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Cytotoxicity Assessment of α Helix Antarctic Yeast Oriented Antifreeze Peptide (Afp1m) on *M. dunni* (Clone III8C) Cells

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ABSTRACT

In order to assess the cytotoxic effects of the cryoprotectant helix Antarctic yeast-orientated antifreeze peptide Afp1m on normal mouse skin fibroblasts, an *in vitro* model was developed for cytotoxicity assessment. In order to evaluate the cytotoxic effects of Afp1m, the cells of M. dunni (Clone III8C) were subjected to various amounts of Afp1m. The cell viability was assessed using MTT Assay (Tetrazolium dye MTT 3-(4, 5 dimethylthiazol-2-yl)-2, 5-10 diphenyltetrazolium bromide) against the positive control cells (Clone III8C) that were cultured with 10% FBS (Foetal Bovine Serum) using an Elisa reader and in medium containing various amounts (10, 5, 2, 1 and 0.5 mg/mL) of Afp1m, the control group (10% FBS) displayed varying survival percentages (78.86 ± 10.17%, 88.38 ± 3.19%, 88.75 ± 7.19 %, 90.61 ± 7.11%, 91.19 ± 4.52%, and 100.00 ± 0.0 %) throughout 24 hr. At 72 hr of treatment, the cell viability scores of Afp1m at 5, 2, 1, and 0.5 mg/mL were significantly higher ($p<0.05$) than those of 10mg/mL, which

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showed 86.73 ± 6.92 % viability of cells (103.9 ± 6.56 %, 104.3 ± 5.13%, 100.9 ± 1.71%, 102.8 ± 1.24%, and 100.00 ± 0.0%). At 24, 48, and 72 hr, retarded development was noted in 10 mg/mL Afp1m. Development was observed, albeit more slowly than in the positive control and treated with lesser concentrations. The findings of this work indicate that Afp1m exhibits cryoprotective properties without inducing toxicity when used for the cryopreservation of *M. dunni* (Clone III8C) cells.

Keywords: Cytotoxicity, fetal bovine serum, *M. dunni*, non-toxic cryoprotective, subzero temperatures, α helix

INTRODUCTION

Previous research has indicated that Anti-Freeze proteins (AFP) have a non-toxic cryoprotective effect on tissues, even at high concentrations. It is attributed to their elevated molecular weight, solubility, and ability to be incorporated into various solutions (Degner et al., 2014; Hirano et al., 2008). Using AFP as a cryopreservative for cells and tissues in hypothermic circumstances presents an alternative to conventional liquid nitrogen storage, which requires extremely low temperatures. Further investigation is warranted to explore the potential of utilising AFPs and their separated peptides for cryopreservation applications in various cell types and tissues and to ascertain their viability for future utilisation.

All types of cell preservation require low temperatures rather than freezing during storage. Effective use of cell culture technology is determined by the reproducible performance of particular cell lines and their continuous availability. Many laboratories rely on the continuous availability of various cell lines with consistent specialised properties, as well as a significant amount of other research resources. This can be achieved by keeping collections and stocks of cell cultures (Grout et al., 1990).

Previous research has failed to find evidence of a direct interface between anti-freeze peptides and cell membranes. In the presence of the liposomes, neither the antifreeze proteins (AFPs) nor the antifreeze glycoproteins (AFGPs) could change the behaviour phase of the phospholipid liposomes. Furthermore, the platelet membrane and the peptide's secondary structure were unaffected. Moreover, AFPs had a slighter protecting effect than other cryopreservatives on phospholipid systems as trans-monounsaturated or fully saturated lipids (Beirão et al., 2012). Cryopreservation of cells using AFP might have a wide range of applications for *in vitro* research and medical technology. It was also thought that AFPs were a non-toxic preservative that would not distress the cells in different solutions and buffers (Adler et al., 1993; Hirano et al., 2008). Due to their higher molecular weight could be soluble in buffer solutions (Baardsnes et al., 2001).

