cells at 24

hours eventually die off at the end of 72 hours, except for DMSO 10% treated cells frozen at -80°C. This demonstrates that although, AFPs may have had an effect on cell membrane protection during cryopreservation, this advantage did not translate into cellular recovery after the cells were thawed. However, trypan blue exclusion assay only roughly assess membrane integrity and therefore does not necessarily suggest absolute cell death (53). There have been cases where cell plasma membrane can become compromised while the cell is still alive. There is even evidence of cells capable of repairing its damaged cell membrane (52). We therefore moved to assess the metabolic activity of post-cryopreserved cells by using MTS assay. Unfortunately, the MTS data reaffirms the trypan blue exclusion data. No significant increase in metabolic activity was observed for cells treated with AFPs (908 and 1643) in any of the temperatures. Cells that were treated with 10% DMSO and frozen at -80°C were the only sample that demonstrated positive metabolic recovery. On one hand, this gives confidence to our assay since 10% DMSO has been used as the standard cryoprotectant for mammalian cell cryopreservation. On the other hand, it confirms that our AFP did not achieve nearly the same metabolic recovery effect. These results indicated that AFP treated cells could not recover or proliferate after freezing and thawing. In other words, solely protecting cell membrane was not enough to ensure cell function recovery. This was expected as we did not observe intracellular delivery of iPTD-engineered proteins. Without cryoprotectants inside the cell, large ice crystals develop and damage organelles. However, we believed that the membrane protective function of our AFPs could still be useful in other biological models.

One such model was the cryopreservation of RBCs. RBCs lack nucleus and other organelles and thus are unable to replicate and renew. RBCs are generally replaced by our body every 120 days

(82). RBCs could not make ATP in large amounts either, due to their lack of mitochondria. Thus, one of the most important aspects of successful RBC cryopreservation lies with its membrane integrity. The current clinical practice of RBC cryopreservation uses a high concentration of glycerol (40%). We decided to add our AFP to glycerol perfused RBCs. This is because glycerol is a penetrative cryoprotectant and therefore can ensure minimal intracellular ice damage while our AFP can enhance RBC membrane protection.

Under physiological conditions, hemoglobin accumulates in the cytosol of RBCs. During cryopreservation, the formation of large ice crystals and intracellular freezing damage RBC membrane. Furthermore, during the process of thawing, solute concentration oscillation also lead to RBC swelling and bursting (56). As a result, intracellular hemoglobin is released into the supernatant. If less hemoglobin is detected in the supernatant, then this suggests that less hemolysis has occurred and therefore more viable RBCs are recovered from cryopreservation.

ATP is the universal energy carrier of cells (58). RBCs are unique in their physiology in that they lack mitochondria and therefore cannot produce ATP through oxidative phosphorylation (64). RBCs can produce a small amount of ATP through glycolysis and lactic acid fermentation; however the amount of ATP yield is far lower compare to oxidative phosphorylation (65, 66). This means that RBCs have a limited stock of ATP in their intracellular space. Once this ATP stock is depleted from cryopreservation induced damages, RBCs quality is reduced due to their inactivity.

When we investigated the effect of AFPs on the current RBC cryopreservation standard (40% glycerol perfusion), we found that they did not significantly reduce the hemolysis caused by cryopreservation. However, we did observe more ATP in the intact RBCs of engineered protein

908 treated RBCs. One possibility is that unlike hemoglobin, ATP is a smaller molecule that can leak out of cells via passive diffusion and through ion channels (85). This means that hemolysis is not necessary for the release of ATP outside of RBCs. The engineered AFP may help in preventing such leakage by coating the RBCs prior to freezing and thus reduce the amount of ATPs that escaped during cryopreservation. In RBCs that were not treated with protein 908, although 40% glycerol could suffice to lower hemolysis, molecules important for post-thawing RBC functions, such as ATP, were not preserved. We then investigated the effect of AFPs on RBCs with reduced glycerol concentration. Surprisingly, when RBCs were perfused with 25% glycerol instead of 40%, the addition of protein 908 resulted in a significant hemolysis reduction. More interestingly, the result of hemolysis was similar to 40% glycerol perfused RBCs. This possibly means that if we use protein 908 as an additive to RBC cryopreservation, we can lower the concentration of glycerol needed to cryopreserve the RBCs. Less glycerol concentration would also shorten the steps and time for washing the glycerol away. This means that more RBCs will survive the strenuous glycerol washing process and be available to function after transfusing to the patient. Furthermore, glycerol is known to interfere with cellular processes and kinetics (59). Glycerol has been shown to interfere with drug functionality and reduce their effects as well (60). Therefore, reduced glycerol concentration could also mean that there is less risk of toxicity to the patient after transfusing cryopreserved RBCs. Protein 908 also had better effect on retaining intracellular ATP with reduced glycerol concentration. Adding protein 908 with 25% glycerol resulted in as much as 5 times ATP retention, while adding to 40% glycerol perfused RBCs only had 2.5 times intracellular ATP. This suggests that treating RBCs with protein 908 and reduced glycerol concentration may result in higher quality of post-thawing RBCs compared to the current cryopreservation protocol.

These results indicate that our engineered AFPs can enhance RBC post cell integrity with reduced glycerol concentration similar to research conducted by several other groups (83, 84). However, in addition to maintaining RBC membrane integrity, our AFP also increased the quality of post-thaw RBCs as shown by their intracellular ATP content. Not only is the total number of post-thaw RBCs increased, but more functional RBCs are available as well. Furthermore, *in vitro* evidences have shown that our AFPs are relatively low in toxicity. These findings are important as the RBCs are eventually transfused into patient to temporarily carry out oxygen transportation to sustain homeostasis.

From the results of this thesis project it can be concluded that iPTD-engineered AFPs quickly attaches to the cell membrane and reduce intracellular component leakage possibly by protecting the cell plasma membrane. However, cell recovery or proliferation was not enhanced. In the RBC cryopreservation model, iPTD engineered AFP significantly reduced the concentration of glycerol necessary to limit hemolysis caused by cryopreservation. In addition, these proteins also increase the quality of RBCs post-cryopreservation.

Chapter 7: Future Directions

The iPTD studied in this thesis project was capable of fast and efficient cell membrane delivery; however, it was not able to deliver the cargo protein into the cell. Therefore, the iPTD tag should be further optimized to allow for intracellular entry of cargo proteins. The selection of AFPs should also be expanded based on their antifreeze and membrane stabilizing activities. In the field of RBC cryopreservation, the quality of post-thawing RBCs can be further evaluated. For example, RBC membrane integrity can be measured also by subjecting RBCs to osmotic stress. Furthermore, other important RBC intracellular organic molecules, such as 2,3-DPG(2,3-diphosphoglycerate) can be analyzed to further assess RBC quality. The safety of AFPs should also be further investigated. Cytokine secretion profile of THP-1 cells should be analyzed to check for potential pro-inflammatory reaction caused by addition of AFPs. AFPs should also be administered to mice to see if there is systematic inflammatory response.

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