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Recent Advances in Cryopreservation

Edited by Hideaki Yamashiro

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http://dx.doi.org/10.5772/56998
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Notice

First published in Croatia, 2014 by INTECH d.o.o.
eBook (PDF) Published by IN TECH d.o.o.
Place and year of publication of eBook (PDF): Rijeka, 2019.
IntechOpen is the global imprint of IN TECH d.o.o.
Printed in Croatia

Legal deposit, Croatia: National and University Library in Zagreb

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Recent Advances in Cryopreservation
Edited by Hideaki Yamashiro
p. cm.
ISBN 978-953-51-1644-8
eBook (PDF) ISBN 978-953-51-5396-2

Meet the editor

Hideaki Yamashiro is a mammalian sperm cryobiologist with more than 10 years of experience in the field. It has been an interesting experience working for Japan International Cooperation Agency (JICA), Japan Overseas Cooperation Volunteers in Botswana, Africa, for developing livestock management by using sperm. After that, he tried to develop the cryopreservation method of the Red maasai sheep sperm at Consultive Group on International Agricultural Research (CGIAR) of the International Livestock Research Institute (ILRI) in Kenya. He received his PhD at Tohoku University in Japan. Postdoctoral training has been done at Institute for Biogenesis Research, Department of Anatomy and Reproductive Biology, John A Burns School of Medicine, University of Hawaii at Manoa, Honolulu, Hawaii, USA. Research interest is now focused on gametes and fertilization biology, assisted fertilization, biotechnology including sperm freezing in mammals at Niigata University in Japan.

Contents

Preface

Cryopreservation allows virtually indefinite storage of biological material without deterioration over a time scale of at least several years. Conservation strategies benefit from advances in cryopreservation and reproductive technologies.

This book addresses a cryopreservation of issues in fish, animal and humans through a series of chapters. The chapters have been contributed by authors from Brazil, China, Spain, USA and Japan. It has provided an updated overview of recent advances of selected topics in the wide discipline of cryopreservation.

We hope the book will be useful to individuals interested in the utilization of cryo- biotechnology for characterization and conservation of genetic resources with regard to aquatic species of fish, domestic animals and humans for humanity of today and tomorrow.

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Chapter 1

Cryopreservation of Adherent Cells on a Fixed Substrate

Lia H. Campbell and Kelvin G.M. Brockbank

Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/58618

1. Introduction

Tissue culture has become the backbone of research in the life sciences. Large numbers of primary cells and established cell lines are available from the American Type Culture Collection (ATCC, Manassas, VA) and various other sources. Some cell types are relatively easy to grow and maintain in culture while other cell lines have specific handling issues that must be learned. These cells come from a variety of animal and human sources and are being used for research as well as the production of enzymes, hormones, and other biological compounds. Toxicology applications range from the use of cells as indicator systems for environmental pollutants to screening cosmetic formulations, new drugs and household chemicals for potential risks. Such biodetector systems may involve cells from almost any vertebrate or invertebrate species. In addition, the universal demand for a reduction of the use of animals in research has prompted the development of alternative methods to determine applicability of new products or procedures. New technologies such as high-throughput screening have been designed to reduce animal testing while increasing the rate at which new drugs or products are brought to market. It has become increasingly clear that cell–based assays will provide the most information for these technologies to be effective in preliminary screening of potential new drugs and products [1].

Convenient cell based systems for a wide variety of applications using a variety of cell types representing key cell-types in various tissues are needed. Readily available cells on a fixed substrate, such as a microtiter plate, that can be used with a minimum of preparation time would not only decrease costs, but also increase the number and variety of assays performed and potential products that are screened in a given amount of time. The idea of cryopreserving cells on a fixed substrate is not new. Researchers have been attempting to cryopreserve cells on a fixed substrate for some time using a variety of cells [2-9]. In general, cell survival in these studies was lower that when the same cells were cryopreserved in suspension. Still, a method has not been developed that can be applied to many different cell types and applications.

Most cells used in research are cryopreserved after addition of 10% dimethyl sulfoxide (DMSO) to cells in suspension in cryovials, slow rate cooling, with or without induced nucleation, and storage at-80°C or below-135°C. For most applications, this procedure is adequate to provide viable, usable cells whatever the yield upon thawing. Often no consideration has been given to how the cryopreservation process affects the cells and could potentially affect their function upon thawing. Not only physical changes, such as water nucleating to form ice, but also chemical changes take place as the temperature is reduced that subsequently affect the viability and survival of cells and tissues upon thawing. As the temperature is reduced, heat is removed and molecular processes are slowed which leads to a variety of structural and functional changes within the cells even before freezing. As a consequence, the cell experiences a cascade of biochemical and biophysical changes that sensitize the cell to further injury and can lead to irreversible damage. For example, the rate at which cells are cooled and rewarmed is known to be a major determinant of cell survival. As the temperature of the system is reduced, ice forms initially in the extracellular space. Pure water separates as ice crystals so that solutes are concentrated in the remaining liquid phase. As a consequence water moves out of the cell across the plasma membrane in an effort to reestablish osmotic equilibrium within the extracellular space. If the cells are cooled too rapidly, less time is allowed for water to move out of the cells and intracellular ice occurs causing irreparable cell damage. If cells are cooled too slowly, more water is allowed to leave the cells increasing the solute concentration within the cell (Figure 1). This increase in solute concentration both inside and outside the cell has been termed "solution effects" injury because it encompasses a number of changes that include increased salt concentrations, which can denature proteins and membranes, precipitation of buffers, induced pH changes, increased concentration of proteins may result in cross linking, or simply removal of structurally important water. Usually an intermediate cooling rate provides the best compromise between cytotoxicity due to cryoprotectants (CPAs) and ice formation. During rewarming the process is reversed, ice is replaced with water, and cryoprotectants (CPA) are removed from the system. However, physical and chemical changes to bring the cells back to physiologic temperature can still cause damage. As the sample is warmed recrystallization can occur. Recrystallization is when metastable ice crystals that formed during freezing are given an opportunity to reform larger crystals during rewarming. These ice crystals can cause damage to the cells in a similar manner as those crystals that were formed during freezing. Another concern during rewarming is the removal of the cryoprotectants. The CPAs were added to the samples prior to freezing and cell permeating compounds like DMSO, replace cell water further decreasing the risk of ice damage. However, during rewarming, DMSO does not move across the cell membrane as readily as water. An imbalance can develop so that the cells will tend to take up water faster than the DMSO is removed causing swelling. Too much swelling can cause irreversible cell damage if the rewarming is not controlled appropriately, even if the freezing protocol worked. All these factors affect the overall survival of cells during cryopreservation. Some cells can be cryopreserved readily, such as hematopoietic stem cells and fibroblasts, while other cell types are much more difficult, such as hepatocytes. Therefore, optimization for a given cell type is usually required, particularly if they are adherent [1,10-12].

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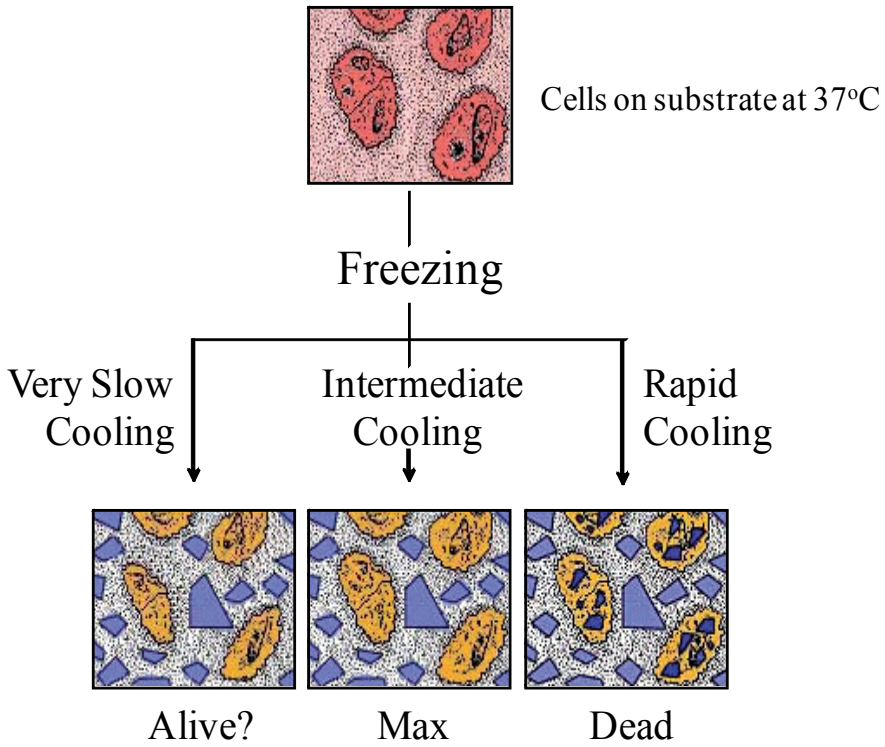

Figure 1. Comparison of differing cryopreservation strategies. If the cells are cryopreserved by freezing ice forms (blue crystals) initially in the extracellular environment and the cells undergo cooling rate dependent shrinkage due to osmotic dehydration. The slower the cooling rate the longer intracellular water has the opportunity to move out of the cell by osmosis due to the increasing osmolality of the extracellular environment as water is incorporated into ice crystals. Maximum cell viability is usually achieved at an intermediate cooling rate that balances osmotic dehydration and the risk of intracellular ice formation. Rapid cooling permits intracellular ice formation and usually leads to cell death upon rewarming. Very slow cooling may lead to excessive cell dehydration and cell death.

In addition to the physical and biochemical changes that take place within the cell during cryopreservation, there is the added parameter of attachment to a substrate that must also be taken into consideration. As described in this chapter, considerable time has been spent developing procedures to cryopreserve cells on a fixed substrate, microtiter plates in particular. However, the protocols described below can be applied to cells fixed on other types of substrates such as a glass slide or a three dimensional scaffold for the purpose of making a tissue engineered construct. The purpose of the CryoPlate™ concept is to provide cells on plates that are ready to use for any number of applications, toxicology testing being one of many. Ideally, any cell type of interest could be cryopreserved on plates and then be ready to use with a minimum of processing time. In practice, no two cell types behave exactly alike when cryopreserved, however, basic criteria and protocols can be established and then adjusted and optimized to fit a specific cell type. While strict criteria have not been established, some basic parameters, specifications, were necessary to guide the development of the CryoPlate™ concept. These are (1) uniform cell number in all the wells within the microtiter

plate (2) >80% of the cells in all the wells are viable (3) the specific activity of a given cell type is intact (4) reproducibility; the retention and viability of the cells is similar in independent experiments after cryopreservation (5) ease of processing; the steps involved in thawing the plate and readying the cells for a specific assay should be user-friendly, not requiring special handling, and not be time-consuming. Potentially any adherent cell type could be established within our system including modified cell lines that may contain plasmid constructs. Such a system would provide cells that are ready to use with a minimum of preparation time and would also be conducive to automation and miniaturization.

2. Materials and methods

Cells: Two cell lines were used in these studies. One comes from rat thoracic aorta called A10 (ATCC# CRL-1476), and the second comes from bovine corneal endothelium, BCE (ATCC# CRL-2048). Both cell types were grown and maintained in Dulbecco's Modified Eagle's Medium (DMEM) with 10% fetal calf serum (FCS) at 37°C with 5% carbon dioxide.

Cryopreservation Methods: For cryopreservation experiments, different cryoprotectant formulations, the plates were placed on ice and increasing concentrations of cryoprotectant(s) added. Then the final cryoprotectant solution was added and left for 10 minutes on ice before being cooled at a controlled -1.0°C/min. rate to –80°C with or without nucleation at-6°C as described in the text. The plates were stored at ≤–135°C in vapor phase nitrogen or in a mechanical storage freezer. The plates were thawed rapidly at 37°C using a water bath for single step thawing. For the two step thawing, plates were left at an intermediate temperature before rapid thawing at 37°C using a waterbath or some other device as described (US Patent #6,596,531) [17]. The cryoprotectant solution was then removed, the wells washed with 0.5M mannitol and culture medium before being left in media for a 1 hour recovery period at 37°C in a tissue culture incubator.

Measurement of Cell Viability and Proliferation: Cell metabolic activity will be assessed using the resazurin reduction assay (alamarBlue™) [13]. Reduction of resazurin to resorufin measures the oxidation/reduction reactions taking place within cells. Resazurin was added directly to the wells in culture medium and incubated for 3 hours at 37°C. Upon reduction of resazurin to resorufin a color change occurs and this color change can be measured and quantified. The culture plates were read using a Gemini EM fluorescent microplate reader (Molecular Dynamics) at an excitation wavelength of 544nm and an emission wavelength of 590nm. Viability is expressed either in percent of untreated controls or after correction, using relative DNA content, for cell losses. In addition to measuring viability after rewarming, the ability of the cells to proliferate was also examined. Because resazurin is non-toxic, it can be used repeatedly without harming the cells [14-16]. Increased metabolic activity over time is indicative of proliferation and decreased metabolic activity is indicative of cell death by apoptosis, subject to verification as described below.

DNA Measurement: The proportion of cells remaining in the well of the microtiter plate after cryopreservation can be assessed by measuring the DNA content of the cells within a given

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well. The Cyquant assay (Molecular Probes) uses a fluorescent dye to label nucleic acids that can then be measured using a fluorescent microplate reader with an excitation wavelength of 485 nm and an emission wavelength of 538 nm. The assay includes a step using RNAse A (Sigma) to eliminate the variable amount of RNA within individual cells and thereby provide a direct measure of the DNA content of the cell alone.

Temperature Measurement: The temperature profiles for individual wells in a microtiter plate were made using thermocouples placed in the middle of wells at the corners of the plate and one well in the center. Several well configurations were evaluated including a 6 well plate where each well contained a thermocouple. The cryoprotectant solution used for these measurements was 1-2M DMSO. Temperature measurements were taken using a datalogger (Omega). Measurements were made every 5 seconds to 5 minutes depending on the rate of warming or cooling being measured.

Evaluation of Apoptosis: Adherent cells were cryopreserved using our protocol and apoptosis was evaluated using Nexin staining. Nexin stain binds to phosphatidylserine that translocates to the outside of the membrane during apoptosis. After thawing, cells were allowed to recover for 1 hour at 37°C. Cells were detached from the bottom of the well and then incubated with nexin stain for 20 minutes at room temperature. Then the samples were run through the Guava cell analysis system which counts the cells and measures the staining in a similar manner as a flow cytometer [16].

Statistical Methods: All experiments were performed several times. The statistical analysis used included t-test, ANOVA and logistic regression methods as appropriate for the type of data being analyzed.

3. Results

3.1. Initial protocol setup

Simple cooling of a commercial microtiter plate by conventional means results in variable thermal profiles. The objective is to have uniform conditions across the plate such that the thermal history of the cell populations in each well of the plate is not significantly different from each other. Conventional wisdom in cryobiology has established that cell survival is markedly influenced by thermal history and the temperature profiles experienced during cooling and warming.

Cryopreservation experiments using adherent A10 cells were initially performed in a fashion similar to that typically done with ampoules of suspended cells and two assumptions were made. First, adherent cells can be cooled successfully at a similar rate as the same cells in suspension,-1.0°C/minute. Second, that thawing should proceed as rapidly as possible at 37°C, the same as for cells in suspension. A10 cells were exposed to 2M DMSO then cooled at -1.0°C/minute to -80°C before being stored overnight at <-135°C. Thawing was performed by placing the plate in a 37°C water bath with the water touching the bottom of the plate. The plate was then placed on ice, DMSO was removed and saved along with subsequent washes to check for

potential detachment of cells and whether detached cells were viable. Both the supernatants and any cells in the wells of the plate after rewarming were checked for viability using the metabolic indicator alamarBlue™.

Measurement of viability after thawing demonstrated essentially no cell survival. When these same cells were cryopreserved as cells in suspension using the same concentration of DMSO their viability was >70%. Several observations were made during this experiment. It was noted that when the plate cooled the plastic constricted and contorted, changing the shape of the plate, which then reversed itself upon rewarming. Most of the cells that had been attached prior to cryopreservation were no longer attached after rewarming and were no longer viable when assessed for metabolic activity.

In an effort to retain cells on the plate and maintain their viability, a two step warming protocol was investigated. This assumed that the differential thermal properties of tissue culture plastic and the adherent cells are influencing the extent of cell recovery. Particular attention was paid to the rates of heat transfer during freezing and thawing. Since, in general, slow cooling is mandatory for optimum survival of cryopreserved mammalian cells, the subsequent experiments focused on the effect of warming conditions following cooling at a standard rate of -1°C/min.

A two-step warming protocol was shown to improve the cell recovery in this adherent cell model [17]. Frozen plates were removed from <-135°C storage and placed at 23°C in air until the cultures reached 0 to -70°C. Sample temperature was recorded in representative wells in the 96 well plates. Plates were then placed into a 37°C water bath to complete the thawing process as rapidly as possible with due care not to allow the wells to warm beyond 0°C. The plates were then immediately placed on ice, washed, and left in culture medium for assessment of metabolic activity. Results from an experiment in which the temperature for transition from step 1 to step 2 was varied are shown in Figure 2. If the plate was rewarmed to -70°C and thawed rapidly at 37°C, there is little difference in viability as compared to a single rapid thawing step. However, allowing the plate to rewarm to progressively higher temperatures resulted in improved viability with the best viability measured when the plate was allowed to warm to -20°C before rapid thawing at 37°C. It was also noted that the more gradual rewarming to -20°C allowed any distortion that occurred due to the plate being cooled to such low temperatures (<-135°C) to subside so that when rapid thawing commenced, no sudden structural changes in the plate occurred.

Using this new two step warming regimen, a preliminary experiment was done to evaluate the effect of DMSO concentration using the same cell model, A10 cells. DMSO (0-5M) was added to wells containing $2.5x10^4$ cells and the plates were cooled and stored as previously described. A representative experiment in which the cells were warmed to –20°C in step 1 of the warming protocol is shown in Figure 3. 1-2M DMSO provided the best protection for adherent A10 cells against freezing-induced loss of viability. These experiments yielded approximately 25-35% of the original cell viability prior to cryopreservation.

While viability improved with 1-2M DMSO, a greater increase was desired. However, this series of experiments did demonstrate that adherent cells could be cryopreserved on plates.

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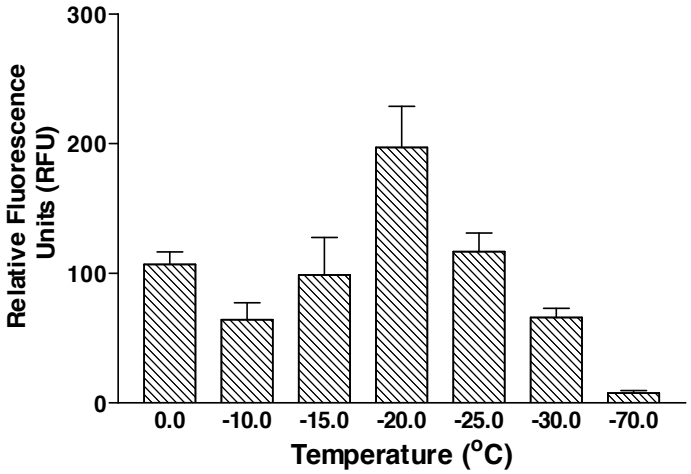

Figure 2. Cell viability after varying the transition temperature from slow to rapid warming of cryopreserved cell cultures. The viability of A10 cells was assessed after cryopreservation in 1M DMSO. Plates were removed from –130°C and placed at ambient temperature (23°C) for slow warming to temperatures ranging from 0 to –70°C, whereupon the plates were transferred to a 37°C water bath for rapid warming to ~ 0-4°C. Then metabolic activity was measured using alamarBlue™.

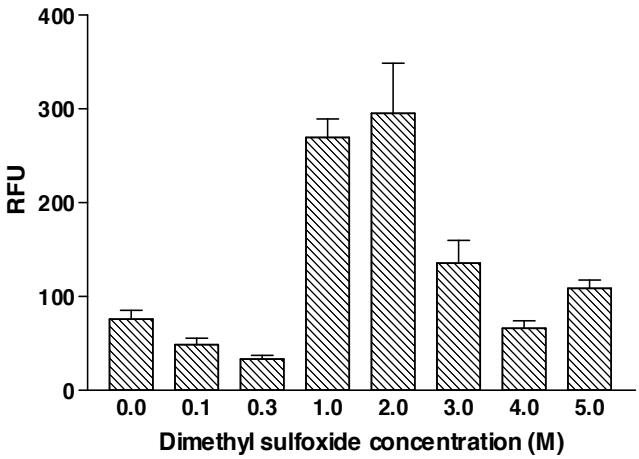

Figure 3. Cell viability of adherent vascular smooth muscle cells cryopreserved in the presence of varying concentrations of DMSO. A10 cells were plated, exposed to varying concentrations of DMSO and cooled at-1°C/min to –80°C then stored at –130°C. The plates were warmed in two steps as described in the text and metabolic activity was measured.