It has been shown that AFP does not contain chemical tissue toxicity at higher concentrations of up to 40 mg/mL. If used in the buffer solution, it is a non-toxic

cryopreservative that will not disturb the cells and tissue. Due to its higher molecular weight, AFP is soluble in buffer solutions (Degner et al., 2014; Hirano et al., 2008).

The utilisation of Antifreeze peptide (Afp1m) involves the application of a compact peptide that imitates the biological functionality exhibited by the parent Antifreeze Proteins (AFPs). The Afp1m protein is constructed using a sequence of 25 amino acids derived from the helix-1 of the helix sections found in Glaciozyma Antarctica. This sequence is modified by introducing specific substitutions. The modifications made to the sequence were implemented to facilitate the creation of helix structures by introducing salt bridges into the peptide sequence. Due to these alterations, Afp1m is expected to form a more stable helix structure than the other isolated peptides. Therefore, it is thought to have higher antifreeze activity (Shah et al., 2012). Afp1m may be useful in the future as a quality storage media of the cells and tissue at low-temperature preservation conditions and can keep the tissue without freezing damage at very low temperatures.

The exact molecular mechanism of action of Afp1m needs to be clearly understood. This study was designed to investigate the interactions of Afp1m in cryomedia and ice crystal effects on post-cryopreservation cell viability. The objectives of this study were to estimate the cytotoxicity level of Afp1m in growing M. *dunni* (Clone III8C) cells at 37°C for 24, 48 and 72 hr.

MATERIALS AND METHODS

All cell culture *M. dunni* (Clone III8C) incubations took place in a 37°C incubator with 5% CO_2 and standard humidity (95%) (Figure 1). Prospectively used solutions were pre-warmed at room temperature before being applied to the cells. *M. dunni* (Clone III8C) (ATCC® CRL-2017™) was purchased from ATCC. *M.dunni* Clone IIIC8 was derived from the dermal layer of the skin of a normal female mouse (*Mus dunni*). These cells resembled fibroblasts. These cells lack most endogenous murine leukaemia virus-linked sequences. The confluent culture of M. dunni (Clone III8C) remains more firmly attached in a growing medium composed of RPMI 1640 or DMEM + 10% of FBS during the assays. These cells are mainly resistant to the infections. The base medium used for this cell line was McCoy's 5A Medium Modified (ATCC-formulated). Fetal bovine serum (FBS) at a concentration of 10% was added to the base medium to modify McCoy's 5A Medium (Weng & Hsu, 2008).

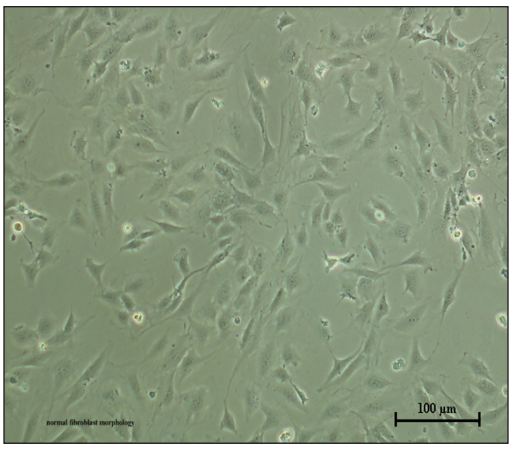

Figure 1. Morphology of normal mouse epidermis fibroblasts M.dunni Clone1118C (Scale bar = 100 µm × 10)

Normal mouse skin fibroblasts (M.dunni Clone1118C) employed in an *in vitro* cryopreservation experiment for the protein Afp1m showed the following morphology.