Further work was required to optimize the protocol to improve cell viability after cryopreservation and also, to evaluate and optimize the viability using an entire plate. With the advancements in high throughput screening and the push to reduce the number of animals used in research, the ability to cryopreserve cells across an entire multiwelll plate would further the development of cell-based assays providing suitable alternatives to animal testing.

3.2. Warming rates

Having developed a warming protocol that retains cells on the plate and maintains their viability, attention was then paid to optimizing the warming protocol for a complete multiwell plate. Initial observations of cell viability across a plate (Figure 4) after rewarming demonstrated regional variations in cell viability with the highest survival being recorded from wells at some edges of the plate.

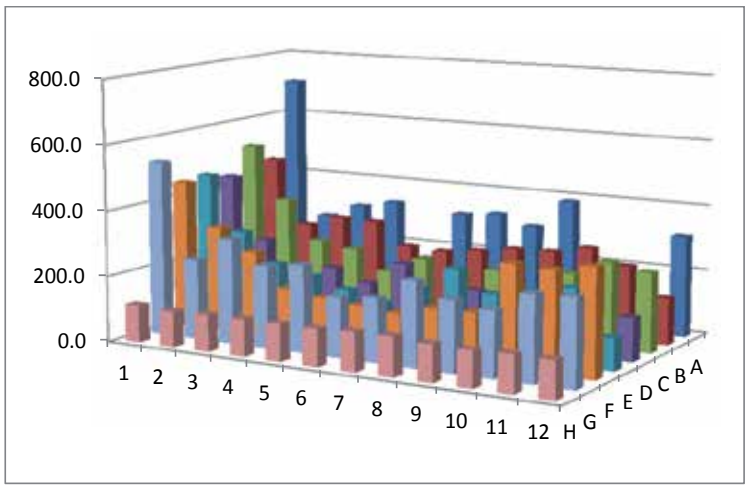

Figure 4. Variability of cell viability in a 96-well plate. Cells were cryopreserved in 1M DMSO. The plate was warmed at 23°C in air to –20°C and then warmed rapidly in a 37°C water bath. The plate was then placed on ice and the cells were washed to remove the DMSO. Cells were left in culture media and alamarBlue™ for assessment of metabolic activity. The results are typical of several experiments. Data from wells with cells minus background in rows A-G. The control wells (background) without cells were in row H.

This effect might be explained by the orientation of the plate during cooling in the control rate freezer leading to differential thermal conditions at various locations in the plate. However, experiments done that evaluated the orientation of the plate within the freezer demonstrated that edge effects were due to the plate and not where the plate was located in the control rate freezer (data not shown). Since warming conditions had already been indicated to be a determinant of cell viability, measurements of the thermal history of different wells in a typical frozen plate during rewarming were measured.

Thermocouples were placed into wells at each corner of the plate and in one well at the center of the plate. Temperature profiles were measured every 15-60 seconds using a datalogger (Omega). The plate was stored at >-130°C overnight. The next day, the plate was rewarmed at ambient temperature (~25°C). The well at the center of the 96-well plate had a different warming profile compared with wells at the outer corners (Figure 5). Uniform conditions of freezing and thawing are necessary in order that optimum conditions for cell survival are applied consistently to each well in the plate.

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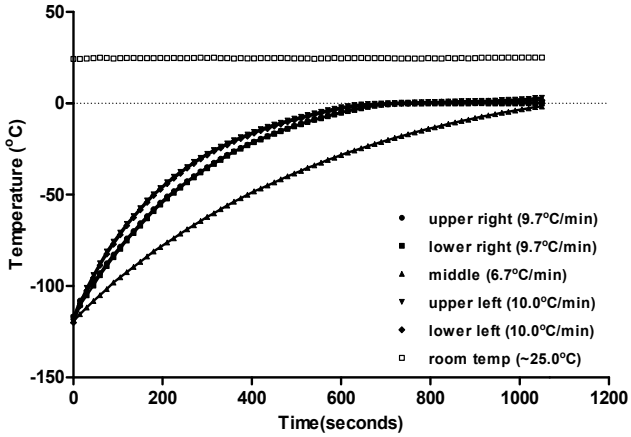

Figure 5. Warming rates at various locations in a 96 well plate. Each well was filled with 150 µl of 1.5% agarose and allowed to solidify. Warming rates were determined using thermocouples placed in the corners and a middle well of a 96-well plate. Warming rates were determined by recording temperatures every 15 seconds using a datalogger (Omega) that records the temperature from all the thermocouples simultaneously.

Warming the plates uniformly proved to be a challenge. An adjustment was made to the initial two-step warming protocol to simplify the procedure. Originally, the two-step protocol called for removing the plate from the cryofreezer and allowing it to warm in air at ambient temperature to –20°C in the first step. This procedure was cumbersome from the point of view of having to carefully monitor the warming process to –20°C in air to ensure there was no thermal overshoot. This problem was solved by placing the CryoPlate™ in a –20°C freezer for the intermediate warming step. An equilibration time of 30 minutes was used and was based on temperature measurements made that defined this time period as the minimum period required to equilibrate the plate to -20°C. The next step was to thaw the plate quickly at 37°C. With this modification, the intermediate temperature of -20°C was reached without the danger of thermal overshoot and it also simplified the warming protocol. It also had the added benefit of establishing a consistent temperature across the plate before the rapid warming step, so that any variations during rapid warming across the plate were kept to a minimum. Steps were also taken to improve thermal conductivity and provide more heat transfer between the CryoPlate™ and the environment by employing custom-made heat sinks and thermal conductive paste. Aluminum heat sinks designed to fit flush with the bottom of the microtiter plate were used for the equilibration at –20°C and for the rapid thawing at 37°C. The wells of the plate were filled with 50 µl of 1M DMSO in PBS and cooled to ~-130°C. The plates were then allowed to equilibrate in a –20°C freezer for up to 30 minutes, and then the plate was placed at 37°C for rapid thawing to the termination temperature of 0°C. Temperature measurements were made and warming profiles were determined as previously described. As mentioned previously, distortion of the plate occurs during cooling. This distortion compromised the tight fit with the platform of the heat sink. To counteract this shape change, we used a thermal conducting compound (Wakefield Engineering) to interface between the plate and the heat sink. The marked effect of this modification is illustrated in Figure 6, where it is seen

that significantly improved uniformity was achieved using the heat sinks in conjunction with the thermal compound. The large disparity in warming rate previously observed between the edges and the middle of the plate was alleviated using the heat sinks with the thermal conduction compound.

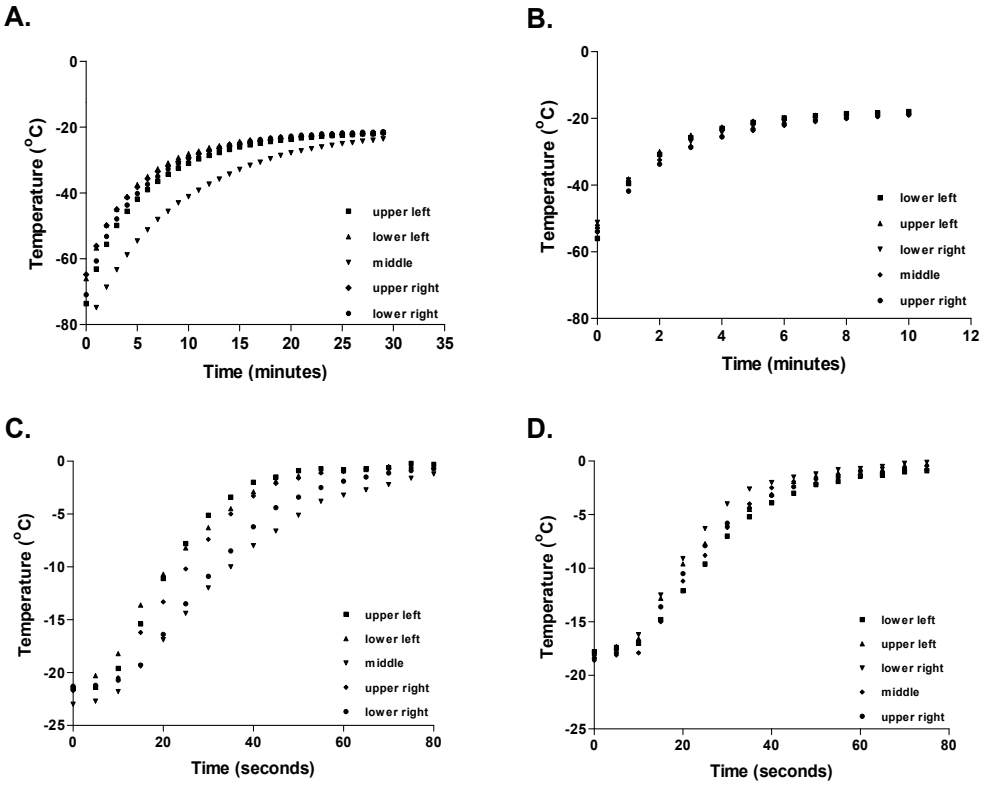

Figure 6. Warming temperature profiles. The wells of a 96-well plate were set up and cooled as described in the text. The plate was then removed from cryostorage and placed in a –20°C freezer until equilibrated before being transferred to 37°C for the second step of the warming protocol. Temperatures were measured every minute while at –20°C and every 5 seconds while at 37°C. Graphs (A) and (C) are without heat sinks while graphs (B) and (D) are done with heat sinks and thermal conducting compound

3.3. Cooling conditions

Having established a protocol that uniformly warms wells across a multiwell plate, attention was focused on defining a uniform cooling profile. Cooling rate is one of several cryobiological variables that can impact cell survival and is usually cell type dependent. Slower cooling rates while discouraging undercooling of the samples and the formation of ice inside the cell do allow for longer exposure times to cryoprotectants and time for osmotic changes to occur that promote cell shrinkage which conceivably have a negative impact on the stability of the cell-substrate interactions. Faster cooling rates reduce exposure to the cryoprotectant and osmotic

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changes to the cell but do allow undercooling to occur and can also allow for the formation of ice inside the cell. A balance between these two extremes has to be optimized in order for maximum cell survival to occur. A system using 96-well plates, presents unique challenges in terms of uniform cooling across the plate as well as providing an optimum cooling rate for a particular cell type. Several cooling rates were examined and included, -0.2°C/min, -0.5°C/min, -1.0°C/min, -3.0°C/min, -5.0°C/min, and -10.0°C/min. Cooling rate measurements for each cooling profile were taken for 3 separate runs. Two different sample volumes were evaluated, 100 µl/well and 50 µl/well. For these experiments, the ambient environment of the microtiter plates was uniformly cooled using a programmable controlled-rate freezer (Planar Kryo 10). Not surprisingly, slower rates, such as -0.2°C/min, provided more uniform cooling as opposed to faster rates like -10.0°C/min (Figure 7). However, increasing the volume of cryoprotectant medium within the wells of the plate did affect the ability of the controlled-rate freezer to cool the samples uniformly.

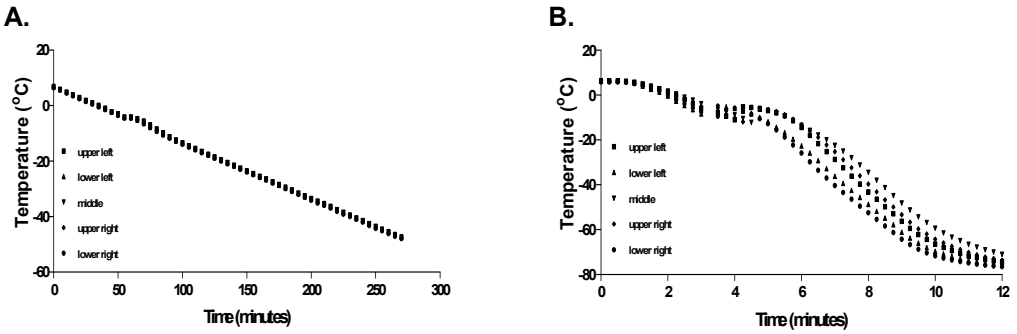

Figure 7. Cooling temperature profiles. All wells of a 96-well microtiter plate (Falcon) were filled with 50 µl of 1M dimethyl sulfoxide (DMSO) in phosphate-buffered saline (PBS). Thermocouples were inserted into the corner wells and a middle well. The temperature was measured every 5 minutes using a datalogger (Omega) for a rate of –0.2°C/min (A) and every minute for a rate of –10.0°C/min (B).

Cooling profiles at the different locations in the plate were more uniformly controlled when the smaller sample volume was employed (Figure 8). Variations between the well locations were greatest in the region of nucleation when the latent heat of crystallization is evolved (referred to as the latent heat bump in the cooling curve and occurring at approx –5°C in this protocol). This is important because varying degrees of undercooling and the consequential variable cooling rates in the immediate post-nucleation phase are known to influence cell survival [18]. Nucleation is a statistical event that is known to depend upon a number of factors, of which sample volume is critical. For example, cryopreservation of cell suspensions is conventionally carried out in ampoules containing sample volumes of 1 mL or greater. In such cases it is usually observed that spontaneous nucleation occurs at widely different temperatures between ampoules. In other words, there is a variable degree of undercooling between

samples before heterogeneous nucleation is initiated. This undesirable phenomenon has led to the common practice of induced nucleation (so called "seeding") to ensure uniform conditions between replicate samples in a cooling run.

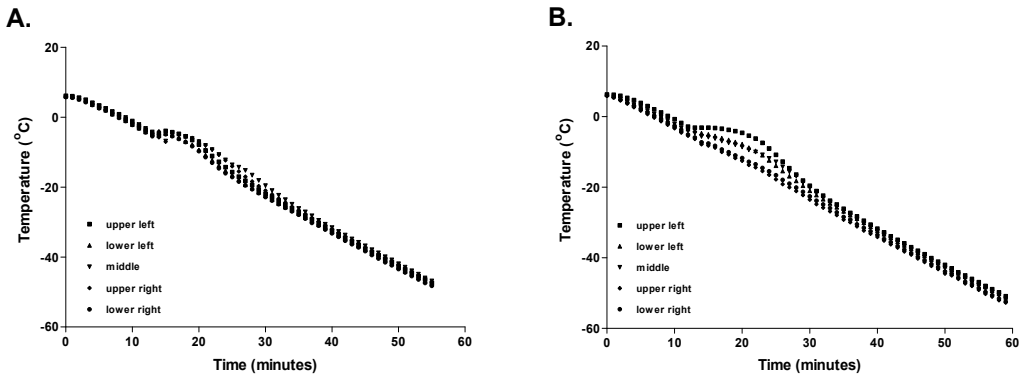

Figure 8. Cooling temperature profiles with different volumes. Plates were set up as described for Figure 1. The plate was cooled at –1.0°C/min to –80°C in a controlled-rate freezer (Planar) and the temperature was measured every minute using a datalogger (Omega). (A) 50 µl/well, (B) 100µl/well.

On the assumption that the five strategic measurement locations in this study are representative of all the wells in a plate, it appears that the spontaneous nucleation of 50µl samples (Figure 8A) is reasonably consistent and more so than the 100µl samples (Figure 8B). Unlike dealing with the freezing of a few ampoules, manual seeding of multiple samples, or simultaneous seeding of all the wells in a microtiter plate, is not practically feasible. Manufacturers of programmed cooling machines have attempted to deal with this by offering the facility for forced nucleation by a momentary sharp drop in ambient temperature initiated by a blast of refrigerant into the cooling chamber. A cooling profile developed in our lab for routine use in screening cryoprotectant solutions on plates that is a modification of a standard –1.0°C/min cooling rate includes a sudden temperature drop to initiate nucleation. It is included here for comparison purposes with regards to conventional cooling profiles, such as –1.0°C/min, and for its potential benefits in terms of cell viability and cell attachment.

From the results presented (Figure 9), it is clear that lower cooling rates provide better cell viability and cell retention ($p<0.001$). For the cells used in these experiments, bovine corneal endothelial cells (BCE), cooling rates above –1.0°C/min demonstrated reduced viability and cell retention. Equivalent cell viability and attachment was observed with cooling rates of –1.0°C/min or less. The modified cooling profile (MP) also demonstrated viability equivalent to a standard –1.0°C/min cooling rate, but better cell attachment after thawing.

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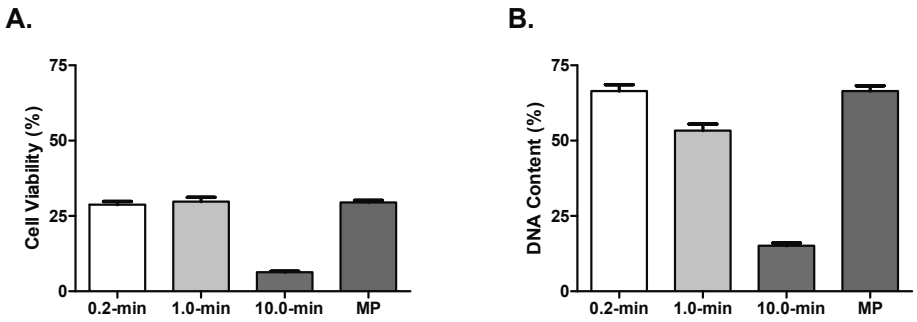

Figure 9. Cell viability and attachment after cryopreservation with varying cooling rates. BCE cells were plated and cryopreserved in 1M DMSO. After thawing metabolic activity (A) and cell attachment (B) via DNA content were measured.

3.4. Cryopreservation in bulk solution vs. air

Another option that was tried was to compare cryopreserving BCE cells in solution versus exposing the cells to CPA and then removing the supernatant to cryopreserve the cells in air. This set of experiments was included on the basis of previous work that showed an improvement in viability of whole corneas when they were cryopreserved in air [19-20]. BCE cells were plated at 20,000 cells/well and cryopreserved in 2M DMSO or 2M 1,2-propanediol (PD). Upon thawing, using the two-step warming protocol, cell viability and cell retention was assessed (Figure 10A). Both DMSO and PD mean cell viability was significantly higher for cells cryopreserved in bulk solution compared with those frozen in air ($p<0.001$). Cell retention determined as DNA content, showed a somewhat similar trend between these two modes of cryopreservation. In the presence of DMSO there was a significant difference between air and bulk medium, with an improvement in cell retention observed when cells were cryopreserved in bulk solution ($p<0.001$). For PD, freezing without bulk medium (in air) resulted in the highest

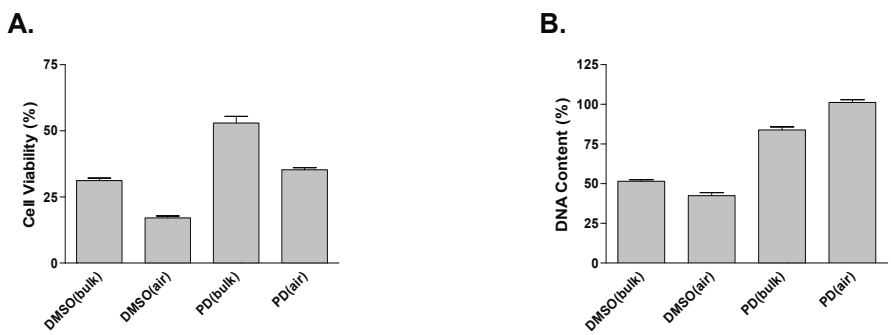

Figure 10. Cryopreservation with and without bulk solution. BCE cells were cryopreserved in 2M DMSO or 2M PD in bulk solution or in air. The graphs for DMSO represent the mean (±SEM) of 252 replicates. Graphs for PD represent the mean (±SEM) of 92 replicates. Statistical analysis done by 2-way ANOVA and presented in the summary.

retention of cells (100%) over freezing in bulk solution ($p<0.001$). It thus appears from these studies that there is a complex relationship between the nature of the CPA and the role of the surrounding medium (liquid or air) in determining both cell retention and cell viability. Moreover, it appears that in this system using BCE cells attached to plastic, a different response is achieved compared with corneal endothelial cells cryopreserved in situ in their native state in whole corneas [20].

3.5. Exogenous nucleating agents

Still another approach was to include an inert nucleating agent within each sample to promote nucleation uniformly at, or just below, the equilibrium freezing point. Such an agent is the commercial product "Snowmax" used effectively in the snow-making industry at ski resorts. We used this product as a tool to compare spontaneous nucleation with enforced nucleation in our system. However, addition of the nucleating agent did not improve the uniformity demonstrated in Figure 7, which was generated in the absence of Snowmax. It is concluded therefore, that slower cooling rates such as -1.0°C/min used with a microtiter plate containing 50μl samples per well yields sufficiently uniform cooling without the need for artificial nucleating techniques.

3.6. Effects on cell survival

Having demonstrated the development of a cooling and warming protocol with satisfactory uniformity across the entire plate, the next step was to examine the effect upon cell survival. To this end, we dispensed BCE cells into each well of a 96-well plate and cryopreserved them in 2M DMSO. The plates were thawed using the modified warming protocol with heat sinks and thermal conduction compound (Figure 11). Recovery of cells and their viability at each location in the plate showed that with the exception of the left edge cell viability was uniform across the entire plate. Interestingly, the left edge of the plate demonstrated viabilities greater than the rest of the plate. Why the other edges do not also demonstrate this effect is unclear. The Cyquant assay measures the DNA content of the cells which is indicative of cell number and is a measure of cell retention on the plate after cryopreservation, was done on the same plates after Alamar Blue. Observation of this data showed that the middle of the plate demonstrated better cell retention than any of the edges.

The data from these experiments was obtained using the modified –1.0°C/min cooling profile (MP) mentioned above that includes a sudden temperature drop to facilitate nucleation. Experiments were also done using other cooling rates such as –0.2°C/min which did not produce more uniform cell viability and cell retention as would be anticipated based on the temperature profile data (see Figure 7). While the temperature profile data showed that a lack of induced nucleation did not affect uniform cooling, nucleation is a statistically random event and does have a significant impact on the survival of the cells being frozen. Based on the cell viability and cell retention data, it is likely that heterogeneous nucleation is taking place even though the temperature profiles of the five wells that were being measured did not indicate it. The data using the modified cooling profile supports this assumption (Figure 9) because it is designed to initiate nucleation across the plate in a limited way and better cell viability was

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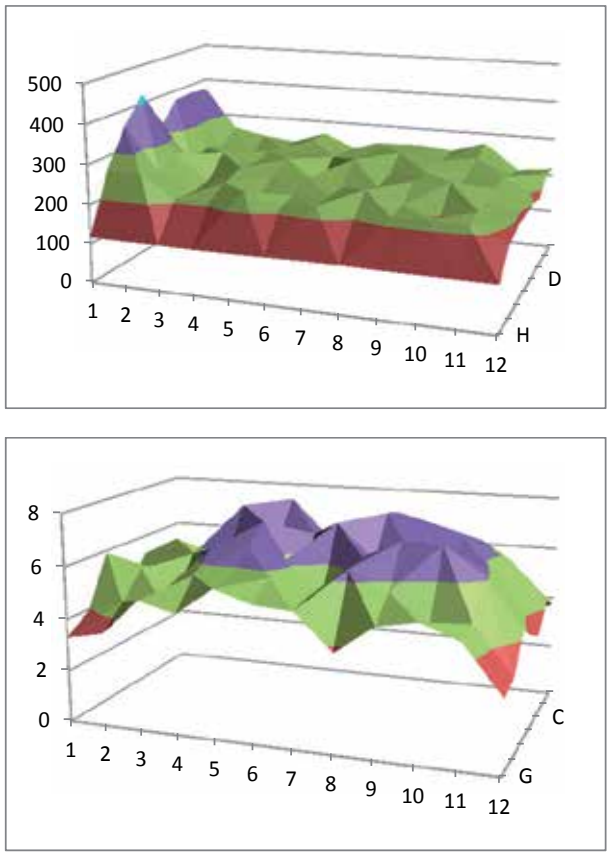

Figure 11. Plate uniformity with cells. BCE cells were plated at $2x10^4$ cells/well and cryopreserved in 2M DMSO. Thawing was done as described in the text. Cell viability (top) and cell retention (bottom) were assayed by Alamar Blue and Cyquant, respectively. The graph represents the layout of a 96-well plate and is the mean relative fluorescence units (RFU) for each well from three experiments. Rows A-G contain cells. Row H represents control wells with no cells and is not shown.

measured when it was used. Therefore, controlled nucleation across the plate is a desirable objective to promote more uniform cell survival.

A system for nucleating all the wells of a 96 well plate was designed. The idea for this system involves using an external liquid nitrogen source. The nucleation manifold consists of spring loaded metal pins, one for each well of a 96 well plate. The plate of pins sits on an aluminum block that allows liquid nitrogen to be pushed through it, thus cooling the block to temperatures well below freezing (~-100°C). Nucleation is achieved by cooling the microtiter plate to a temperature near the freezing point of the solution then pressing the plate onto the spring loaded pins that have been cooled to a much lower temperature. Once nucleation occurs the plate is removed from the pins and the cooling cycle can continue until complete. A representative profile using the manifold and showing nucleation of the corner wells and middle wells of a 96 well plate is presented (Figure 12). Note that each well nucleates within the time span

of ~30-45 seconds and that the variation in the temperature at which nucleation is initiated and the amount of latent heat released is minimal.

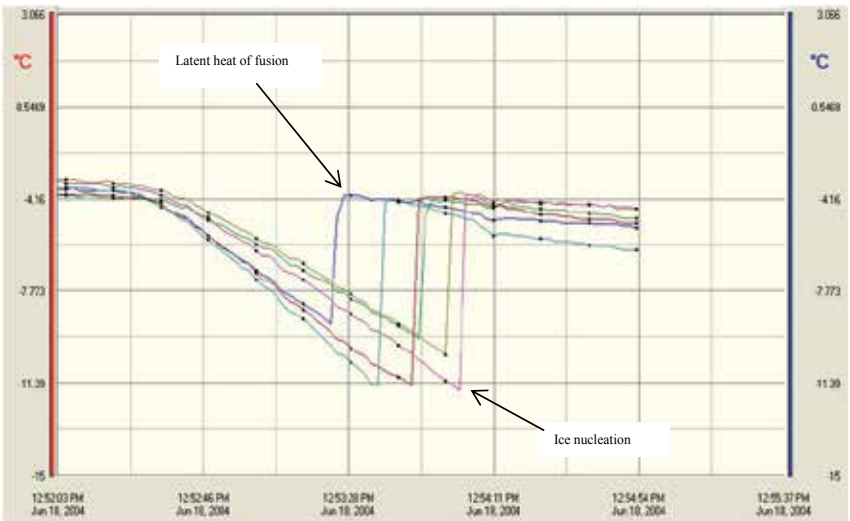

Figure 12. Temperature profile using nucleation manifold. Thermocouples were placed in the corner wells and middle wells of a 96 well plate. Temperatures were recorded every few seconds.

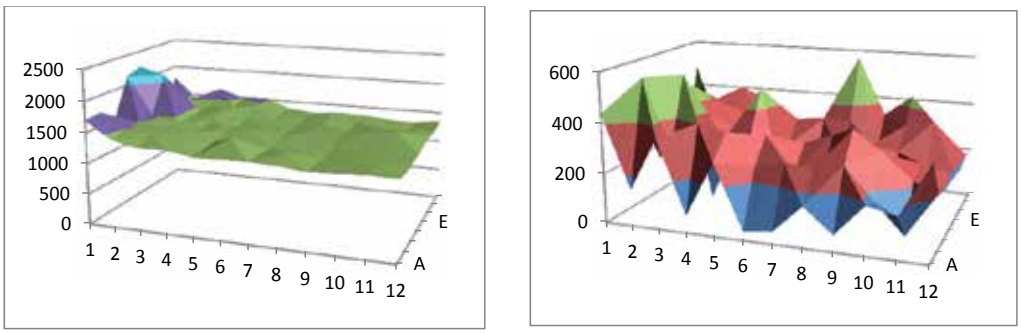

Figure 13. Cell viability and cell attachment of BCE cells after cryopreservation using the nucleation manifold. BCE cells were plated in each well of a 96 well plate and left in 2M PD-EC before being cryopreserved using the nucleation manifold. Viability was measured by alamarBlue™ and DNA content was measured by the Cyquant assay. (left) cell viability. (right) cell attachment.

Experiments were then done to determine whether or not more uniform viability and cell attachment was achieved using this nucleation device. This experiment was performed many times using both DMSO and PD as the CPA. Figure 13 is a representative experiment where viability and cell retention were measured. It was easier to achieve more uniform viability then cell retention. Having done this experiment many times we have noted user variation. Pressure

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applied to the plate sitting on the pins is applied strictly by the user using a lever. It has been noted that variations in the handling of the lever produced variations in the uniformity of nucleation across the plate. The experiments to date certainly support the mechanism of nucleation that has been developed, but it is obvious that further optimization of this apparatus is required to improve the uniformity of viability and cell retention across the plate.

4. Other parameters

In natural tissues, or engineered tissue constructs, as well as cell monolayers, that could conceivably be used for cell based assays, maintenance of cell-cell interactions as well as cell-substrate adhesion will be important for full function after cryopreservation. Therefore, the effect of cell confluency on the ability of cells to remain attached to a substrate during cryopreservation was evaluated. Some research groups have suggested that a monolayer reacts differently than subconfluent cell populations and demonstrated a loss of cells upon rewarming. It is hypothesized that because the cells have contacts with each other, when one cell becomes detached it will cause surrounding cells to detach also, even if those surrounding cells are still viable [3]. Other groups agree that monolayers behave differently from subconfluent cell populations when they are cryopreserved, but argue that optimization of cryopreservation variables such as cooling rate can produce cell viability and attachment values consistent with subconfluent cell populations [21]. Various cell densities, both subconfluent and a monolayer, were assayed for cell viability and cell attachment after cryopreservation. BCE cells were plated on tissue culture plastic at varying densities up to a monolayer of cells that consisted of 80,000 cells/well. After exposure to 2M DMSO, the plates were cryopreserved at one of two cooling rates, either -1.0°C/min or with the modified -1.0°C/min cooling profile (MP). The results presented in Figure 14 demonstrated similar cell viabilities regardless of the cell density assayed. More confluent cell populations showed better cell retention compared with more sparsely populated wells (i.e: 2,500 cells/well vs 20,000 cells/well). Slower cooling rates were also evaluated (data not shown) with similar results. It can be concluded, that in this system, the degree of confluency does not greatly impact cell viability and cell retention after cryopreservation. However, different cell types may behave differently when cryopreserved as a monolayer. In addition, the previous studies cited used glass cover slips and microtiter plate filter inserts to study the cryopreservation of a monolayer while viability and cell retention were evaluated on tissue culture plastic in the present study.

In a related experiment, BCE cells were cryopreserved in two different cryopreservation solutions, 2M DMSO and 2M PD, at a subconfluent density of 20,000 cells/well and as a monolayer at 80,000 cells/well (Figure 15). No significant differences in viability or cell attachment were observed. While initial viability was only 50%, the cells were able to initiate proliferation within 24 hours post thaw and continued to proliferate at 4 days post thaw. Cell attachment was very good with values that were 75% or greater based on an untreated control demonstrating that the monolayer was just as robust as the subconfluent population after cryopreservation.

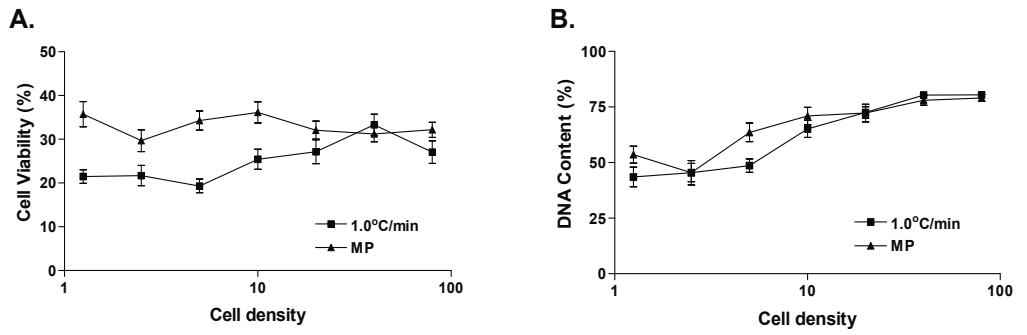

Figure 14. Cell viability and attachment after cryopreservation for varying cell densities. BCE cells were plated from 0-80,000 cells/well and cryopreserved as indicated in the text. Cell viability (A) and cell retention (B) were assessed and the data represent the mean (±SEM) of 12 replicates. Cell density is expressed using a log scale.

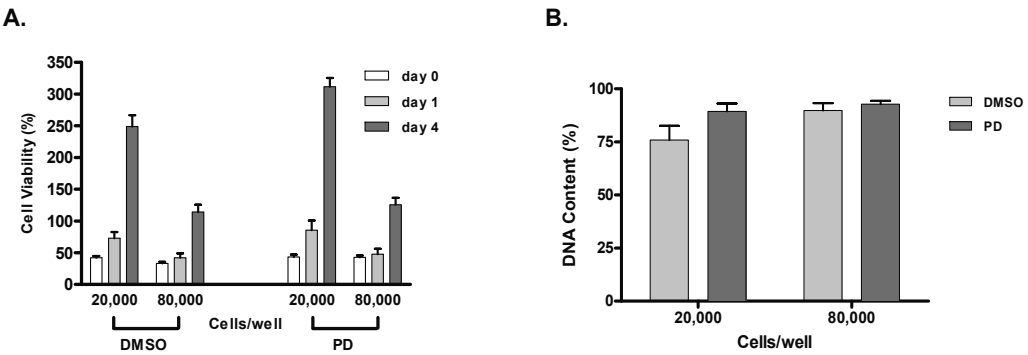

Figure 15. Percent cell viability and DNA content of a subconfluent cell population and a cell monolayer after cryopreservation. BCE cells were cryopreserved in 2M DMSO in HBSI or 2M PD in EC at the indicated cell densities. Viability was measured by alamarBlue™ and DNA content was measured by the Cyquant assay (Molecular Probes). Percent viability and DNA content is the mean (±SEM) of 10 replicates.

4.1. Optimized system

Having optimized the cooling and warming protocols for cryopreserving cells in a multiwell plate, experiments were then done to evaluate the system and how well it could be used to measure cell viability in cryopreservation experiments. Two commonly used cryoprotectants, DMSO and PD were assessed in Euro-Collins (EC), a well established vehicle solution used in cryopreservation, at concentrations of 0-4M. BCE cells were plated at a density of 20,000 cells/well and exposed to DMSO and PD before being cryopreserved using the modified –1.0°C/min protocol. The graphs depict cell retention for both CPA and their viability after cryopreservation (Figure16). For both CPAs, concentrations of 1-2M demonstrated the best cell viability and attachment. Higher CPA concentrations showed viability and attachment that was significantly worse ($p<0.01$). At a concentration of 2M PD, cell viability of ~77% was observed versus only ~50% for the same concentration of DMSO. For both cell viability and

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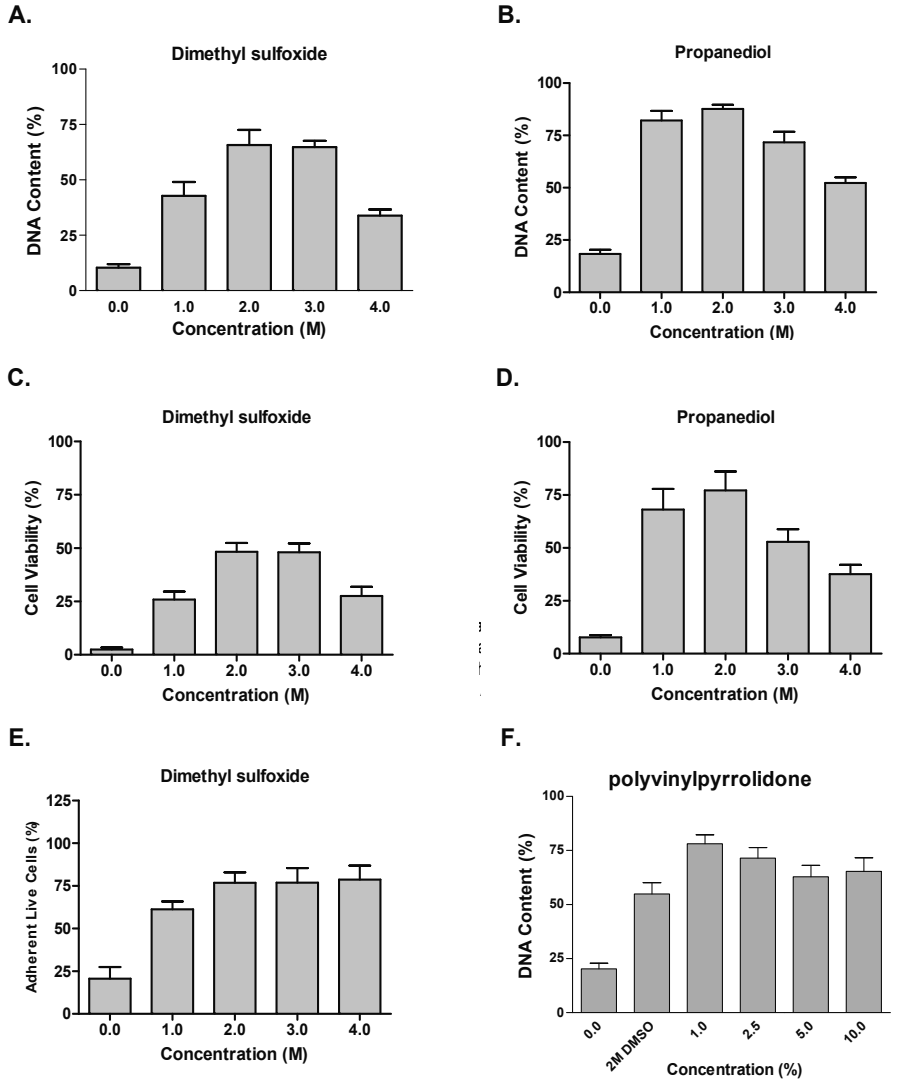

Figure 16. Cell viability and retention after cryopreservation. BCE cells were plated and cryopreserved as described in the text using the modified –1.0°C/min cooling profile. Graphs A & B show DNA content which is indicative of cell retention. Graphs C & D show cell viability. Graphs E & F show viable adherent cells. All graphs represent the mean (±SEM) of 12 replicates. Statistical analysis done by 2-way ANOVA and presented in the summary.

cell retention after cryopreservation, PD demonstrated better values over DMSO at all concentrations evaluated ($p<0.001$). DNA content values of >75% were achieved using 2M PD in EC over the same concentration of DMSO which achieved values of only ~60-65%. Taking this a step further, an evaluation of the viability of those cells still attached to the plate was done, expressed as adherent live cells (Fig. 16 E and F). It was observed that adherent cells were >60% live (viable) for all concentrations of DMSO tested and >70% for all concentrations of PD. So, cells that remained attached after cryopreservation had a high level of viability at

optimal CPA concentrations. Further study of other cryoprotectants could yield even better viability and cell attachment values. In our experience to date, the CryoPlate™ protocol works for most cells we have evaluated but the optimal CPA requirement varies.

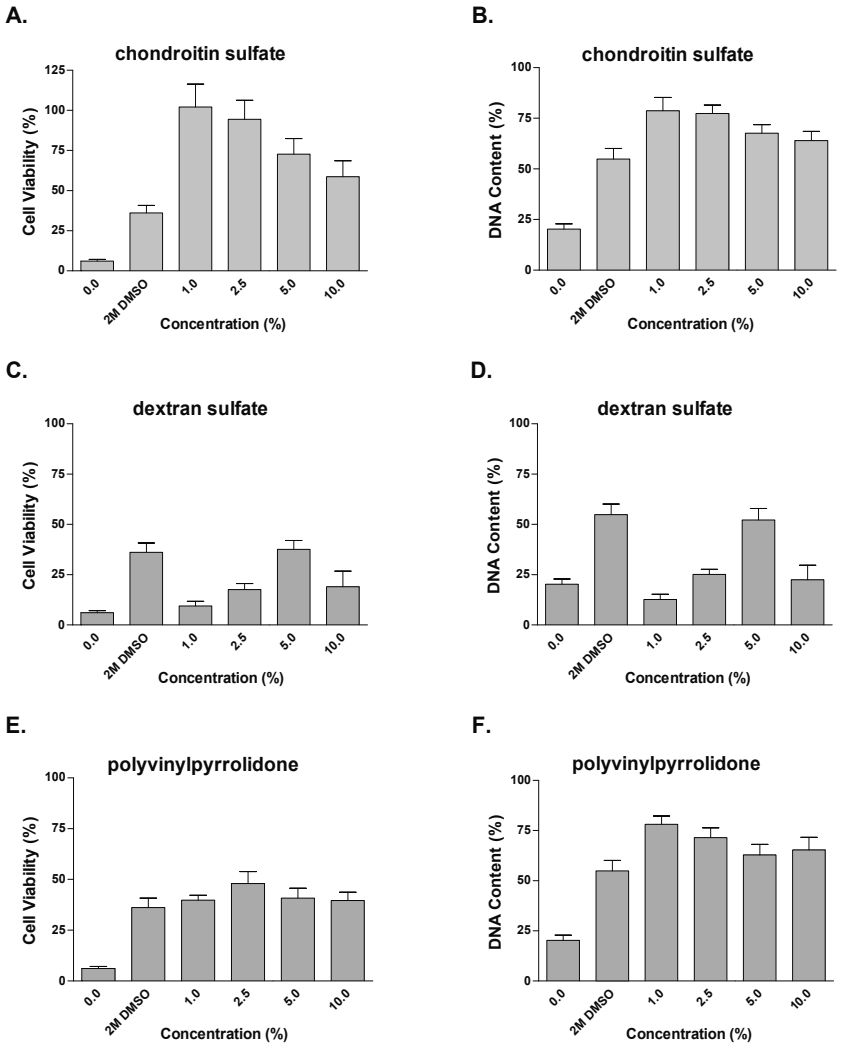

Figure 17. Cell viability and retention after cryopreservation using macromolecular CPAs. BCE cells were plated at 20,000 cells/wells, exposed to the above compounds at the indicated concentrations with 2M DMSO in EC and cooled using the modified –1.0°C/min cooling program. Graphs A, C & E show cell viability and graphs B, D & F show DNA content. All graphs represent the mean (±SEM) of 12 replicates. Statistical analysis done by 2-way ANOVA.