Subculture Procedure

A cryovial of *M. dunni* (Clone III8C) cells was rapidly thawed by gentle spinning in the 37°C water bath. The cap and the O-rings of the cryovial were kept out on the water surface to reduce the risk of contamination. The cryovial was disinfected by spraying with 70% ethanol in the biohazard safety cabinet type II. The cell suspension of *M. dunni* cells was transferred from the cryovial into a 15 mL conical tube containing 10 mL of ATCC-formulated McCoy's 5A Medium supplemented with 10% fetal bovine serum (FBS). Cells were pelleted using a centrifuge at 200 × g for 5 minutes at room temperature. After pelleting, the supernatant was removed, and cells were resuspended in 5 to 10 mL McCoy's 5A Medium, 10% FBS supplemented and transferred into a 25 cm^2 tissue culture flask (T25) by adding 5 mL of McCoy's 5A medium. Suspension was transferred to tissue culture flasks and vented caps. The flask was incubated in a 37°C incubator with 5% CO_2. Cells were monitored daily or every other day up to the time until they reached 90% confluence. Media was changed every 3 days until the cells reached 80%–90% confluence and were passed into the new flasks (Freshney, 2005; Ho et al., 2012).

Maintenance of Cell Culture

M. dunni (Clone III8C) cells stopped growing and started to die after they reached confluence; therefore, it was extremely important to monitor cells and subculture them until they reached confluent monolayers. Actively grown cells usually double roughly in numbers every 24 (Chen & Okayama, 1987). The frequency of cell passaging, often ranging from 2 to 3 times each week, is contingent upon the initial quantity of cells planted in the flask as well as the size of the flask.

The growth medium was removed from a confluent monolayer of *M. dunni* (Clone III8C) cells. Cells were washed twice with 5 mL of Phosphate-Buffered Saline (PBS) to remove serum containing trypsin inhibitors. 5 mL of 1× trypsin-EDTA was added, and the cells were incubated at 37°C for 2 to 3 minutes until they started to detach from the surface of the flask.

Moderate shaking and tapping of the flask were performed on the flask surface to help the cells detach from the flask. McCoy's 5A Medium (5 mL) was added with 10% FBS to inactivate the trypsin-EDTA. The cell suspension was pipetted up and down to break up any clump of cells. Cells with media were removed from the flask, transferred to a sterile 15 mL falcon tube, and then centrifuged at 200 × *g* at room temperature for 5 minutes. The supernatant was removed, and cells were resuspended in 10 mL McCoy's 5A Medium with 10% FBS. The desired dilution of cells in 12 to 20 mL of McCoy's 5A Medium with 10%

FBS was added to 75 cm^2 cell culture flasks with vented caps. Cells were monitored daily or every alternate day. Media was changed after every 3 to 4 days. When cells reached 80%–90% of the confluent monolayer level, by repeating this protocol, cells were passaged again (Ammerman et al., 2008).

Cytotoxicity Determination

M. dunni (Clone III8C) cells suspended in McCoy's 5A medium, which was modified with 10% FBS, cells were plated before treatment. About 1 x 10^5 cells/mL were seeded in the 96-well titration plate (Becton Dickinson, USA), and the plate was kept at 37 °C with a 5% CO_2 incubator for 24 h. On the following day, the cells were treated with different Afp1m concentrations. The old medium was removed, and the sub-diluted extracts were then transferred into a 96-well titer plate using sterile pipette tips. The concentrations of both types of cells were determined using a haemocytometer. The cells were suspended in supplemented McCoy's 5A medium, containing the different concentrations of Afp1m and 10 mg/mL.

A 4 mg/mL was prepared and then diluted by serial dilution in 96-well microtitration plates to achieve the required concentration of a 20 mg/mL stock solution. The titration plate was placed for incubation at 37°C with 5% CO_2 for 24, 48 and 72 h. Cells were suspended in a growth medium containing McCoy's 5A supplemented with 10% FBS for positive control.

After 24, 48 and 72 hr incubation at 37°C with 5% CO_2, MTT reagent (20 µL) was added to each well, and the plate was incubated for 3 hr in an incubator at 37°C with 5% CO_2. The generated purple formazan was dissolved by adding 150 µL of dimethyl sulfoxide (DMSO) in a dark environment at room temperature. The solution was then agitated on an orbital shaker for approximately 15 minutes. The absorption measurement was conducted using a microplate reader, with the recorded values at 570 mM and a reference value at 630 mM.

The trials were conducted in triplicate, resulting in three sets of data. After the incubation period, the fraction of survived cells was determined relative to the untreated cell population by the MTT assay. The data were analysed using Graph Pad Prism Version 9, which showed the graph of viability percentages after cytotoxicity tests and cryopreservation.