In a second series of experiments, non-permeating macromolecular cryoprotectants were also examined in combination with the cell permeating cryoprotectants DMSO and PD. Chondroitin sulfate (CS), dextran sulfate (DS) and polyvinylpyrrolidone (PVP) were assayed at various

concentrations (0-10%) in the presence of 2M DMSO for their ability to enhance viability and cell retention. Concentrations higher than 10% for each of the CPAs suggested proved to be difficult to get into solution above 10%, hence this defined the upper limit of the concentrations tested. BCE cells were assayed for cell viability and DNA content as described above and the results are presented in Figure 17. Using 2M DMSO as a baseline, which demonstrated a cell viability of ~40% with a DNA content of ~55%, enhancement of viability ($p<0.001$) and cell retention ($p<0.05$) was observed with CS. A concentration of 1% CS in 2M DMSO demonstrated cell retention at ~100% with viability at ~75%. Dextran sulfate did not improve viability or cell retention over 2M DMSO alone. At the best concentration of dextran sulfate (5%), viability and cell retention were equivalent to using just 2M DMSO. Cell retention was improved over 2M DMSO alone with all concentrations of PVP examined ($p<0.05$) but only a slight improvement in cell viability at a concentration of 2.5% PVP. In summary the only macromolecular cryoprotectant that demonstrated benefits for cells with regards to cryopreservation was chondroitin sulfate. In fact this combination provided results that were not significantly different compared with untreated controls. Chondroitin sulfate is readily found in association with the extracellular matrix of cells and this may explain why greater viability and cell retention were observed compared with the other macromolecular CPAs that were evaluated.

4.2. Carrier solution

Different carrier solutions were evaluated to determine if they had an impact on cell viability during cryopreservation. Others have reported the significance of the cryoprotectant carrier solution to overall viability after preservation [22-24]. In this experiment, four carrier solutions were used, Dulbecco's Modified Eagles Medium (DMEM), Euro-Collins (EC), a well established organ preservation solution, Hepes-buffered saline (HBSI), and Unisol (UHK), a preservation formulation developed in our labs. BCE cells were exposed to 2M DMSO in the carrier solutions listed and then cryopreserved using the optimized cooling and warming conditions described above, Cell viability was assessed by measuring metabolic activity immediately after thawing and for several days post thaw. The best viability was observed when cells were cryopreserved in either HBSI or UHK. Viability was as high as 70% and there was evidence of recovery and proliferation by day 3 post thaw with an increase in viability close to 100% of untreated controls. The least viable cells were observed while cryopreserved in EC and their recovery post thaw was also minimal. Using this system, it was observed that the choice of carrier solution can have a significant impact on cell survival. It is likely that different vehicle solutions may combine effectively with other CPAs.

4.3. Apoptosis

Using our system for cryopreserving cells on plates, apoptosis was evaluated. The nexin staining assay was performed and measured using the Guava Cell Analysis System on cells that had been cryopreserved as a monolayer and as a subconfluent cell population in 2M DMSO or 2M 1,2-propanediol (Table 1). Apoptosis was evaluated up to 2 days post thaw to determine if apoptosis was occurring. Immediately after thawing, there was little if any evidence for apoptosis. At day 1 post thaw, some apoptosis was occurring so that the percent

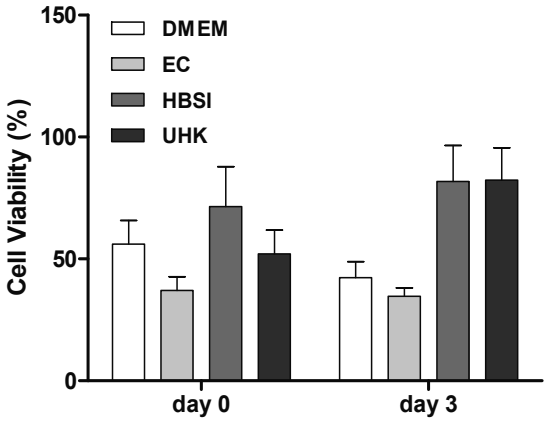

Figure 18. Cell viability of BCE cells after cryopreservation in different carrier solutions. Cells were cryopreserved in 2M DMSO in the indicated carrier solutions. Percent cell viability is the mean (±SEM) of 12 replicates

of viable cells had dropped to between 80-90%. However by day 2 apoptosis was detectable at very low levels if at all.

Cell density	CPA	Day 0		Day 1		Day 2	
		Viable†	Apoptotic*	Viable†	Apoptotic*	Viable†	Apoptotic*
20,000 cells/	DMSO	97.9	2.1	80.8	19.2	99.3	0.7
well	PD	94.2	5.8	82.7	17.3	95.5	4.5
80,000 cells/	DMSO	98.3	1.7	91.9	8.1	99.8	0.2
well	PD	97.7	2.3	88.5	11.5	98.9	1.1

† Viable cells are calculated as a percentage of the total cells counted: average number of cells counted is 1000

*Apoptotic values include early and late apoptosis events and any resulting dead cells

Table 1. Apoptosis in BCE cells after cryopreservation

4.4. Well configurations

In addition to the 96 well plate configuration, four other different well configurations were evaluated for their ability to produce viable cells after cryopreservation using the cooling and warming protocols established. As a first step, temperature profiles were measured for each configuration. Six thermocouples were placed into wells at each corner of the plate and in two wells at the middle of the plate. For the 6-well plate, a thermocouple was placed in each well. Temperature profiles were measured every minute using a datalogger (Omega) during a cooling profile of -1.0°C/minute in a controlled rate freezer (Planar). While it was anticipated that for most of the cooling regime, the plate would follow the same cooling pattern as the freezer, part of this process was to determine when the latent heat of fusion occurs, when water changes to ice, and how significant the release of heat was. Using this information, a modified protocol can be developed that would help alleviate the latent heat of fusion or at least reduce

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it to a level that causes minimal disruption to the cooling profile of the sample. In addition to the 6-well and 384-well plates (Figure 18), 12-well and 24-well configurations were also evaluated. Each well configuration demonstrated varying degrees of latent heat. The 384-well demonstrated the most variable release of latent heat while the 6-well plate release of heat did not appear to be as abrupt and was not as distinct as for the other well configurations.

This did not mean that the cooling profile for the 6-well plate was actually better, in fact, when viability was measure for each of the well configurations (Table 2), the 6-well plate demonstrated the lowest cell viability and cell attachment. Cell attachment for all the well configurations except the 6-well plate demonstrated attachment values of at least 90% or greater as compared with an untreated control. Viability was much more variable and was indicative of the degree of latent heat observed during the recording of the temperature profiles for each well configuration. Surprisingly, the 384-well plate demonstrated cell viability that was as good as that observed with the 96-well plate. Every well of the 384-well plate was not evaluated during these experiments and so while these results demonstrated that high viability values are achievable, there is still may be an issue with uniform viability and attachment in every well of a plate regardless of the well number and configuration.

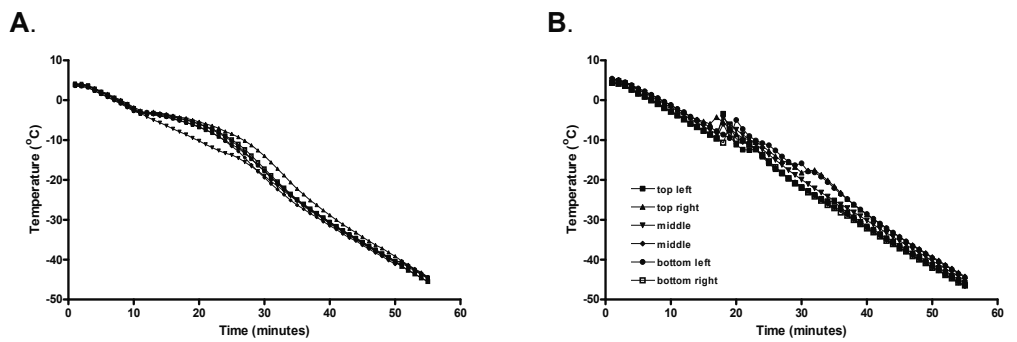

Figure 19. Temperature profiles for a 6-well and a 384-well microtiter plate. Thermocouples were placed into the corner wells and middle wells of each plate. Temperatures were measured every minute with a datalogger during a cooling profile described in the text. The graphs represent one of several experiments performed for each well configuration. (A) 6-well. (B) 384-well.

	Cell Viability %		Cell Attachment %	
Well configuration	**Day 0**	**Day 3/5**	**Well configuration**	**Day 0**
6-well	25.845±1.04	127.81±6.66	6-well	43.12±8.79
12-well	24.53±1.20	125.25±3.72	12-well	90.32±3.93
24-well	43.73±1.50	185.35±6.60	24-well	94.66±3.05
96-well	71.52±16.28	81.76±14.84*	96-well	89.76±3.57
384-well	72.62±5.50	169.02±7.46	384-well	125.61±2.08

*Only the 96-well configuration viability is from day 3. All others are from day 5.

Table 2. Cell viability and attachment with differing well configurations

5. Discussion

In this chapter, development of a method for cryopreserving cells on a fixed substrate has been described (US Patent #6,596,531) [17]. Specifically, a CryoPlate™ protocol has been developed to freeze cells on microtiter plates. This protocol was developed in our labs as a method for the rapid screening of cryoprotectant solutions. However, its application for other cell-based assays is far reaching. Potentially, any adherent cell can be cryopreserved using this system for a variety of applications upon thawing.

Initial experiments using a traditional approach for cryopreserving cells in suspension that involved slow rate cooling and rapid thawing at 37°C produced no viable cells. It was noticed that as the polystyrene plastic plate cooled, the plastic constricted and the plate contorted or bowed at its edges. It was also noticed during the initial experiments that the cells came off the plate. One can speculate that the sudden change in temperature causes stresses resulting in the plastic changing shape, similar to what occurs when an ice cube is placed in a warm liquid and it develops cracks. The sudden temperature change and abrupt changes to the plastic culture plate as it warms causes the cells to be forcefully detached and this detachment may trigger the death of the cell via apoptosis or other cell death mechanisms. Instead, a two step warming protocol was tried in which the plates were warmed to an intermediate temperature before being rapidly thawed at 37°C. The idea being a more gradual warming would allow the plate to readjust to warmer temperatures without abrupt changes in its shape which would prevent the cells from coming off during warming, promoting greater cell viability and attachment after thawing. The first protocol involved removing the plates from cryostorage and leaving them in air (~25°C) until they reached ~-20°C before undergoing rapid thawing at 37°C. Plates were then placed on ice for cryoprotectant removal and subsequent assessment of viability. Using this protocol, viable cells were obtained after freezing.

Focus then shifted to applying these conditions across an entire plate. Preliminary measurements of temperature at strategic locations within the plate demonstrated differential warming profiles. Uniform warming was achieved by removing the plate from cryostorage and leaving the plate in a -20°C freezer for 30 minutes followed by rapid thawing at 37°C using an aluminum heat sink with thermal conducting compound prewarmed to 37°C [17]. As mentioned previously, the intermediate incubation at -20°C allowed the plate to warm enough to relax the plastic so that no distortion remained and also allowed all the wells to equilibrate to a similar temperature before the rapid thawing facilitating uniform warming conditions. As was observed in Figure 6, uniform warming conditions were achieved.

Uniform cooling was also sought. In particular, synchronizing ice nucleation in each well was a primary objective as this would insure that all the wells of a plate had a similar cooling profile which should in turn produce similar cell viability upon thawing. A survey of various cooling rates demonstrated that slower cooling rates were better at achieving similar profiles across wells including more synchronous nucleation than faster cooling

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rates. It was also noted that the volume of solution in the wells impacted the cooling profiles of measured wells. More similar cooling profiles were possible when smaller solution volumes were used. However, completely removing all solution once the cells had been exposed to cryoprotectants prior to freezing did not improve viability and in fact impacted cell viability negatively. Nucleation is a random event and so further steps were taken to control nucleation so that all wells of the plate were nucleating together. The use of an exogenous nucleating agent, such as Snomax, was investigated and did not improve ice nucleation uniformity. A modification to the -1.0°C/minute cooling profile was made that included a sudden drop in temperature which promoted more uniform ice nucleation across the plate. This improved the consistency of viability and cell retention across the plate, but some variations still existed predominantly along the left edge of the plate.

Efforts were then made to further improve synchronous ice nucleation across the plate by the application of a nucleation manifold. Described in the text, this device was designed to promote ice nucleation across the plate in a controlled and timed manner. Further improvement in uniform viability was observed, but uniformity of cell retention did not show any significant improvement. As the nucleation manifold was considered a prototype, further improvements are envisioned that would automate the process and remove variation cause by user inconsistencies. There is also the possibility that cell retention is actually improved as well and that the decreased uniformity of cell retention has to do with inconsistent cell plating prior to cryopreservation. Further experiments are in progress to answer these questions and promote more uniform cell retention that is consistent with cell viability values.

In addition to cooling and warming, other parameters were considered that might affect the viability of cells attached to a plate. Cell confluency was felt to play an important role in viability and retention of cells on a fixed substrate with the thought that subconfluent populations would have better retention than a monolayer of cells. Monolayers have developed cell to cell connections similar to cell-cell and cell-matrix connections that are present in tissues. It was hypothesized that when one cell detached that would cause neighboring cells that had developed connections to the detaching cell to come off the plate as well [21]. Experiments done using our two step warming protocol demonstrated no difference between confluent and subconfluent cell populations. Factors that promoted the detachment of the cells using a one step thawing regime have been alleviated using a two step protocol so that confluency does not have the same influence on outcomes as noted previously by other groups [3]. Maintenance of a monolayer and its cell-cell interactions strongly suggests that our protocol could readily be applied to tissue constructs such as skin equivalents or other types of three dimensional constructs that would be used for drug testing and other applications.

Taking this a step further, nexin staining was performed on subconfluent cells and a monolayer for several days after cryopreservation and thawing on plates to check for the induction of apoptosis. Viability values remained high, ~95% of the counted population, even after 2 days in culture following thawing and no differences were observed between subconfluent and monolayer cell populations. Apoptosis induction was minimal demonstrating that our process promotes cell viability and that the cells are able to function and are available for further assays.

Application of our cryopreservation protocol to microtiter plates of differing well configurations showed variation of cell viability and cell retention that was not necessarily based on the consistency of their cooling profiles. For example, while the 6 well plate demonstrated a relatively consistent thermal profile across its wells, cell viability and cell retention were not as good at 25% and 43% respectively. However, the best viability was observed using the higher well configurations, 96 well and 384 well with cell viability at ~70% and cell retention at >90% even though their cooling profiles were not as consistent or uniform as that observed in the 6 well plate. Other factors are likely involved that influence cell viability during cryopreservation in plates. The volume of the wells is one factor as was observed when thermal profiles were compared using 50µl and 100µl in a 96 well plate. Smaller volumes produced better thermal profiles. With regards to the 6 well plate, the larger volume in each well means it is easier to cool each well consistently particularly at slower rates. However, at faster rates a larger volume is harder to cool or rewarm while keeping the conditions consistent across each well. This is likely why viability and cell attachment values are lower in a 6 well plate versus a 96 well plate. Modifications to the two step warming protocol might be needed to accommodate the changes in volume as the wells get larger so that better viability and cell retention can be maintained.

The initial purpose behind the development of this protocol for cryopreserving cells on plates was to develop a higher throughput system for screening possible cryoprotectant formulations. An example CryoPlate™ protocol, based on the studies presented here, is illustrated in Figure 20. The plates are placed on ice and cryoprotectant(s) added either in a single step or multiple steps of increasing concentration. When the final cryoprotectant solution has been added it is incubated for 10 minutes on ice. Then it is placed in a precooled (4°C) control freezer and once the temperature has returned to 4°C it is cooled at -1°C/min. rate to -80°C with nucleation at -6°C. Then the plates are stored at ≤ –135°C in a vapor phase nitrogen or mechanical storage freezer. The plates are thawed in at least two steps. First by placing in a –20°C freezer for 30 minutes and second, by rapid warming, ~20°C/min. to 0°C, at 37°C. Rapid thawing has been performed using aluminum heat sinks and thermal conduction compound as well as a waterbath, both prewarmed to 37°C with similar results. This warming method minimizes detachment during thawing (Figure 2). The cryoprotectant solution is then removed in one or more washing steps and the plates are incubated for at least 1 hour at 37°C in cell culture medium in a tissue culture incubator to allow cell recovery to occur. We usually utilize rewarmed cell cultures after completion of a 1 hour recovery period.

A survey of several cryoprotectants, penetrating and non-penetrating, and cryoprotectant carrier solutions was done (Figure 16) that demonstrated the utility and versatility of our system allowing rapid selection of different cryoprotectants and carrier solutions as well as their concentration with regards to the the specific cell type assessed. In this particular case excellent cell viability was obtained combining 2 M DMSO and 1% (w/v) chondroitin sulfate in EC solution (Figure 16A). We have since employed the CryoPlate™ method to cryopreserve adherent endothelial cells with trehalose [15] and endothelial cells, smooth muscle cells and heart valve leaflet myofibroblasts using either DMSO or PD formulations with acceptable high

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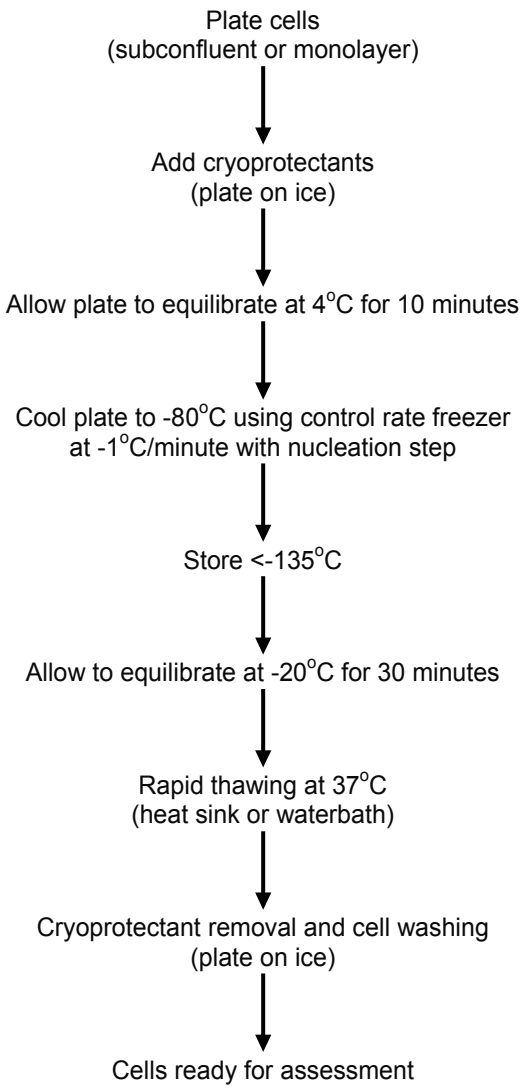

Figure 20. Example CryoPlate™ protocol for cryopreserving cells on microtiter plates.

cell viabilities in combination with other carrier solutions [25-26]. Preliminary results also indicate that our protocol is effective for preservation of human mesenchymal stem cells [28].

Based upon our experience to date, we anticipate that our CryoPlate™ protocol in combination with various CPAs and carrier solutions can be adapted for many, if not all, cell-based assay systems, and possibly tissue constructs. Cell-based assay systems and tissue constructs are currently replacing animal testing, so methods that can preserve and facilitate the accessibility of these types of systems are needed. Our protocol cryopreserving attached cells on microtiter plates fulfills this need and is versatile so that it can be applied to a wide variety of cell types for different applications.

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Chapter 2

Effects of Slow Cooling Methods and Vitrification Methods on Red Seabream (*Pagrus major*) Embryos

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Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/58830

1. Introduction

Fish gametes cryopreservation would benefit global germplasm shipping and supply, aquaculture, aquatic resources conservation and scientific research [1]. In fish sperm cryopreservation, more than 200 species have been successfully cryopreserved [2]. However, the cryopreservation of fish embryo has not been successful due to its complex multi-compartmental system, large content of water, high sensitivity to chilling, large amount of egg-yolk and low membrane permeability [3,4]. In recent years, many researches have been carried out on cryopreservation protocols [5] and mechanism of cryoinjuries in fish embryos [6].

Conventional slow cooling and vitrification are commonly used methods for long term storage of mammalian embryos. Conventional slow cooling method has been widely used in various species, but it suffers from several limitations such as chilling injury, ice formation damage, expensive equipment and tedious cooling protocols [7]. Vitrification, a solidification of a liquid without crystallization, seemed to be a promising approach. It is an extreme increase of viscosity and requires either rapid cooling rates or high concentration cryoprotectants [8], and can greatly simplify the process of cooling, avoids physical damage, and lessens the chilling injury to embryo [7]. However, the embryo cryopreserved by vitrification may still be injured by toxicity and osmotic effects of cryoprotectants [9].

Despite the fact that cryopreservation of embryos for some fish species have been attempted, successful results were not achieved [10]. Slow-cooling method had been approved to be not suitable for cryopreservation of zebrafish embryos, starfish oocytes and *Xenopus laevis* oocytes [11,12]. Therefore, some researchers suggested that vitrification would be a good option for its absence from ice crystal formation. In olive flounder, some researchers reported survival embryos were obtained after vitrification [13], however, it could not be replicated by others

[14]. In spite of this, as a promising method, attempts have been made on various fish species by vitrification.

Red seabream (*Pagrus major*) is one of the most important cultured fish species in China, Japan and South Korea. We have carried out some researches on red seabream embryos in cryopreservation protocols [15] and mechanism of cryoinjuries [6]. Potential protocols of slowing cooling and vitrification (sorts of cryoprotectant, programs of cooling and thrawing) have been screened [15]. However, little was known upon the morphological changes issue during cooling and thawing process by the two methods. The objective of the present study was to investigate the effects of two conventional slow cooling methods (S1 and S2) and two vitrification methods (V1 and V2) on red seabream embyos. The main topics are as follows:1) the effect of different cryoprotectant solutions on hatching rate of red seabream embryos; 2) the morphological changes during exposure to different cryoprotectant solutions; 3) and the changes of embryos during cooling and thawing process under cryomicroscope.

2. Materials and methods

2.1. Fish breeding and embryo collection

Sexually mature red seabream (8 female, 12 male; body weight, 3–4 kg) were maintained in a 12 m^3 concrete rearing pond (temperature: 16-18 °C) with filtered seawater changed two times a day and pumped air supply. The photoperiod was fixed at L: D=16 h: 8 h. They were fed twice a day with cooked meat of mussel. Naturally fertilized embryos were collected each morning before feeding and then incubated in filtered seawater with pumped air supply at 18 ± 1 °C in a small plastic barrel. Embryos developed to heart-beat stage (heart rate: 60–90 beats/min (fig.1); approximately 36 h after fertilization) were used for experiments. The developmental stages of the embryo were determined morphologically using a light microscope (Nikon-YS100).

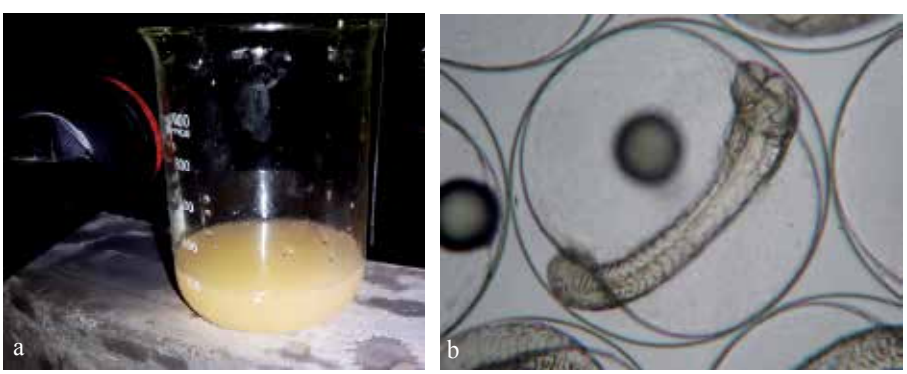

Figure 1. The collected fresh embryos of red seabream. Naturally fertilized embryos were collected each morning before feeding and then incubated in filtered seawater with pumped air supply at 18 ± 1°C in a small plastic barrel. Embryos developed to heart-beat stage were used for experiments. a. The collected fertilized embryos. b. The heart-beat stage embryo.

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2.2. Chemicals and solutions

Dimethyl sulfoxide (DMSO) was purchased from Sigma-Aldrich, methanol (MeOH), 1,2-propylene glycol (PG) and the other chemicals were purchased from Beijing Chemical Agents Ltd. All the chemicals were analytical grade. The cryoprotectant solutions used in the following experiments were made with Hank's solution [16] (8 g/L NaCl, 0.4 g/ L KCl, 0.14 g/ L $CaCl_2$, 0.1 g/ L l $MgSO_4$ $7H_2O$, 0.1 g/ L $MgCl_2$ $6H_2O$, 0.06 g/ L Na_2HPO_4 $12H_2O$, 1g/ L glucose, 0.35 g/ L $NaHCO_3$) as the extender. The concentration of cryoprotectant was expressed as the percentage of volume (v/v).

2.3. Effect of cryoprotectant solutions on the embryos

For each exposure test, approximately 50 embryos were exposed to 10 mL cryoprotectant solutions with various concentrations for different exposure time (Tab 1) at room temperature (18 ± 1 °C). Three replicates were taken for each experiment group.

	Cryoprotectants	extender	Exposure time(min)		
S1*	5%DMSO+5%PG	Hank's solution	10	30	60
S2*	8%MeOH				
V1**	40%DMSO		5	10	15
V2**	40%PG				

* For conventional slow cooling methods

** For vitrification methods

Table 1. Cryprotectant concentrations and treatment time used in cryopreservation of red seabream.

After immersion the embryos were first removed from the cryoprotectant solution using a nylon mesh, and carefully washed three times with fresh seawater. Then the embryos were transferred to a 100 mL beaker containing 80 mL fresh seawater for incubation with a change of seawater an hour later. Control groups were incubated in the filtered seawater at room temperature. The toxicity of cryoprotectant was assessed by the hatching rate which was calculated as the percentage of hatched larvae (48 h after fertilization) in relation to the total number of each group.

2.4. Morphological changes during exposure to cryoprotectant solutions

One embryo with seawater was loaded on a concave slide under a light microscope (Nikon-YS100). We removed the seawater with filter paper and added 100 μl cryoprotectant solution. Morphological changes were observed by taking pictures using a digital camera (Nikon CoolPix 4500) under microscope with 40× magnification and the interval was about 1 min. 5 embryos were observed for each cryoprotectant solution.

2.5. Changes of embryos during cooling and thawing process

The embryos were immersed in the four cryoprotectant solutions for different time (Tab 2), respectively. After immersion, the embryos suspended in 20 μll of cryoprotectant solution were loaded into a small quartz holding vessel and placed onto a Linkam Cryostage (Linkam-THMS600, UK). The embryos were cooled with different methods (Tab 2). After thawing, the embryos were transferred to a 100 mL beaker containing 80 mL filtered seawater for incubation. Each experiment was repeated three times. The morphological changes during the cooling-thawing process were recorded using a microscope (Olympus BX-51, Japan) with a video attachment and monitor (Nikon-E200, Japan). In addition, two temperature values, T_{EIF} and T_{IIF} were recorded. They were identified as the temperature when a flash appears in the field of view and the temperature when the embryo suddenly blackens, respectively. The temperatures reported were obtained by the Linkam cryostage thermocouple.

	Cryoprotectants		Cooling methods	Thawing methods
S1	5%DMSO+5%PG	Immersed for 50 min	0°C for 1 min, to -15.5°C with -3.5 °C /min, to -30°C with 3 °C /min, then to -150°C with -130°C /min and for 1 min;	to -80°C with 30°C/min, to 20°C with 130°C/min
S2	8% Methanol	Immersed for 30 min	0°C for 1 min, to -20°C with -2°C /min and for 5 min, to -60°C with -2°C/min and for 5 min, then to -150°C with -10°C/min and for 1 min;	to -70°C with 30°C/min, to 20°C with 130°C/min
V1	40%DMSO	Immersed for 5 min	cooling to-150°C with -130°C /min and for 1 min;	to 20°C with 130°C/min
V2	40%PG	Immersed for 10 min	cooling to-150°C with -130°C /min and for 1 min;	to 20°C with 130°C/min

Table 2. Cooling methods used in cryopreservation of red seabream.

After thawing, the morphological changes of embryos were observed and recorded by the video attachment and monitor under the microscope. The proportion of morphologically intact embryos was calculated as the percentage of embryos with normal morphology in relation to the total number of each group.

2.6. Statistical analysis

Percentage data were normalized through arcsine transformation and analyzed by one-way ANOVA with SPSS software (SPSS Inc., USA). The results were expressed as means ± SD. The significant differences between treatments in the different experiments were detected using an SNK (Student Newman Keuls) statistical test ($P < 0.05$). The percentages of embryos with intact morphology were arithmetical means.

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3. Results

3.1. Effect of cryoprotectant solutions on the hatching rate of red seabream embryos

The hatching rates of embryos treated with cryoprotectant solutions are shown in Table 3. After exposure to S1 and S2 solutions, the hatching rates of embryos showed no significant decrease compared to control except for S2 solution with 60 min exposure. However, the hatching rates of embryos exposed to V1 and V2 solutions decreased sharply, only the embryos immersed in V2 solution for 5 min and 10 min had higher hatching rates (>80%). So in the later cooling experiment, we choose 60 min, 30 min, 5 min and 10 min as exposure time for S1, S2, V1 and V2, respectively.

	Cryoprotectants	Exposure time	Hatching rate (%)
Control	—	—	99.67±0.82^{a}
S1	5%DMSO+5%PG	10 min	98.00±2.00^{a}
		30 min	100.00±0.00^{a}
		60 min	100.00±0.00^{a}
S2	8%MeOH	10 min	98.67±2.31^{a}
		30 min	96.67±2.31^{a} 65.67±5.13^{bc}
		60 min	
V1	40%DMSO	5 min	59.33±14.19^{c}
		10 min	15.33±5.03^{d}
		15 min	15.00±15.52^{d}
V2	40%PG	5 min	96.67±5.77^{a}
		10 min	81.33±22.74^{ab}
		15 min	65.00±27.73^{bc}

Values with different letters are significantly different (P<0.05) (means ± SD)

Table 3. Hatching rates of embryos treated with the four selected solutions

3.2. Morphological changes during exposure to cryoprotectant solutions

The morphological changes of the embryos in S1 and V1 are shown in Figure 2. The morphological changes in S2 and V2 were similar with those in S1 and V1, respectively. No obvious change was found after immersed in S solutions (after exposure for 30 min (Fig. 2-S-2). But there was an obvious dark strip around the yolk sac in V groups as soon as embryo was exposed in the cryoprotectants (Fig. 2-V-2), and the dark strip became narrower until disappeared gradually at the time of 10 min (V1, Fig. 2-V-4)/13 min (V2). At the end of immersion, the

embryos presented different extent of degeneration (Fig. 2-S-6), and the degeneration in S2 was more serious (Fig. 2-S-7).

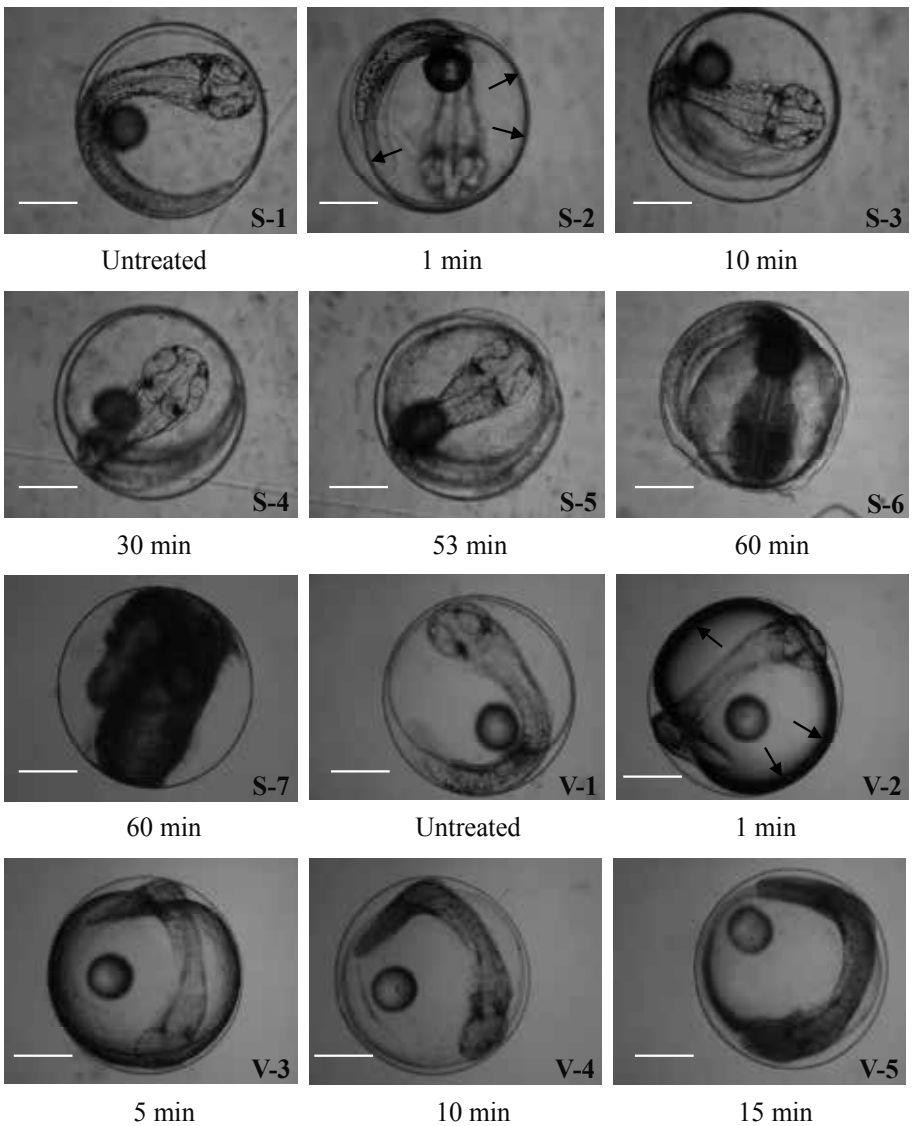

Figure 2. Representative morphological changes of embryo immersed in cryoprotectant solutions. This figure shows the morphological changes of red seabream embryo immersed in cryoprotectant solutions. (S-1) Untreated embryo for S1 group. (S-2~6) Morphological changes of embryo immersed in S1 for different times. (S-7) Degeneration of embryo immersed in S2 for 60 min. (V-1) Untreated embryo for V1 group. (V-2~5) Morphological changes of embryo immersed in V1 for different time. Scale bar=300.00 μm.

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3.3. Changes of embryos during cooling and thawing process

The representative changes of embryos during cooling and thawing process under cryomicroscope are shown in Figure 3 and Figure 4. Intra-cellular ice formation occured in both group (Fig. 3-S-4,5; Fig. 4-V-2,3) when the embryos suddently blacken. According to the extent of blackening, we separated the intra-cellular ice formation temprations (T_{IIF}) into two categories, high temperature blackeners (T_{IIF1}) and low temperature blackeners (T_{IIF2}). The values of T_{EIF} and T_{IIF} in the four groups are shown in Table 4.

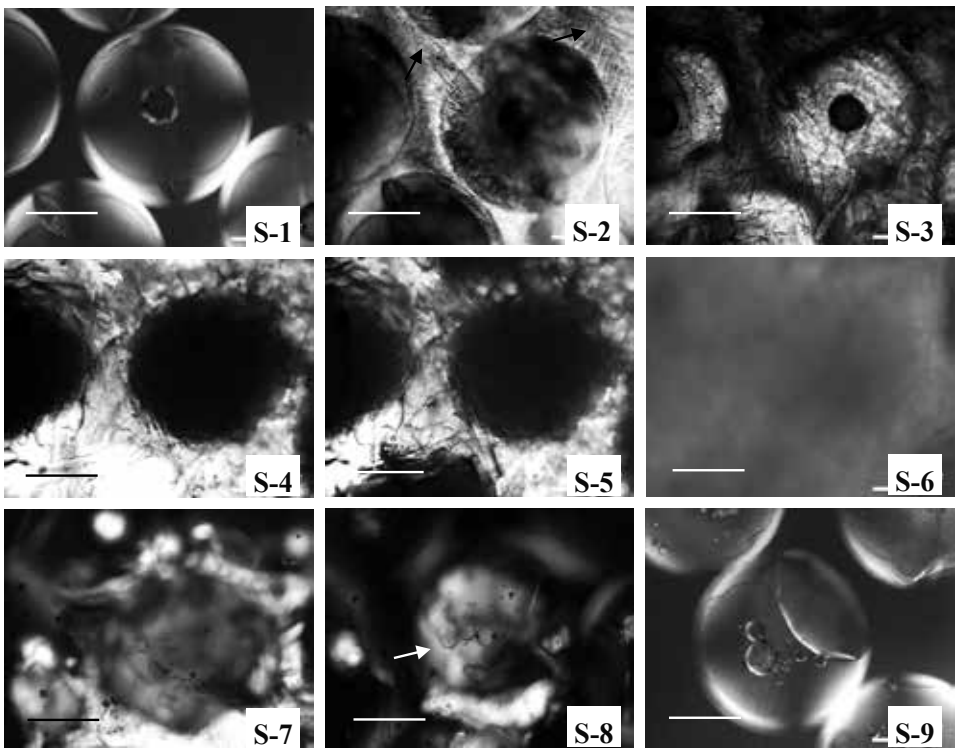

Figure 3. The representative changes of embryos during cooling and thawing in conventional slow cooling groups. This figure shows the representative changes of embryos during cooling and thawing in conventional slow cooling groups. (S-1) Embryos before cooling. (S-2) Extra-cellular ice formation: a flash appears and dendritic spears of ice (arrow) project in the field of view. (S-3) Extra-cellular ice formation. (S-4) Intra-cellular ice formation in partly embryos. (S-5) Intra-cellular ice formation in all embryos (S-6) Completely frozen embryos. (S-7) Embryo with extra-cellular ice thawing. (S-8) Embryo with intra-cellular ice thawing. (S-9) Completely thawed embryos. Scale bar=400.00 μm.

Both T_{EIF}s and T_{IIF}s in S groups were significantly higher than those in V groups. In V groups, T_{IIF} was higher than T_{EIF}, indicating intra-cellular ice formed earlier than extra-cellular ice (Fig. 4-V-2), opposite to S groups (Fig. 3-S-2). And the intra-cellular ice firstly formed in the yolk and developing embryo (Fig. 4-V-3), then the perivitelline space (Fig. 4-V-4). Besides, dendritic ice crystals were observed in S groups (Fig. 3-S-2, arrow), and no big ice crystals were observed in V groups.

When the embryos cooled to-150 °C, the phenomena was similar in S groups and V1, the whole field of view was obscured by the extra-cellular ice (Fig.3-S-6, Fig.4-V-5). In V2, the embryos were obscure, while the solutions outside the embryos were transparent, just like solid glass (Fig.4-V-9). The transparent spaces among embryos were obscured during the thawing process (Fig.4-V-10).

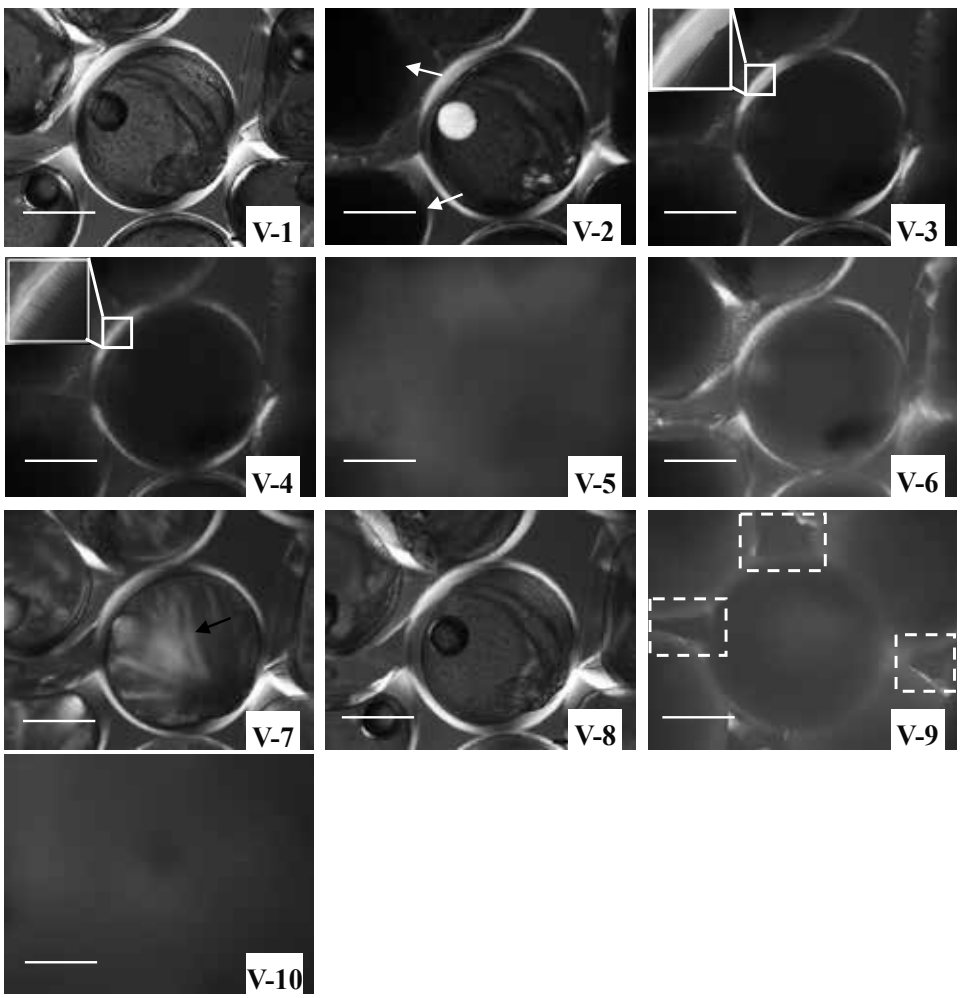

Figure 4. The representative changes of embryos during cooling and thawing in vitrification groups. This figure shows the representative changes of embryos during cooling and thawing in vitrification groups. (V-1) Embryos before cooling. (V-2) Intra-cellular ice formation in partly embryos. (V-3,4) Intra-cellular ice formation: the intra-cellular ice firstly forms in the yolk and developing embryo (arrow), then the perivitelline space (arrow). (V-5) Completely frozen embryos in V1 group. (V-6) Embryo with extra-cellular ice thawing. (V-7) Embryo with intra-cellular ice thawing. (V-8) Completely thawed embryos. (V-9) Vitrification in the solutions outside embryos in V2 group. (V-10) Devitrification during the thawing process in V2 gourp. Scale bar=400.00 μm.