Statistical Analysis

The dose-response of each concentration, temperature and period was determined from those summarised and presented by bar graphs with a mean (±) standard deviation after analysing data as depicted in Figures 2 to 5**.** The score data were evaluated parametrically using one-way ANOVA and two-way ANOVA tests. The multiple comparison tests of Sidak and Tukey were conducted, and statistical significance was determined based on the observed p-value ($p< 0.05$) (Parvaneh et al., 2017).

RESULTS

Cytotoxicity Determination

This study employed the MTT test to evaluate the cytotoxic effects of Afp1m on *M. dunni* (Clone III8C) cells. The cells were cultured with Afp1m in a 37°C incubator with 5% CO2, and the percentage of viable cells was assessed at various time intervals (24, 48, and 72 hr).

Cytotoxicity After 24 hr Treatment with Afp1m at 37°C (Cell Viability %)

The % values of live cells following 24 hr of treatment with different concentrations of Afp1m, *i.e.* 10, 5, 2, 1 and 0.5 mg/mL with positive and negative controls were 78.86 ± 10.17%, 88.38 ± 3.19%, 88.75 ± 7.19%, 90.61 ± 7.11%, 91.19 ± 4.52%, and 100.00 ± 0.0%, respectively.

One-way ANOVA observed a significant difference ($p<0.01$) among different concentrations following 24 hr treatment at 37°C (Figure 2). There was a significant difference ($p<0.05$) between 10 mg/mL and all lower concentrations, *i.e.* 5, 2, 1, 0.5 mg/mL and control percentages. A significant difference ($p<0.05$) was observed between the control and 5, 2, 1 and 0.5 mg/mL percentages. There was no significant difference ($p>0.05$) between 5 mg/mL and lower concentrations.

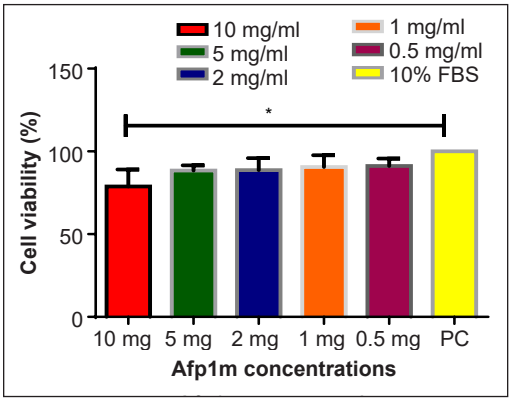

Figure 2. Cell viability for different concentrations of Afp1m incubated for 24 hr at 37°C (A statistically significant difference ($p<0.01$) was discovered using a one-way analysis of variance (ANOVA) when examining the percentage of reported viable cells following an incubation time of 24 hours at 37°C with 5% CO_2)

Cytotoxicity Following a 48-hour Afp1m Therapy at 37°C (Cell Viability %)

The % of live cells following 48 hr of treatment with different concentrations of Afp1m with positive and negative controls were 83.37 ± 8.74%, 97.46 ± 1.71%, 95.80 ± 6.24%, 94.2 ± 4.81%, 95.29 ± 5.53%, and 100.00 ± 0.0%, respectively. A significant ($p<0.01$) difference was observed between different concentrations at 48 hr.

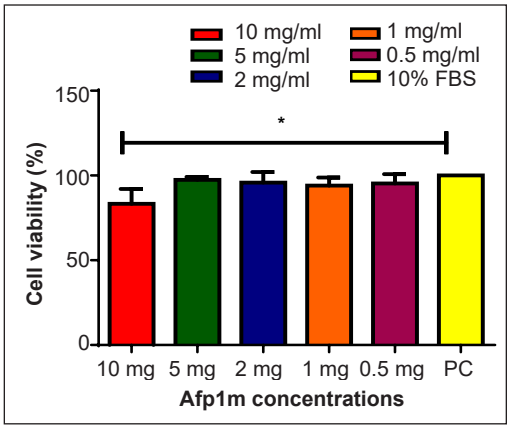

Figure 3. Cell viability for different concentrations of Afp1m incubated for 48 hr at 37°C (Following a 48-hour incubation period at 37 degrees Celsius and 5% carbon dioxide, a One-way Analysis of Variation (ANOVA) was performed. The results showed a statistically significant difference (p 0.01) in the percentage of cell survival.)

Cell viability in 10 mg/mL was significantly lower ($p<0.01$) than all lower concentrations, i.e. 5, 2, 1, and 0.5 mg/mL with control percentages. There was no significant difference ($p>0.05$) between 5 mg/mL and lower concentrations, i.e. 2, 1 and 0.5 mg/mL with control percentage (Figure 3).

Cytotoxicity Following a 72-hour Afp1m Therapy at 37°C (Cell Viability %)

The % of live cells following 72 hr of treatment with different concentrations of Afp1m, *i.e.* 10, 5 ,2 ,1 and 0.5 mg/mL with positive and negative controls were 86.73 ± 6.92%, 103.9 ± 6.56%, 104.3 ± 5.13%, 100.9 ± 1.71%, 102.8 ± 1.24%, and 100.00 ± 0.0%, respectively. One-way ANOVA suggests a significant difference ($p<0.01$) between different concentrations at a 72 hr time point. Tukey's multiple comparison tests revealed a noteworthy distinction ($p<0.01$) between 10 mg/mL and all lower concentrations, i.e. 5, 2, 1, 0.5 mg/mL and control percentages. There was no significant difference ($p>0.05$) between 5 mg/mL and lower concentrations of 2, 1 and 0.5 mg/mL with control percentage (Figure 4).

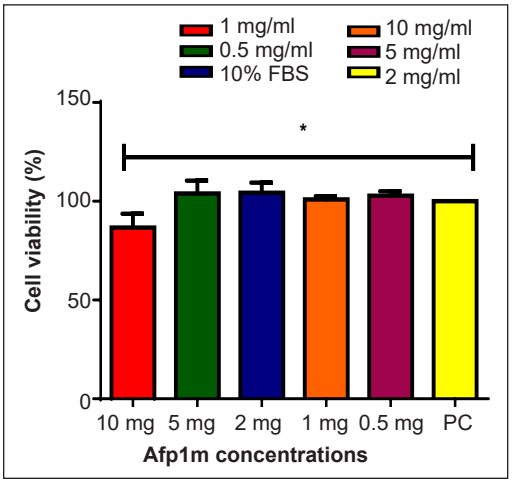

Figure 4. Cell viability for different concentrations of Afp1m incubated for 72 hr at 37°C

Cytotoxicity Comparisons Between Different Afp1m Concentrations at 72 hr (Cell Viability %)

One-way ANOVA showed a significant difference ($p <0.01$) between 24, 48 and 72 hr of cell viability percentages. Multiple comparison tests depicted a significant difference at 24 and 48 hr, 24 and 72 hr, and 48 and 72 hr. Cell number was significantly increased from 24 to 48 hr and 48 to 72 hr (Figure 5).

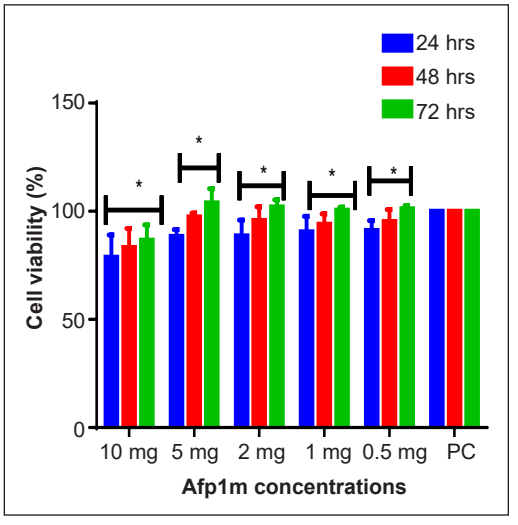

Figure 5. The viability of cells at 37°C for different amounts of Afp1m at different times

Afp1m Concentration 10 mg/mL

A statistically significant variation ($p<0.01$) was seen in the viability of cell percentages

between the time points of 24 and 72 hrs. No statistically significant difference ($p>0.05$) was seen between the 24-hour and 48-hour time points. At the same time, a significant difference ($p<0.05$) was also observed between 48 and 72 hr (Figure 5).