During the thawing process, the ice of the outside solution thawed firstly and then thawed the ice inside the embryo with the embryo brightening (Fig. 3-S-8, Fig. 4-V-7) in all four groups. After thawing, the percentage of embryos with intact morphology in the four groups were S1, 10.26%; S2, 8.19%; V1, 55.26%; and V2, 70.00%, respectively. In total, the percentage of embryos with intact morphology in V groups (62.82%) was significantly higher than that in S groups (9.21%).

	Cryoprotectants	T_{EIF} (℃)	T_{IIF1} (℃)	T_{IIF2} (℃)	$T_{EIF} - T_{IIF2}$ (℃)
S1	5%DMSO+5%PG	-5.00±0.17^{a}	-17.74±0.59^{a}	-21.40±0.31^{a}	16.40±0.47^{a}
S2	8%MeOH	-4.93±0.35^{a}	-16.34±0.08^{a}	-17.51±0.48^{b}	12.58±0.74^{b}
V1	40%DMSO	-70.30±2.72^{b}	-56.83±1.48^{b}	-64.33±0.51^{c}	-5.97±2.21^{c}
V2	40%PG	—	-46.23±0.31^{c}	-53.70±1.00^{d}	—

Values with different letters within the same row are significantly different (P<0.05) (means ± SD)

Table 4. The values of T_{EIF} and T_{IIF}

4. Discussion

4.1. Morphological changes during exposure to cryoprotectant solutions

Fish embryo is composed of three membrane-limited compartments: a large yolk surrounded by the yolk syncytial layer, a developing embryo limited by its own cell plasma membranes, and perivitelline space surrounded by chorion [17]. The different layers and membranes represent permeability barriers hindering the movement of water and cryoprotectants, and consequently, making the balance of cryoprotectant in the whole embryo difficult [18].

In the observation of morphological changes, we found a dark strip around the yolk sac after immersion in the V solutions. This may ascribe to the different refractive indexes between outer solution and perivitelline space. With the time going on, the strip became narrower and eventually disappeared. It indicated that the chorion of red seabream embryo was permeable to the cryoprotectant solutions. Similar results were found in studies on zebrafish embryos and medaka oocytes [18,19]. In V groups, the dark strip was obvious, while in S groups, the dark strip was not observed. This may be due to higher concentrations of V solutions than S solutions, which caused higer refractive index difference.

4.2. Changes of embryos during cooling and thawing process

In our cooling experiment, we have observed some different phenomena between the two methods. The first representative phenomenon in our experiment was the large dendritic ice crystals observed in S groups, but not in V groups. High cooling rate and cryoprotectant

concentration were reported to be prone to vitrify and avoid dendritic ice crystal formation [20], which may explain the phenomenon in this study.

Secondly, in our result, T_{IIF}s in S groups were significantly higher than those in V groups. This may be because the nucleation temperature of red seabream embryos is too high to achieve sufficient cell dehydration to avoid IIF [21]. T_{IIF} is a main factor many researchers focus on in the study of cryoproservation. Slow cooling usually causes dehydration and decreases T_{IIF} largely in mammal oocytes or embryos, and contributes to successful cryopreservation [22]. However, it was not the case in red seabream embryos, zebrafish embryos [23] and starfish oocytes [24] mainly due to the high water content and multi-membrane structures which inhibit sufficient dehydration of fish embryos.

Thirdly, intra-cellular ice formed earlier than extra-cellular ice in V groups, which is opposite to the phenomenon in S groups. And the intra-cellular ice in V groups firstly formed in the yolk and developing embryo, then in the perivitelline space. Based on the result of section 3.2 and previous research [19], the cryoprotectant could penetrate into the chorion but hardly penetrate into the yolk syncytial layer and the developing embryo. So the concentration of cryoprotectant outside the chorion was the highest, followed by the perivitelline space, and the yolk and developing embryo was the lowest. We speculated that the sequence of ice formation was determined by the different cryoprotectant concentration in the different parts.

At the point of-150 °C, the phenomena in the four groups were different. In the group of S groups and V1, the whole field of view was obscured by the extra-cellular ice. However, in V2 group, the solutions outside embryos were transparent, like solid glass. We inferred that the outer solution vitrified. The difference of cryoprotectants caused the different phenomena between V1 and V2, because PG (used in V2) vitrifies more easily than DMSO [25]. The embryos in V group did not vitrify, which indicated that the cryoprotectant concentration of inner embryo was not high enough to form vitrification at this cooling rate. It indicated that limited or no cryoprotectant penetrated into the yolk and developing embryo. Yolk syncytial layer (YSL) was the major obstacle for the penetration of the cryoprotectant into the embryo. This was coincide with many researches [26].

In the thawing process, devitrification or/and recrystallization usually take place. Devitrification is the transition of glassy to crystalline state and recrystallization is the growth of existing ice crystals. In our experiment, the transparent space in V2 group was obscured during thawing, which indicated that devitrification had occurred in extra-cellular ice. Recrystallization had also occurred within the embryos in all four groups, for the embryos were all turned bright in the thawing process. Devitrification and recrystallization were thought to be the major reason for cellular damage associated with the thawing process of cryopreserved cells, for they could cause ice crystals formation and larger ice crystals [27]. To avoid devitrification and recrystallization, the specimens must be warming at higher rate than the critical warming rate [23]. However, there are no effective means to avoid them at the present time because of the technique limitation.

http://dx.doi.org/10.5772/58830

5. Conclusion

In the present study, we compared conventional slow cooling method and vitrification method in red seabream embryos cryopreservation. In V groups, the T_{EIF}s and T_{IIF}s were significantly lower than those in S groups, and no big ice crystals formed. Besides, after thawing, the percentage of embryos with intact morphology in V groups was significantly higher than that in S groups. All the results indicated that vitrification method would be a good option for red seabream embryos cryopreservation, though the embryos were better tolerant of S solutions than V solutions. Therefore, further study is still required to optimize the cryopreservation protocol for reducing toxicity of cryopreservation as well as improving the internal cryoprotectant concertration in red seabream embryos.

Acknowledgements

This work is supported by Regional Demonstration of Marine Economy Innovative Development Project (No. 12PYY001SF08)" and National Infrastructure of Fishery Germplasm Resources.

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Chapter 3

Cryopreservation of Embryos and Oocytes of South American Fish Species

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Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/58703

1. Introduction

The importance of animal genetic resources for wildlife maintenance as well as farming production has become more and more evident in recent years. Fish stocks are globally threatened mainly due to overfishing and environmental pollution. Cryopreservation of aquatic germplasm brings the possibility of preserving the genome of endangered species, increasing the representation of genetically valuable animals for farming purposes and avoiding genetic losses through diseases and catastrophes.

In fish, successful cryopreservation of semen from many species has been well documented and cryopreserved semen has been used for reproduction of many wild and farmed species. Attempts to cryopreserve fish embryos have been conducted over the past three decades, nevertheless successful cryopreservation protocol for long-term storage still remains elusive.

In this chapter we cover the cryobiology applications on assisted reproduction for fish farming, focused on embryo and oocyte cryopreservation. Our research group has been working in this area for more than 10 years and the chapter shows the main results that we have achieved from researches on embryos cryopreservation. The barriers that have been identified as a hamper for successful cryopreservation and the sensitiveness of tropical fish embryos to low temperature exposure are also detailed here.

Recently, researches have reported that the use of oocytes may offer some advantages when compared to fish embryos, improving the chances of a successful cryopreservation. On this point, the chapter presents the most recent achievements in oocytes cryopreservation from South American fish, new trends and future works on this area.

2. Brazilian aquaculture growth and researches in cryobiology area

The history of Brazilian aquaculture dates back to 1930's when the first scientific studies related to fish farming were carried out by Rodolpho von Ihering [1], however, the farming activities had bit higher impulses only from the 1970's by creation of research center facilities like CEPTA (Center for Research and Training in Aquaculture) and the insertion of academic activities related to aquaculture at universities. From the mid 90's the entry of some private companies boosted the sector, and from the 2000's aquaculture started to represent an important activity of the Brazilian agribusiness.

Fish farming is the sector of the Brazilian animal production industry which has displayed the highest growth in recent years, reaching 544,490 t in 2011, representing an increase of 38.1% compared to its production in 2010 [2]. From the total fish farmed in 2011, about 46% (250,046 t) of the production was from South American species [2].

The growth in fish production has called attention of numerous companies on planning the fish farming development, and biotechnology area has contributed decisively to this. Germplasm cryopreservation brings the possibility of preserving specific species or strains of particular interests, increasing the representation of genetically valuable animals, extending the reproductive life of a particular animal, and avoiding genetic losses through disease, catastrophe, or transfer between locations [3]. In fish farming, the successful cryopreservation of gametes, eggs, and embryos would offer new commercial possibilities, allowing the unlimited production of fry and potentially more robust fish as required by the market [4]. Germplasm cryopreservation also provides a secure method for preserving the genomes of endangered species, having an important role in conservation and preservation of genetic biodiversity of aquatic resources [5].

Research on cryopreservation of gametes and embryos from South American species focused initially on those fish with commercial interest, and more recently has also focused on species which have environmental importance. The first record published in the literature regarding semen cryopreservation from South American species was in 1984 [6] using *Prochilodus scrofa=Prochilodus lineatus* and *Salminus maxillosus*. Afterwards, papers started to be published involving other species such as: *Rhamdia hilarii* [7]; *Leporinus silvestrii* [8]; *Piaractus mesopotamicus* [9, 10]. In the 2000's studies on semen from South American species took different lines of research, not only investigating other species such as *Leporinus macrocephalus* [11], *Brycon cephalus* [12], *Brycon orbygnianus* [13], *Brycon nattereri* [14], but also testing different protocols for semen cryopreservation [15, 16], and assessing fertilizing capability after thawing [17, 18].

In the 2000's researches on cryopreservation of fish embryos began in Brazil, with the first tow thesis performed on the subject in the country [19, 20] and having the first papers published in 2007 (*Piaractus mesopotamicus*) [21] and in 2008 (*Prochilodus lineatus*) [22]. Currently, three research groups have been working on fish embryo cryopreservation research: *Aquam* Group (Federal University of Rio Grande do Sul-UFRGS), PeixeGen Group (Maringá State University-UEM) and the Cryopreservation of Gametes and Embryos of Neotropical Fish Group (Universidade Estadual Paulista Júlio de Mesquita Filho-UNESP). However, other groups have

started activities in this research area, as NAQUA Group (Federal University of Lavras) and certainly new scientific papers related to this area will begin to be published in the next years.

3. Main difficulties in cryopreserving fish embryos

Fish, reptiles, birds and amphibian embryos contain a considerable amount of yolk and represent a complex multicompartmentalized biological class, whose cryopreservation has constantly failed [22]. Rall [23] pointed out five features that would hinder the cryopreservation of embryos from teleosteans:

- Cells of great size and a large final size of the embryos: In general, fish embryos are much larger than most mammalian embryos. Compared to a human zygote, which has about 100 μm in diameter, a fish zygote (> 1 mm in diameter) comes to be a thousand times larger. This large size of fish embryos results in a low surface/volume ratio and lower membrane permeability to water and cryoprotectant solutions;
- Distinct osmotic properties for each compartment of the embryo: the complex membrane structures hamper considerably the transport between each other;
- Chorion: the low permeability of this membrane envelope makes it almost impassable;
- High sensitivity at low temperatures: fish embryos are too sensitive to sub-zero temperatures exposure.

Within this context, several studies have been carried out in order to overcome the pointed difficulties: Microinjection of cryoprotectants directly into the cytoplasm [24], use of negative pressure to increase permeability of the cryoprotectant agents (CPAs) [25], chorion removal [26] and microinjection of anti-freeze proteins (AFPs) [27]. AFPs are a class of polypeptides produced by certain vertebrates, plants, fungi and bacteria that permit their survival in subzero environments. AFPs bind to small ice crystals to limit the growth to manageable sizes. Application of hydrostatic pressure [25, 28-31] was also tested. Nevertheless, all these attempts have failed. Some researchers state that precise knowledge of embryo-permeability would be one of the keys for designing and achieving a successful cryopreservation protocol.

4. Results achieved according to the cryopreservation technique employed

The initial focus of the earlier studies on cryopreservation of South American fish embryos was to assess the embryo sensitivity to different CPAs and exposure times, as well as the best stage of embryonic development to be used. As there was no reference to undergo cryogenic experiments with embryos of South American fish species, the initial studies were based on researches using *Cyprinus carpio* [29] and *Danio rerio* [28].

In early tests it was identified that for embryos of South American species, the intermediate stages of development, post-morula 8 hours post fertilization (hpf) were less susceptible to

injuries caused by CPAs [20]. Methanol, dimethyl sulfoxide (DMSO) and ethylene glycol as permeating and sucrose as non-permeating CPAs led to better results, then started to be used to compose protocols of further researches [19, 20].

4.1. Chilling

To the South American species, designing a practical and efficient chilling protocol was the main objective attempted by the research teams. Unlike freezing technique where specific cooling rates can be achieved by using liquid nitrogen (LN), and once frozen in liquid nitrogen samples can be stored for extended periods of time; the chilling technique consists of exposing cryoprotected fish embryos to subzero temperatures (normally using a refrigerator) followed by short storage periods [32, 33]. This method can be very useful for hatchery management since it allows synchronizing the development of embryos collected from different spawning events and may optimize the use of hatchery facilities, especially in remote areas where facilities are very limited.

We chilled *Piaractus mesopotamicus* embryos using a chilling solution composed by 0.5 M (17.1%) sucrose and 2.81 M (9%) methanol for six hours at-8 °C, which resulted in a hatching rate of 69.2% [21]. The same protocol was used for other South American fish embryos, achieving good results as for *Brycon orbygnianus* with hatching rate above 62% [34], *Rhinelepis aspera* upper 50% [35] (Figure 1), for *Salminus brasiliensis* with hatching rate of 67.06 % [36], and for *Colossoma macropomum* achieving a hatching rate of 77.6% [36].

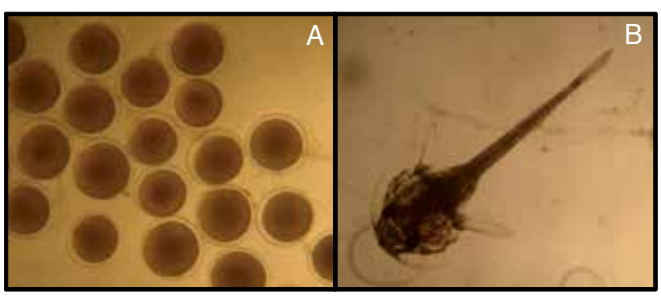

Figure 1. (A) *Rhinelepis aspera* embryos at blastoporous closing stage (75% epiboly movement) 9 h post-fertilization and (B): newly hatched larva of *Rhinelepis aspera* after exposure to a short-term cooling protocol carried out by Fornari et al. [35]. Digital images (7.2 megapixels) obtained by using a stereomicroscope. A: ×30 magnification. B: ×10 magnification.

Increasing the exposure time of embryos to chilling was a study carried out by Digmayer [37] with *Piaractus mesopotamicus*. In this study the author increased the sucrose concentration from 0.5 to 0.73 M and methanol concentration was also increased from 2.81 to 3.12 M. There was reported some hatching rate within 24 hours exposure, but not after 36 hours.

Lopes et al. [38] using the protocol set by Digmayer [37] assessed the chilling of *Piaractus mesopotamicus* embryos at four stages of development and confirmed to be the post-morula stage (8 hpf) as the best one in terms of hatching rate.

The exposure time (3, 6, 9, 12, 18 and 24 hours) was also tested to embryos of another South American species, *Prochilodus lineatus*, using the standard chilling protocol (0.5 M sucrose and 2.81 M methanol) employed by Streit Jr. et al. [21]. In this study, it was possible to estimate that at every hour under chilling the hatching rate decreased by 6.58% and after 12 hours none larvae hatched.

The embryos of *Piaractus mesopotamicus* were exposed by Fornari et al. [33] to-8 °C for 6, 12, and 24 hours, using a fixed methanol concentration of 2.81 M combined with different sucrose concentrations (0.25, 0.50, 0.75 and 1 M). There was no influence among the cryoprotectant combinations on the hatching rate up to 12 hours chilling, except for the treatment combining 2.81 M methanol with 0.99 M sucrose, in which no larvae hatched after this exposure time.

The combination of permeating and non-permeating cryoprotectants can be a research line that may produce good results. Working with *Brycon orbignyanus* Paes [39] exposed the post-morula stage embryos to a chilling solution composed by 0.5 M sucrose and methanol (2.81, 1.40 and 0.94 M), ethylene glycol (1.45, 0.72 and 0.48 M), and DMSO (1.15, 0.57 and 0.38 M) at tow controlled temperatures (0.0 and 8.0 °C) for 6, 10, 24, 72 and 168 hours. According to the author, the best result was observed when combining 1.4 M methanol and 0.57 M DMSO associated with 0.5 M sucrose, independent of the tested temperature. According to Milliorini [39] *Brycon orbignyanus* embryos are less sensitive to chilling than *Salminus brasiliensis* embryos. This is another important aspect, the range of sensitivity from some South American fish species when exposed to low temperatures.

Results obtained from these researches on embryos cryopreservation by chilling have great importance and can help in developing a successful freezing protocol which would allow preserving embryos for longer periods.

4.2. Freezing

The knowledge on developmental stage which South American fish embryos are less sensitive to cryoprotectants and low temperature exposure was the starting point to initiate the studies on embryos freezing in Brazil. The choice by intermediate stages of development, morula and post-morula (8hpf) was based on information reported by Gwo et al. [41], when working with red drum (*Sciaenopus ocellatus*) found that embryos at morula stage showed higher resistance to chilling than the eight-cell stage (embryos at earlier stages) and gastrula (embryos at later stages). Ninhaus-Silveira et al. [22] reported that *Prochilodus lineatus* embryos at morula stage were more resistant than those which had between 4 and 6 somites. This information corroborated the observation made by Streit Jr. [20] pointing 8hpf as the embryonic stage that best survived to exposure to different cryoprotectants.

Pre-cooling in cell cryopreservation is essential for the success of the process. When the cooling is sufficiently slow, cells are able to lose water rapidly by osmosis, tolerating dehydration and maintaining the equilibrium of the water chemical potential of the intracellular and extracellular solutions [42]. However, the author shows that even then the exposure of cells to cryoprotectant solution causes an osmotic stress, which depending on the concentration, time and temperature of exposure to the solution can lead the cell to collapse. On the other hand, if cooling is too fast, the chemical potential of water and extracellular solution decreases faster

than the intracellular water, resulting in remaining intracellular water that eventually will form intracellular ice crystals, which are often lethal to cells [3].

Lopes et al. [43] using the protocol set by Digmayer [37] tested three cooling curves for *Piaractus mesopotamicus* embryos at post-morula stage (8hpf) at-8 °C and concluded that 1 °C/s is better than 0.5 °C/s and 35 °C/s. This result corroborates the observations made by Zhang et al. [30] with embryos of the model species *Danio rerio* and testing the effect of slow (1 °C/min), intermediate (30 °C/min) and fast (300 °C/min) cooling curves, using methanol as cryoprotectant.

Likewise studies with other fish species, successful results for long-term storage of South American fish embryos is still remains elusive. However, researches have been focused on trying to understand the extent of injuries in embryonic tissue during the freezing process. In the freezing protocol tested for *Prochilodus lineatus* by Ninhaus-Silveira et al. [22], it was possible to observe some evidences of the problems caused by freezing. Although authors have found intact blastoderm, after scanning microscopy analysis they verified nuclei with disorganized chromatin and cracks in the plasma membrane, which compromised the microvilli, causing embryo death.