Afp1m Concentration 5 mg/mL

At a dosage of 5 mg/mL, there was a statistically significant difference ($p <0.01$) in cell viability percentages between the 24 and 48-hour and 24- and 72-hour time points. Furthermore, after 48 and 72 hr, a statistically significant difference was seen ($p <0.05$) (Figure 5).

Afp1m Concentration 2 mg/mL

The proportion of cells that survived between the time points of 24 and 48 hr, as well as between 48 and 72 hr, showed statistically significant differences (p 0.05) at a dose of 2 mg/mL. Furthermore, a highly significant (p 0.01) difference was discovered between the 48- and 72-hour intervals (Figure 5).

Afp1m Concentration 1 mg/mL

No significant difference ($p>0.05$*)* was observed in cell viability percentages at 1 mg/mL at 24 h. In contrast, a significant difference was observed in cell viability percentages between 1 mg/mL between 48 and 72 h, while a high significant difference ($p<0.01$) was also observed between 24 and 72 hr (Figure 5).

Afp1m Concentration 0.5 mg/mL

No statistically significant differences ($p>0.05$) were found in the cell viability percentages at a concentration of 0.5 mg/mL between the time points of 24 and 48 hr, as well as between 48 and 72 hr. On the other hand, a highly significant difference was seen at the time points of 24 and 72 hr ($p < 0.01$) (Figure 5).

DISCUSSION

Different Afp1m treated cells showed different survival percentages after growing the cells in media containing different Afp1m concentrations at 37°C with 0.05% CO_2. The percentage of cell viability in different concentrations, i.e. 10 mg/mL, was increased from 78.86% to 86.73%, 5 mg/mL (from 88.38% to 103.9%), 2 mg/mL (from 88.75% to 104.3%), 1 mg/mL (90.61% to 100.9%), and 0.5 mg/mL (from 91.19% to 102.8%). At the 72-hour mark of treatment, the concentrations of Afp1m at 2 and 5 mg/mL showed notable differences, surpassing the scores obtained by the concentrations of 1 mg/mL and

0.5 mg/mL. Impaired growth was found in the 10 mg/mL AFP solution at 24, 48, and 72 hr. The growth rate of the samples treated with a concentration of 10 mg/mL was slower than that of those treated with lower concentrations.

These results were in partial agreement with the studies in which it was mentioned that protective effects may be narrowed at higher Afp1m concentrations and were in agreement with the study that the maximum concentration of AFP1 may produce damage to cryopreserved tissue (Chao et al., 1996). These findings also align with a study by Liu et al. (2007), which used human liver and kidney cells treated with AFGP8 and produced different results. The study showed that a higher concentration of more than 2 mg/mL AFGP8 showed higher toxicity, subsequently 20 hr of incubation at 37°C (Liu et al., 2007). Findings also showed similarity to the study where the cryoprotectant like DMSO was the most toxic of the cryoprotectant to zebra fish blastomeres when exposed at room temperature (Ritar, 1999). These results agree with previous findings where an anti-freeze peptide from *Pleuronectes americanus* was successfully used as a cryoprotectant at 37°C. They gave 50% viability when Vero cells were grown with different concentrations of Afp1m from *P. americanus* (Migliolo et al., 2012).

Previous studies reported using anti-freeze glycopeptides as cryoprotectants characteristically worked at concentrations greater than 10 mg/mL. One of these reports designated the cytotoxicity in spinach thylakoid membranes (Biggs et al., 2017; Hincha et al., 1993; Sun et al., 2022). In this study, Afp1m has been mentioned to be a good vitrification agent that protects cells and enhances growth at subzero temperatures and lower temperatures like 0°C and in fresh cell suspensions (Bang et al., 2013; Katkov et al., 2012).