Piaractus mesopotamicus embryos were exposed by Neves et al. [44] to six freezing protocols: 0.1 M sucrose plus ethylene glycol (1.20, 1.61 and 2.09 M) or methanol (2.18, 3.12 and 4.06 M). After thawing the chorion was observed to be broken and missing blastoderm in all treatments, which was crucial to the embryos death.

4.3. Vitrification

Vitrification was first developed by Rall and Fahy [45] to overcome the problems of intracellular ice formation and has the advantage of minimize the procedure time and eliminate the need for a programmed freezing machine. Vitrification is the solidification of a liquid brought about not by crystallization but by an extreme elevation in viscosity during ultra-rapid cooling rates [4].

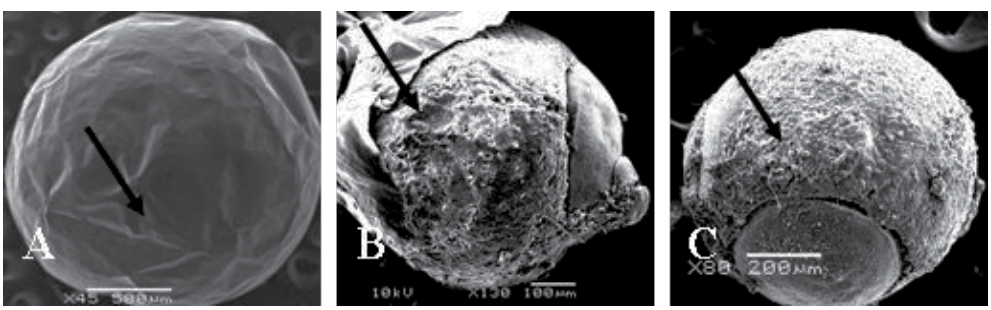

Figure 2. Scanning electron micrograph of *Colossoma macropomum* embryo after exposure to a vitrification protocol carried out by Fornari [46]. (A): embryo displaying intact chorion; (B): embryo with manually removed chorion, displaying damaged blastoderm; (C): embryo at blastoporous closing stage displaying cell injuries.

Vitrification of *Colossoma macropomum* embryos (8hpf) was carried out by Fornari [46] using methanol, DMSO, ethylene glycol and glycerol at concentrations of 10, 20 and 30%. Although hatching did not happen, when using scanning electron microscopy (SEM) (Figure 2), it was noted that the embryos exposed to methanol treatments had the chorion and some cellular structures preserved.

5. Cryopreservation of fish oocytes

Sensitivity of fish oocytes and embryos to low temperatures exposure is specific for each species and for each cell types [30, 31]. The assessment of damages caused by cryopreservation is of great importance as it can be used to characterize the intensity of cell damage, which can incapacitate the cells to overcome such injuries and eventually lose their viability [47].

The difficulties in cryopreserving fish embryos, previously reported in this chapter seem to be reduced when dealing with oocytes due to its smaller size and less sensitivity to low temperature and CPAs exposure, besides the absence of fully formed chorion, which makes them more permeable to water and solutes. Fish oocytes have some advantages over other species, especially at early stages of development [4, 48, 49].

Recently, Digmayer [50] assessed the sensitivity of *Colossoma macropomum* oocytes by exposing to cryo-solutions containing 1.6 M methanol and two concentrations (0.25 and 0.50 M) of non-permeating cryoprotectants (glucose, sucrose, trehalose and fructose) using a slow cooling protocol (1 °C/min until seeding), following storage in liquid nitrogen. After thawing, in the protocol consisting of 1.6 M methanol and 0.25 M sucrose it could be observed oocytes with intact zona radiata (Figure 3); however a displacement of this structure from the oocyte membrane was also observed. None fertilization success was achieved.

Figure 3. Morphological structure of *Colossoma macropomum* oocytes after exposure to a slow cooling protocol carried out by Digmayer [50]. (A and B): yolk (y) and yolk granules (ygr) heterogeneous in size; (C): zona radiata (zr) displacement. Oocytes were stained with hematoxylin and floxin.

Digmayer [50] also worked with *Piaractus brachypomus* oocytes at room temperature (28.4 ºC) combining methanol (1.6 and 3.1 M) and DMSO (0.7 and 1.3 M) with 0.25 M sucrose diluted in 50% L-15 medium or Hanks' solution. In this study, the least toxic combination of CPAs was

1.6 M methanol with 0.25 M sucrose diluted in Hanks' solution, which allowed that 17.3% of those oocytes after being fertilized developed into a health larva.

Studies on cryopreservation of South American fish oocytes are too recent and several points should be further clarified in order to help in achieving a successful cryopreservation protocol in the future.

6. Germplasm banking and genetic improvement programs in fish farming

In Brazil the use of gene banks starts driving to a professionalism level and an example serves very well to illustrate this situation. The use of a germplasm bank was crucial to the operation of the first genetic improvement program for native fish species created in the country. The important increase of aquaculture led the Brazilian government to create a national program for research and development of aquaculture, named AQUABRASIL. Among the AQUABRASIL's research areas, the genetic improvement program (GIP) for two South American native species tambaqui (*Colossoma macropomum*) and cachara (*Pseudoplatystoma reticulatum*) has been considered as great importance. It is important to highlight that this genetic improvement program was developed based on a similar program carried out by the World Fish Center (Malaysia) and implemented for tilapia in Brazil in 2005.

In the first four years of the GIP, the genes supplier populations were identified and the families were formed, for later fingerlings production. The semen bank enabled the required mobility, since the distances between base populations were higher than 2000 km. Transporting breeders for a long distance would be costly and risky, requiring a logistics that would involve land and air transportation, beyond too time to get the program's deployment and also the possible loss of these specimens. It was much easier to transport cryopreserved semen in liquid nitrogen dewars.

The protocols used for semen freezing were: YEAR 1-Cryopreservation protocol suggested by Carolsfeld et al. [15] for Characidae: DMSO+glucose+egg yolk; and for Siluridae: powder milk +methanol. Egg yolk and powder milk were thereafter replaced by BTS™ (Beltsville Thawing Solution). YEAR 2 and 3-Cryopreservation protocol from studies carried out by Murgas et al. [51] using semen of South American species; YEAR 4-Cryopreservation protocol employed only to composition of *C. macropomum* families, replacing DMSO by dimethylformamide (DMF), based on studies performed by Varela Júnior et al. [52].

Currently the GIP is still being developed and germplasm bank acts as a reservoir of genetic material for an eventual replacement or change in focus of the desired genetic trait.

7. New trends and future works in the area

Future studies in the area should try to find solutions that allow the cryopreservation of fish embryos. The path for future researches with South American species unfailingly passes

through a better understanding of the injuries caused to the cell when exposed to CPAs and sub-zero temperatures in different protocols (slow, rapid or ultra-rapid cryopreservation). Advances in devices development as well as biochemical and molecular techniques can help in elucidating some questions and allow being closer to a successful cryopreservation.

A new research line aims to cryopreserve fish ovarian tissue instead of oocyte or embryo and seems to generate encouraging results. Recently, Godoy et al. [4] using *Danio rerio* as a model observed that 60% of the membranes of stage III ovarian follicles in tissue fragments remained intact after vitrification. This research line is currently under investigation by our research group involving South American species.

Another point that we should pay greater attention is related to genetic and behavioral changes induced in the larvae, juveniles and even in adult life after being exposed to cryo-solutions. Studies on this subject may lead to changes in cryopreservation procedures, including to other animal species.

Acknowledgements

We thank Boa Esperança Fish Farm (Rondônia-Brazil), Buriti Fish Farm and the "Delicious Fish" Farm (Mato Grosso-Brazil) for the partnership in research. The studies currently under development have been financially supported by CAPES and CNPq via the Science without Borders Program-PVE-Project N° 105/2012.

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Chapter 4

Cryopreservation of Sperm in Brazilian Migratory Freshwater Fish

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Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/58685

1. Introduction

The Brazilian freshwater fish diversity is the richest in the world. The majority this fish species migrate during the spawning season (a phenomenon known as piracema). Urbanization, pollution, hydroelectric dams and deforestation are some of the causes of stock depletion or even local extinction of some of these species [5].

The disappearance of native fish species and the decrease of genetic stocks undertake the aquatic ecosystem and commercial food production. Cryopreservation of semen is a biotechnology that has the ability to minimize these problems, increasing flexibility and operationalization of the reproductive period and improving assisted reproduction programs in native fish [1,2]. This also enables the biotechnology application of new techniques such as transplantation of gametes to form chimeras [3].

May be cited as advantages of semen cryopreservation: (i) sincronyzation of the gametes release from both sexes, (ii) semen economy, (iii) easeness of the management with breeders, (iv) transport of gametes from different locations and (v) genebanks for genetic selection programs and species conservation [4] and decreased need for males breeders from nature.

However, the heterogeneity of semen responses after freezing has hampered the standardization of a protocol for the different species of fish [5]. Protocols have been tested for *Salminus*

maxilosus and *Salminus brasiliensis* [6,7], *Colossoma macropomum* [8], *Brycon orbignyanus* [6,9], *Brycon nattereri* [2], *Brycon amazonicus* [10], *Leporinus elongatus* [11], *Prochilodus lineatus* [12,13,14], obtaining different results.

2. Sperm characteristics essential for freezing

Due to the great variation in semen quality is extremely important to make a preliminary evaluation of fresh semen to be cryopreserved by determining a set of qualitative and quantitative variables. Traditionally, the quality parameters evaluated are sperm motility (percentage of motile cells, duration and speed), sperm concentration and sperm morphology [15].

In most teleost fish immobility sperm are present in the male genital tract and their motility is acquired from the moment that there is contact with water [7], being the difference in osmolarity between the semen and the solution responsible for activating.

The seminal plasma is the substance responsible for the suppression of testicular sperm motility, being a component of the isotonic semen [16]. The seminal fluid is rich in nutrients and ions, and for the activation, external environmental factors such as pH, osmolarity, temperature and ions can affect the quality and motility of sperm [17].

For the activation of the sperm and motility two fundamental changes must occur, the first is related to the fluidity of the plasma membrane [18], while the second is related to the activation of ion channels, increasing calcium intracellular generation of cyclic adenosine monophosphate (cAMP) [19].

The activation of ion channels and the passage of water, whether for water channels or aquaporins mediated the activation of second messengers, particularly the cAMP pathway that are essential for signal transduction. The activation of the cAMP pathway determines the activation of flagellar proteins by phosphorylation / dephosphorylation [20,21,22].

These phosphorylation and dephosphorylation activities are mainly related with a primer motion phosphoproteins of 15 kDa (kilodalton) and dynein light chain of 22 kDa that are associated with microtubules of the sperm tail [23,24].

Sperm motility is one of the most important factors when you want to analyze fish semen quality and evaluate the effect of biotechnologies such as cryopreservation [25]. According to [14] the minimum rate of sperm motility necessary for the semen processing for cryopreservation is 80%, therefore, should be considered some factors such as temperature, nutritional status and health, activating solutions employed, studied species and reproductive season in which the breeder is [26].

The sperm concentration is also an indicator that should be considered to evaluate the quality of semen. However, despite being traditionally used, sperm concentration is not as very sensitive and specific, since shows great variability among species. For example, see [27] observed a negative correlation between sperm concentration and sperm cryopreservation

potential of *Gadus morhua*, probably related to variations in the composition of the extender and seminal plasma among evaluated males.

Indirectly, the fertilization capacity can also be used as a parameter of sperm quality. However, this analysis may not be reliable since the quality of the oocytes is variable and may affect the success of fertilization. Another important aspect is that eggs for fertilization sometimes are not available, which can limit this procedure and sometimes the posterior development cannot occur [28]. Along with the fertilization rate, embryo survival is the parameter used to evaluate the ability of the fertilized egg to develop successfully and thus assess the quality of gametes [29].

The morphological study of the sperm cell and its relationship with semen quality is also extremely important and increasingly has become an integral part of the routine semen analysis. The morphology evaluation permits inferences about the potential fertilization and assists in the characterization of samples of semen cryopreservation [30].

3. Extenders used in freezing semen of fish

Cryopreservation involves the dilution of semen in a cryoprotectant solution, which must provide appropriate nutritional and osmolarity, avoiding damages of the semen [2] and activation of sperm. If the semen is activated, the energy sources of the cell will be scarce, thus shortening their activity [31]. Typically the extenders present in the composition carbohydrates or salts, isotonic stable and high thermal conductivity [32].

Some media have been tested as extenders, where we can cite from simple solutions such as distilled water [33] with 3% glucose [34] and 5% glucose [35] by diluting synthetic and complex commercially available as Betsville Thawing Solution (BTS-MinitubTM) [12], M IIITM (Merck III) or AndrostarTM (MinitubTM) [34]. In addition, they should exhibit pH and osmolality similar to plasma and after freezing, must support the sperm while maintaining viability and the ability of these cells to fertilization [36].

The proportions of semen dilution may vary according to the species of fish, may influence the quality sperm after thawing. For some species such as *Piaractus mesopotamicus, Salminus brasiliensis*, *Leporinus obtusidens* and *Brycon orbignyanus* dilutions can be made in the ratio of 1:3 (semen / extender) [6]. For *Prochilodus lineatus,* 1:4 [12,14] and *Clarias gariepinus* 1:10 [37].

4. Cryoprotectant solutions and packages of semen doses

Cryoprotectants prevents formation of crystal ice within the spermatozoids, which are considered lethal, but in high concentrations can be toxic, even during the input or output of cells [38]. There are intern and extern cryoprotectants, the first reduce intracellular cryoscope point, reducing the formation of ice microcrystals and external cryoprotectants stabilize the outer membrane, preventing cell disruption [39].

The association between intracellular and extracellular cryoprotectant is indicated by [40] and [41] and have been tested for the native species rheophilic, of which we can mention the *Prochilodus lineatus* [12,13] and *Brycon orbignyanus* [9].

Among the most commonly used internal cryoprotectants can be cited glycerol [5], ethylene glycol [42], methanol [13,9,12] or dimethylsulfoxide DMSO [10,12] and methyl cellolose [43,44]. Among external cryoprotectants are used coconut water [13], egg yolk [45], glucose [46] and milk powder [6].

The semen storage can be accomplished in different types of straw which vary the volume conditioning (0.25 ml, 0.5 ml, 1.0 ml, 1.2 ml, 2.5 ml and 5 ml.) According [47] the fertilization rate using 1.2 mL straw was similar to 0.5 ml straw for salmonids by using low freeze temperature and high thawing temperature. The 5 mL straw resulted in successful fertilization of only 40% comparing to fresh semen control. Straws of 0.5 ml are the most commonly used among researchers in the freezing of fish semen, since higher caliber straws do not provide a uniform thawing, because the surface thawed more quickly than the central portion [40].

5. Freezing and thawing semen

The success of freezing semen with liquid nitrogen requires cooling rates between 10°C and 50°C min-1 [48]. [49] froze semen of *P. lineatus* using temperatures that range from 17.9 to 52.9°C min-1. The use of cylinders containing only liquid nitrogen vapor, such as those used by [40], provides cooling rates within this interval.

Most of cells support a rapid thaw, even if she does not fully hydrate. The speed after thawing is necessary to prevent recrystallization, which forms small ice crystals that regroup to form large crystals that are lethal to the cell [50]. The frozen semen straw must be removed from the liquid nitrogen and gently shacked in a water bath for a few seconds to thaw uniform [49].

6. Sperm activators

The use of appropriate activating solutions, which mimic the seminal plasma and do not compromise the quality of semen, can increase the duration and intensity of sperm motility, both contributing to higher survival and fertilizing capacity of the sperm, thus counteracting the deleterious effects of exposure to the aquatic environment or the activator sperm [51,52]. Moreover, it can help to improve semen cryopreservation.

The ionic composition of the activator, although important, has less influence on the sperm compared osmolality, since nonionic solutions are also capable of activating sperm motility. However, the addition of ions brings additive effects on motility parameters [53].

The sperm activation can be performed with different solutions, influencing the rate of semen motility. Can be include as solutions that can activate sperm motility distilled water, calcium bicarbonate (NaHCO3) [54] and potassium chloride (KCl) [55], and these solutions may

promote increased time rate and motility in various tropical species [56,14]. According [57] the osmolarity and pH of the activating solution were the main factors that affected the rate and duration of sperm motility after semen activation of *P. lineatus*. For this same species [58], verify that besides the osmolarity the dilution ratio (semen: activator) can also influence the rate and duration of motility.

For *Brycon orbignyanus* water was considered a good activator after thawing, causing high rates of hatch compared to a solution of NaCl (50 mM) at a ratio of 1:5 [9]. In *P. lineatus* was observed that different solutions can influence the activating sperm motility and fertilization rate of cryopreserved semen, being considered the best activator solution was sodium bicarbonate (60 mM), providing higher motility duration rates than in the activated semen with distilled water. However, this solution significantly reduced the fertilization rate, suggesting a deleterious effect to spermatic cell [30].

7. Cryopreservation effect on semen quality

The success of cryopreservation may be affected by the high variability in semen quality to be used. This variability is mainly associated with intrinsic factors that determine cryoresistance of the sperm cell, which is nothing more than the ability of sperm to preserve their morpho-functional characteristics after cryopreservation. The cryoresistance is a function of the resistance of cell membrane and the ability to maintain their intracellular structures and basic functions [15].

In the cryopreservation process, the sperm cells are exposed to external conditions that are not physiologically appropriate and this ultimately affect many parameters of sperm activity, such as motility, morphology, and composition of seminal plasma antioxidant activity [59].

Among the causes of this deleterious effect, the exposure of sperm to the cryoprotectant medium can be highlight, which causes cell osmotic stress and consequently damage to sperm function. The formation of internal ice microcrystals during freezing is also deleterious to sperm which can cause dehydration of cells and osmoconcentration [60]. Metabolic and oxidative damage caused by ROS (reactive oxygen species) are also highly detrimental to sperm events [61].

Among the parameters affected by cryopreservation, sperm motility can be citing, which is significantly reduced when the cell is exposed to low temperatures. As an example, see [12] showed that cryopreservation of *P. lineatus* semen progressively decreased their motility. According [20], the reduction of the motility parameters can be associated with damage to the flagellum, reduced mitochondrial function and degradation of proteins.

Sperm morphology can also be affected by the cryopreservation process. Ultrastructural alterations in sperm may occur due to changes in the osmolarity of the medium surrounding them. These changes may be due to membrane disruption, reduced mitochondrial function, spiralization, breakage or adhesion of the axoneme, and functional abnormalities which are responsible for reduced motility and fertilization ability [62].

Species	Solution*	Dilution (semen: solution)	Motility (%)	Motility duration (s)	Abnormal sperm post-freezing (%)	Thawing rate (°C) - time (s)	Reference
P. lineatus	Propylene glycol 10% + BTS5%	1:4	64.3 ± 1.6	29.7 ± 21.3	27.71 ± 1.87	40 – 8	Andrade et al. (UD)
	Methanol 10%+ BTS 5%	1:4	74.6 ± 5.6	50.1 ± 6.9	30.57 ± 3.90	40 – 8	
	Glycerol 10%+ BTS 5%	1:4	71.8 ± 3.6	52.2 ± 5.4	15.28 ± 6.90	40 – 8	
	DMSO 10%+ BTS 5%	1:4	52.1 ± 10.2	127.3 ± 47.7	25.85 ± 0.56	40 – 8	
	Ethylene glycol 10%+ BTS 5%	1:4	70.3 ± 2.6	39.7 ± 14.2	25.85 ± 0.56	40 – 8	
	DMSO 8% + egg yolk 5% +BTS 5%	1:5	60.8 ± 24	58.2 ± 49	20.6 ± 6	60 - 8	Felizardo et al. [12]
	DMSO 8% + lactose 5% +BTS 5%	1:5	64.1 ± 26	70.8 ± 53	30.8 ± 10	60 – 8	
	Methanol 8% + egg yolk 5% +BTS 5%	1:5	67.8 ± 25	74.3 ± 59	21.4 ± 6	60 – 8	
	Methanol 8% + lactose 5% +BTS 5%	1:5	58.9 ± 26	75.3 ± 44	27.3 ± 6	60 – 8	
	Methanol 5% + BTS 5%	1:4	63	40	41.3	60 – 8	
	Methanol 7.5% + BTS 5%	1:4	43	43	30.4	60 – 8	Miliorini [30]
	Methanol 10% + BTS 5%	1:4	47	47	25.6	60 – 8	
	Methanol 12.5% + BTS 5%	1:4	53	53	30.2	60 – 8	
	DMSO 5% + BTS 5%	1:4	76	48	31.2	60 – 8	
	DMSO 7.5% + BTS 5%	1:4	78	54	27	60 – 8	
	DMSO 10% + BTS 5%	1:4	85	62	26.1	60 – 8	
	DMSO 12.5% + BTS 5%	1:4	69	83	24.3	60 – 8	
B. orbignyanus	Propylene glycol 10% + BTS 5%	1:4	21.4 ± 9.1	34.4 ± 16.6		60 - 5	Andrade et al. (UD)
	Methanol 10% + BTS 5%	1:4	0	0		60 – 5	
	Glycerol 10%+ BTS 5%	1:4	0	0		60 – 5	
	DMSO 10%+ BTS 5%	1:4	0	0		60 – 5	
	Ethylene glycol 10% + BTS 5%	1:4	13.6 ± 3.5	15.6 ± 3.3		60 – 5	
	DMSO 8% + egg yolk 5% +BTS 5% DMSO	1:5	28	63.2	22.8	60 -8	Felizardo et al. [12]
	8% + lactose 5% +BTS 5%	1:5	28	69.6	18.6	60 – 8	
	Methanol 8% + egg yolk 5% +BTS 5%	1:5	22.5	50.5	26	60 – 8	
	Methanol 8% + lactose 5% +BTS 5%	1:5	30	66.2	23.4	60 - 8	
P. mesopotamicus	Propylene glycol 10%+ BTS 5%	1:4	67.1 ± 12.6	71 ± 5.8		60 -8	Andrade et al. (UD)
	Methanol 10%+ BTS 5%	1:4	61.6 ± 8.8	91.4 ± 20.2		60 -8	
	Glycerol 10%+ BTS 5%	1:4	22.5 ± 18.8	33.9 ± 20.4		60 -8	
	DMSO 10%+ BTS 5%	1:4	52.1 ± 2.1	71.7 ± 6.3		60 -8	
	Ethylene glycol 10% + BTS 5%	1:4	42.5 ± 4.7	46 ± 11.9		60 -8	

*DMSO: dimethyl sulfoxide, BTS: Beltsville Thawing Solution.
UD=unpublished data

Table 1. Cryopreservation protocols and parameters found in various fish species.

http://dx.doi.org/10.5772/58685

The sperm abnormalities can be divided into two groups, the minor or larger ones. Minor abnormalities occur during spermatogenesis due to factors that affect breeding. The largest abnormalities are related to handling procedures during collection [30]. For fish, the acceptable limits of sperm abnormalities have not yet been established, however, during cryopreservation of *P. lineatus* semen abnormalities observed was 20-30%, which was associated with decreased motility rate post-thaw [12].