CONCLUSION

The finding of the current study revealed that cryopreservation with Afp1m was safe. Cryopreservation of cells with the Afp1m concentration like 10 mg/mL and above and very low concentrations like 2 mg/mL and 1 mg/mL may need further studies to confirm the effects of peptide on the cell morphology. Using damage-specific N-glycosylases will help researchers assess the various types and amounts of damage. It will allow researchers to compare the role of numerous DNA repair pathways in DNA damage caused by tissue cryopreservation. Therefore, the use of Afp1m in the cryopreservation of cells and tissue should be further investigated.

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REFERENCES

Adler, K., Arkona, C., Manteuffel, R., & Süss, K. H. (1993). Electron-microscopical localization of chloroplast proteins by immunogold labelling on cryo-embedded spinach leaves. *Cell Biology International, 17*(2), 213-220. https://doi.org/10.1006/cbir.1993.1057

Ammerman, N. C., Beier-Sexton, M., & Azad, A. F. (2008). Growth and maintenance of Vero cell lines. *Current Protocols in Microbiology, 11*(1), A.4E.1-A.4E.7. https://doi.org/10.1002/9780471729259.mca04es11

Baardsnes, J., Jelokhani-Niaraki, M., Kondejewski, L. H., Kuiper, M. J., Kay, C. M., Hodges, R. S., & Davies, P. L. (2001). Antifreeze protein from shorthorn sculpin: Identification of the ice-binding surface. *Protein Science, 10*(12), 2566-2576. https://doi.org/10.1110/ps.ps.26501

Bang, J. K., Lee, J. H., Murugan, R. N., Lee, S. G., Do, H., Koh, H. Y., Shim, H. E., Kim, H. C., & Kim, H. J. (2013). Antifreeze peptides and glycopeptides, and their derivatives: potential uses in biotechnology. *Marine Drugs, 11*(6), 2013-2041. https://doi.org/10.3390/md11062013

Beirão, J., Zilli, L., Vilella, S., Cabrita, E., Schiavone, R., & Herráez, M. P. (2012). Improving sperm cryopreservation with antifreeze proteins: effect on gilthead seabream (Sparus aurata) plasma membrane lipids. *Biology of Reproduction, 86*(2), 59-1. https://doi.org/10.1095/biolreprod.111.093401

Biggs, C. I., Bailey, T. L., Graham, B., Stubbs, C., Fayter, A., & Gibson, M. I. (2017). Polymer mimics of biomacromolecular antifreezes. *Nature Communications, 8*(1), Article 1546. https://doi.org/10.1038/s41467-017-01421-7

Chao, H., Davies, P. L., & Carpenter, J. F. (1996). Effects of antifreeze proteins on red blood cell survival during cryopreservation. *Journal of Experimental Biology, 199*(9), 2071-2076. https://doi.org/10.1242/jeb.199.9.2071

Chen, C., & Okayama, H. (1987). High-efficiency transformation of mammalian cells by plasmid DNA. *Molecular and Cellular Biology, 7*(8), 2745-2752. https://doi.org/10.1128/mcb.7.8.2745-2752.1987

Degner, B. M., Chung, C., Schlegel, V., Hutkins, R., & McClements, D. J. (2014). Factors influencing the freeze-thaw stability of emulsion-based foods. *Comprehensive Reviews in Food Science and Food Safety, 13*(2), 98-113. https://doi.org/10.1111/1541-4337.12050

Freshney, R. I. (2005). Culture of specific cell types. In *Culture of Animal Cells: A Manual of Basic Technique* (pp. 375-420). John Wiley & Sons. https://doi.org/10.1002/0471747599.cac023

Grout, B., Morris, J., & McLellan, M. (1990). Cryopreservation and the maintenance of cell lines. *Trends in Biotechnology, 8*, 293-297.

Hincha, D. K., De Vries, A. L., & Schmitt, J. M. (1993). Cryotoxicity of antifreeze proteins and glycoproteins to spinach thylakoid membranes - comparison with cryotoxic sugar acids. *Biochimica et Biophysica Acta (BBA)-Biomembranes, 1146*(2), 258-264. https://doi.org/10.1016/0005-2736(93)90364-6

Hirano, Y., Nishimiya, Y., Matsumoto, S., Matsushita, M., Todo, S., Miura, A., Komatsu, Y., & Tsuda, S. (2008). Hypothermic preservation effect on mammalian cells of type III antifreeze proteins from notched-fin eelpout. *Cryobiology, 57*(1), 46-51. https://doi.org/10.1016/j.cryobiol.2008.05.006

Ho, W. Y., Yeap, S. K., Ho, C. L., Rahim, R. A., & Alitheen, N. B. (2012). Development of multicellular tumor spheroid (MCTS) culture from breast cancer cell and a high throughput screening method using the MTT assay. *Plos One, 7*(9), Article e44640. https://doi.org/10.1371/journal.pone.0044640

Katkov, I. I., Bolyukh, V. F., Chernetsov, O. A., Dudin, P. I., Grigoriev, A. Y., Isachenko, V., Isachenko, E., Lulat, A. G. M., Moskovtsev, S. I., Petrushko, M. P., Pinyaev, V. I., Sokol, K. M., Sokol, Y. I., Sushko, A. B., & Yakhnenko, I. (2012). Kinetic vitrification of spermatozoa of vertebrates: What can we learn from nature? In *I. I. Katkov (Ed.), Current Frontiers in Cryobiology (pp.3-40).* INTECH Open Access Publisher. https://www.intechopen.com/chapters/31227

Liu, J. X., Srivastava, R., Che, P., & Howell, S. H. (2007). An endoplasmic reticulum stress response in Arabidopsis is mediated by proteolytic processing and nuclear relocation of a membrane-associated transcription factor, bZIP28. *The Plant Cell, 19*(12), 4111-4119. https://doi.org/10.1105/tpc.106.050021

Migliolo, L., Silva, O. N., Silva, P. A., Costa, M. P., Costa, C. R., Nolasco, D. O., Barbosa, J. A. R., Silva, M. R. R., Bemquerer, M. P., Lima, L. M. P., Romanos, M. T. V., Freitas, S. M., Magalhães, B. S., & Franco, O. L. (2012). Structural and functional characterization of a multifunctional alanine-rich peptide analogue from *Pleuronectes americanus*. *Plos One, 7*(10), Article e47047. https://doi.org/10.1371/journal.pone.0047047

Parvaneh, S., Mohler, J., Toosizadeh, N., Grewal, G. S., & Najafi, B. (2017). Postural transitions during activities of daily living could identify frailty status: Application of wearable technology to identify frailty during unsupervised condition. *Gerontology, 63*(5), 479-487. https://doi.org/10.1159/000460292

Ritar, A. J. (1999). Artificial insemination with cryopreserved semen from striped trumpeter (*Latris lineata*). *Aquaculture, 180*(1-2), 177-187. https://doi.org/10.1016/S0044-8486(99)00109-X

Shah, S. H. H., Kar, R. K., Asmawi, A. A., Rahman, M. B. A., Murad, A. M. A., Mahadi, N. M., Basri, M., Rahman, R. N. Z. A., Salleh, A. B., Chatterjee, S., Tejo, B. A., & Bhunia, A. (2012). Solution structures, dynamics, and ice growth inhibitory activity of peptide fragments derived from an antarctic yeast protein. *Plos One, 7*(11), Article e49788. https://doi.org/10.1371/journal.pone.0049788

Sun, Y., Maltseva, D., Liu, J., Hooker, T., Mailänder, V., Ramløv, H., DeVries, A. L., Bonn, M., & Meister, K. (2022). Ice recrystallization inhibition is insufficient to explain cryopreservation abilities of antifreeze proteins. *Biomacromolecules, 23*(3), 1214-1220. https://doi.org/10.1021/acs.biomac.1c01477

Weng, C. H., & Hsu, M. C. (2008). Regeneration of granular activated carbon by an electrochemical process. *Separation and Purification Technology, 64*(2), 227-236. https://doi.org/10.1016/j.seppur.2008.10.006