The exposure of sperm cells to cryoprotectant medium is responsible for causing sperm damage. In addition to osmotic shock, dilution in extenders alters the constitution of seminal plasma, diluting major components such as proteins and antioxidants enzymes. Therefore, when seminal plasma constituents present in suboptimal conditions, may impair the protective function of the spermatozoa and thus the potential for freezability [63].

Although these observed changes in semen quality after cryopreservation, this is a safe method to be used for the preservation of genetic resources, since, after the process of freezing and thawing sperm motility and fertilization capacity can be recovered. However, for the maintain of the sperm in low temperature is necessary to use a suitable cryopreservation protocol which depends on the quantity and quality of the extender, type and concentration of internal cryoprotectants and the sample volume and sperm cell characteristics [64].

8. Semen vitrification

Vitrification is a process of ultra-fast freezing characterized by the use of high concentrations of cryoprotectant (40-60%) and high cooling rates (up to 1000 ° C min-1) by immersion of semen directly into liquid nitrogen. This method inhibits the formation of hexagonal ice crystals and induces a glassy state, which differs from traditional methods in which the permeability of cryoprotectants and dehydration occur before starting the freezing [65].

A number of advantages of ultra-fast freezing can be listed, among them, does not require expensive equipment, is a quick and simple technique allows preservation of samples in the field. Despite these advantages, the addition of cryoprotectants in high concentrations can be toxic and cause osmotic damage sperm cell, being necessary find alternatives that overlap this limitation.

The possibility to obtaining a glassy state result in improved survival of sperm is the main starting point for using the vitrification technique [66]. Although it represents a viable alternative to semen preservation of fish native species of Brazil, there are no reports on the use of this technique to date.

In conclusion we believe that there are protocols defined for the different stages of cryopreservation in different species of native fish in Brazil. Moreover, vitrification semen offers a new option for conservation of native species. However, it is still necessary to improve the studies in this area, to find favorable results.

Acknowledgements

The authors would like to thank FAPEMIG (Fundação de Amparo à Pesquisa do Estado de Minas Gerais), CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior) and CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico) for funding this research.

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Chapter 5

Cryopreservation of Cattle, Pig, Inobuta Sperm and Oocyte after the Fukushima Nuclear Plant Accident

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Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/58629

1. Introduction

After the Great East Japan Earthquake on 11 March 2011, the Fukushima Daiichi Nuclear Power Plant (FNPP) accident led to a discharge of a tremendous amount of radioactive substances [1, 2]. On 22 April 2011, the evacuation zone was set to a 20-km radius surrounding the FNPP, leaving approximately 3,400 cows, 31,500 pigs, and 630,000 chickens behind within the zone. On 12 May 2011, the Government of Japan ordered Fukushima prefectural government to euthanize unleashed livestock within the evacuation zone. Abandoned animals now have formed an invaluable model for studying the effects of chronic radionuclide intake. A comprehensive assessment of the effect of long-term exposure to internally deposited radionuclides on surviving domestic animals in the evacuation area is therefore urgently needed for the benefit of the livestock industry, as well as for human health. Radiobiological data from the FNPP accident could help to develop a set of internationally harmonized measures to protect domestic animals in the event of a future nuclear or radiological emergency.

Exposure to a large dose of ionizing radiation can cause irreparable damages to multiple organ systems, particularly those with highly proliferative cells, such as the skin, the hematopoietic and gastrointestinal system [3]. The testis and ovary are relatively radiosensitive organs [4], composed of a series of spermatogenic cells such as stem cells, spermatogonia, spermatids,

spermatocytes, sperm, and oogonium, primary oocyte, secondery oocyte and ovum, respectively. These different types of germ cells differ remarkably in their susceptibility to radiation-induced effects according to their level of reproductive activity [5]. The effect on reproductive organs and behaviour by chronic exposure to radionuclides is one of major concerns. Furthermore, radiation-induced genomic changes, occurring in germ cells may have hereditary effects, including carcinogenesis, congenital malformation and growth retardation in offsprings. A germ cell is the only cell that can produce next-generation. Therefore, greater use of cryopreservation of germ cells provide an essential resource to preserve their genetics and foetuses obtained by fertilization using those of the freezing sperm and oocytes for further studies on the effect of ionizing radiation on the next generations.

We have collected and cryopreserved the sperm and oocytes from three spices of domestic animals in the FNPP evacuation zone between 27 September 2011 and 31 March 2013. In this chapter, we introduce approaches to cryopreserve germ cells from the cattle, the pig, and the inobuta which is a mongrel of the wild boar and the pig for further research of radiobiology.

2. Reporting studies of Chernobyl for domestic animals and human

Data used for estimating the risk associated with exposure to ionizing radiation have been primarily obtained from epidemiological studies of survivors of the atomic bombing of Hiroshima and Nagasaki [6], the Chernobyl nuclear accident [7], and some complementary animal experiments [8–10]. However, reports of the effect of chronic low-dose radiation on livestock animals are limited.

Direct radiation injury to animals was reported only in local areas within the 30-km exclusion zone in Chernobyl nuclear power plant [11]. In some cases, chronic dose rates may have reduced the fertility of some animal species inside the zone. Recently, review of Russian language studies on radionuclide behavior in agricultural animals has been published [12, 13]. There are several important animal pathways for radionuclide transfer to the diet of humans. The important for many contamination scenarios for radiologically important radionuclides (^{90}Sr, ^{131}I and ^{137}Cs) is muscle (meat) consumption. The information presented this review has reported values are for Cs due to the Chernobyl accident in cattle, sheep, goat, rabbits, and chicken.

On the other hand, irradiation damages the ovaries and testes as direct effects of radiation, and indirectly affect through hormonal disruption. In human and wild animals, several studies of the functional changes in the reproductive tract have been made as a result of Chernobyl accident, abnormalities in spermatozoa and reproductive failures have been described [7]. Additionally, Weinberg et al. reported some genetic changes of unclear importance in offspring of Chernobyl accident liquidators [14, 15]. Although there are some claim that its changes is caused by the psychological factors (stressful conditions), it does not yet have enough information to explain all of the serious changes.

http://dx.doi.org/10.5772/58629

3. Cryopreservation of cattle, pig, inobuta sperm

The Japanese government ordered Fukushima prefecture to euthanize cattle in the evacuation zone on 12 May 2011 to prevent radio-contaminated livestock products from entering the human food chain. We obtained testes and ovaries from the euthanized cattle, pigs and inobutas collected by the combined unit of veterinary doctors belonging to the Livestock Hygiene Service Center of Fukushima prefecture.

Almost bulls and boars were castrated. Therefore, we could only collect testes from 11 euthanized Japanese black beef bulls, 3 boars, and 1 male inobuta between 29 August 2011 and 28 February 2013 (Figure 1). Testes were collected in Kawauchi village located 15 km southwest of FNPP: the air dose rate was 0.5 μSv/h, Naraha town located 17 km south of FNPP: the air dose rate was 2 μSv/h, Tomioka town located 7 km south of FNPP: the air dose rate was 20 μSv/h.

Figure 1. Animals in the evacuation zone of the Fukushima Daiichi Nuclear Plant. A: Japanease black beef cattle B: Pig C: Inobuta

In bull, sperm from two caudae epididymides were collected. Immediately after collection, sperm were diluted with a Triladyl freezing extender containing egg yolk at natural temperature (Mini Tube, Germany). The tubes containing sperm were transferred to the Niigata University within 6-8 h after collection. Semen samples were cooled to 5-10°C during transferring. Aliquots of 0.5 ml of sperm suspension were individually placed in straws and ends were sealed. The straws were then placed in liquid nitrogen vapor for 10 min and then plunged directly into liquid nitrogen. In boars and male inobuta, freezing protocol was performed as described above. The semen extender, Modena extender containing egg yolk was used for freezing epididymal sperm. Total number of frozen sperm was 507 straws from bulls, 160 straws from boars and 83 straws from inobutas (Table 1).

Animal	Number of animals	Number of cryopreserved sperm
Bull	11	507
Boar	3	160
Inobuta	1	83

Table 1. Total number of cryopreserved straws contained sperm from bulls, boars, and male inobuta in the evacuation zone of the Fukushima Daiichi Nuclear Plant.

4. Cryopreservation of cattle, pig, inobuta oocytes

Oogenesis is associated closely with folliculogenesis in mammals. Oogenesis begins in the fetal ovary when the primordial germ cells arrive in the gonad of a genetic female and become oogonia. These cells proliferate via mitosis during fetal development. When proliferation ceases and the cells enter meiosis either before birth (human, cows, sow) or shortly thereafter (mice, rats, hamster) [16, 17], they are defined as primary oocytes arrested in the first meiotic prophase. Primordial follicles are formed in which the primary oocytes are surrounded by single layer of flattened granulose cells. Although the primordial follicles remain in this state of suspended animation for long time, oocytes and follicle resume the development near the time of ovulation. When follicles enter the development phase, they develop into primary and subsequent secondly follicles, along with proliferation of granulose cells and the oocytes growth. Primary and secondly follicle have cuboidal single layer and multiple layers of granulose cells, respectively. During the next phase, a fluid-filled cavity is formed adjacent to the oocyte in the follicle defined as antral follicle. Finally, one follicle growth rapidly and become the ovulatory follicle (maturation). In most mammals, the oocytes resume and complete the first meiotic division at ovulation.

Ionizing radiation may affect infertility or genetic disorders in subsequent generation induced by DNA damage in the germ cells and follicular cells. Many experiments have shown that radiosensitivity of follicle/oocyte varies widely according to the developmental stage of them and species [18]. In mice, the genetic sensitivity of oocyte in early stages of follicle development is a relatively high, and which decreases during the last week before ovulation. However, the sensitivity increases around the time of ovulation again. In contrast, oocytes in primordial follicle show a very low genetic sensitivity, and which increase with follicle development thereafter in guinea pigs. However, the knowledge in livestock is limited. In order to reveal the affect of exposure to low-dose of radiation on germ cells, it is necessary to study carefully at long term, including the influences on subsequent generations.

The aim of our studies is to examine of development of female germ cells in livestock within the evacuation zone and to preserve of female gametes for future studies. We collected ovaries from 36 cows, 12 sows, and 2 female inobutas. In cows, collected ovaries were washed and stored at 20°C in physiological saline containing antibiotics, and were transported to the laboratory within 7 h after the collection. Cattle cumulus-oocyte complexes (COCs) were

aspirated from small ovarian follicles and incubated in *in vitro* maturation (IVM) medium for 22 h. After maturation, the COCs were fertilized by co-incubation with thawed sperm. Then presumptive zygotes were cultured for *in vitro* development to the blastocyst stage. As a result, total 493 of morphologically normal COCs were recovered, and 40 blastocysts were yielded from 9 donors following *in vitro* embryo production. The bovine blastocysts produced were cryopreserved by vitrification using a nylon mesh method described in the previous report [19] (Table 2). On the other hand, pig and inobuta ovaries were transported to the laboratory at 37°C. Pig and inobuta COCs aspirated were carried out IVM for 44 h, and the presumptive matured oocytes were vitrified by similar method above. Total number of vitrified oocytes was 371 from sows and 64 from inobutas.

Animal	Number of animals	Developmental stage for cryopreservation	Number of cryopreserved egg
Caw	9	Blastocyst	40
Sow	12	Metaphase II	371
Inobuta	2	Metaphase II	64

Table 2. Total number of cryopresearved oocytes and embryos from caws, sows, and female inobutas in the evacuation zone of the Fukushima Daiichi Nuclear Plant.

5. Concluding remarks

Germ cells in the testis show one of the highest mitotic activities of any tissue in the body, so that in the human adult about 100 million new cells are produced each day [20]. Spermatogenesis is highly regulated, starting with spermatogonial stem cells and ending with differentiated, motile spermatozoa. The testis is one of the most radiosensitive tissues, with very low doses of radiation causing significant impairment of function. It is well known that immature cells are more radiosensitive to doses as low as 0.1 Gy, causing morphological and quantitative changes to the spermatogonia in human testis. Doses of 2–3 Gy result in overt damage to spermatocytes, leading to a reduction in the spermatid numbers. At doses of 4–6 Gy, the numbers of spermatozoa significantly decrease, implying damage to the spermatids. A recent study in mice showed that low-dose-rate radiation exposure (3.49 mGy/h) did not cause adverse effects at dose levels of ≤2 Gy, but the testis weight, sperm count and motility decreased at a dose of 2 Gy [21].

We needed to overcome a number of obstacles while working in the evacuation area, that is, dissections under the sun in the summer and snow in the winter, gathering the dissectors, as well as drive 400 miles a day. We also had restrictions on the time that we were allowed to stay in the area and the radiocontaminated materials to bring out of the area. We recently reported radionuclide deposition in organs of abandoned cattle following the FNPP accident. The deposition occurred in an individual radionuclide and in an organ-specific manner, and

radioactive Cs was detected in all the organs examined [22]. Discharge of ^{134}Cs and ^{137}Cs that emit γ-and β-rays is of primary concern, because they were released in a large amount and have a long half-lives [23]. Furthermore, we have investigated the effect of chronic radiation exposure on bull testes to ^{134}Cs and ^{137}Cs associated with the FNPP accident. Adverse radiation-induced effects, so far, have not been observed in bull testes following chronic exposure to the above levels of radiation for up to 10 months [24].

The paternal and maternal genomes are not equivalent and both are required for mammalian development. The difference between the parental genomes is believed to be due to gamete-specific differential modification, a process known as genomic imprinting, suggesting that DNA methylation may play a role in genomic imprinting. Lie et al, have examined the expression of these three imprinted genes in mutant mice that are deficient in DNA methyl-transferase activity [25]. Results demonstrate that a normal level of DNA methylation is required for controlling differential expression of the paternal and maternal alleles of imprinted genes. Few animal studies have investigated the possible link between paternal exposures and effects on genomic imprinting. Chronic treatment of male rats with 5-azacytidine, a drug that alters DNA methylation resulted in abnormalities in male germ cells and early embryo development but no increase in the incidence of congenital malformations [26]. This is an important area with potential consequences for the offspring of exposed males, and warrants further study [27].

In addition, questions regarding the effect of log-term exposure to radiation on the genetic damage to next generation are now being raised, but no clear evidence for this has been reported, except laboratory animals. Genetic analysis of foetuses obtained by fertilization using cryopreserved sperm and oocytes from cattle in the evacuation zone, is underway in our laboratory.

In conclusion, cryopreservation of germ cells has potential applications not only for production of next generations of animals but also for general reproductive biology including the field of radiation biology.

Acknowledgements

We express our gratitude to the Iwaki Livestock Hygiene Service Centre in Fukushima Prefecture, especially to DVM. Yuji Kobayashi and livestock farmers in the 20-km FNPP evacuation zone. This work was partly supported by a grant for Manabu Fukumoto of the Japan Society for the Promotion of Science, and the Emergency Budget for the Reconstruction of Northeastern Japan, MEXT, Japan, Discretionary Expense of the President of Tohoku University and Nippon Life Insurance Foundation supported this study. This work was also supported by the Programme for Promotion of Basic and Applied Researches for Innovations in Bio-oriented Industry.

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Chapter 6

The Maining of Cryopreservation for in-vitro Fertilization Patients

Enrique Criado Scholz and Cristina González Navas

Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/58746

1. Introduction

The necessity to cryopreserve certain tissues and cell types for their use in assisted reproduction has allowed technique developments that improve the quality of treatments and help both professionals and patients to perform these techniques. In this chapter we will talk about the importance that cryopreservation has meant to assisted reproduction techniques and about the benefit for patients of the advance and improvement of different cryopreservation techniques, for example in the recent and increasingly demanded technique Egg Vitrification, to preserve female fertility.

From the cryopreservation point of view sperm, oocytes, embryos and ovarian tissue will be analyzed, by reviewing how different cryopreservation techniques have evolved up to reaching the techniques used nowadays giving, furthermore, a vision of how they will be in the future to optimize even further, the procedure increasing survival rates and viability of gametes, embryos and ovarian tissue to 100%.

The Development of cryopreservation techniques, the increase in demand for cryopreserved cells or tissues and the use of these techniques in cells or tissues from patients with infectious diseases, has forced us to reduce the risk of contamination during the freezing process and the risk of cross-contamination during the storage of this material. Recent publications that demonstrate the survival of pathogens at low temperatures and possible contamination of the cells or tissues stored have changed the laws of each country and the customs and protocols used so far in the cryopreservation.

To understand the problem of contamination in cryopreservation we need to have an overview of the current problem in which all researchers are concerned about, seeking a cryopreservation protocol with good results but without contamination problems. Discussing the cryopre-

servation's different techniques such as slow freezing, vitrification, kinetic vitrification (extra-, hyper-, super-, ultra-fast vitrification) and the various components that help us understand the difficult balance between technique, device used and the risk of contamination.

The device used, the protocol used and the cooling solution used can change the outcome of cryopreservation and therefore we have to find a protocol for cryopreservation with a cooling solution and a secure device to provide us good results free of contamination.

2. Slow freezing and Vitrification

Two are the most utilized methods for gamete cryopreservation: slow freezing and

vitrification. Slow freezing uses low concentrations of cryoprotectants which are associated with chemical toxicity and osmotic shock. Vitrification is a rapid method that decreases cold shock, without the risks of solution effects or crystallization, and uses high cooling rates in combination with a high concentration of cryoprotectant [1].

Slow freezing is a conventional cryopreservation process in which a relatively low concentratiton of cryoprotectant is used (1.5 M), it shows little toxicity to cells or tissue and requires expensive equipment. As the cryoprotectant is added to cells, it results in initial cellular dehydration followed by a return to isotonic volume with the permeation of cryoprotctant and water. Generally, cells are cooled slowly using a controlled rate freezing machine, which allows samples to be cooled at various rates; ovarian tissue is generally cryopreserved at 2° C/min prior to ice seeding and 0.3° C/min after crystallization to ensure the tissue is dehydrated before intracellular ice formation occurs. Optimal rates to minimize intracellular rates formation vary among cells and tissue types [2].

It is generally believed that cell injury at low cooling rates is principally due to the concentration of both intracellular and extracellular electrolytes and that cryoprotectants act by reducing this build-up. Experimental data support this explanation, in fact the extent of damage to human red blood cells during freezing in solutions of sodium chloride/glycerol/water can be quantitatively accounted for by the increase in solute concentration. Furthermore, a given degree of damage occurs at lower concentrations of solute in the presence of higher concentrations of glycerol; it appears that glycerol contributes as element of damage itself [3].

Although initially reported in 1985 as a successful cryopreservation approach for mouse embryos, vitrification has taken a backseat in human assisted reproduction. However, the practical advantages of this cryopreservation method have more recently caught the attention of many ART laboratories as a feasible alternative to traditional slow freezing methods. Since 1985 more than 2,100 publications can be found referring to the topic of "vitrification", which is further evidence of the burgeoning growth of interest in this cryopreservation technology. One "drawback" considered by embryologists who are not familiar with the vitrification technique, is the use of higher concentration of cryoprotectants, which does potentially mean that the vitrification solutions are more toxic than their counterpart solutions used for conventional slow freezing. However, with better understanding of the physical and biological

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principles of vitrification this has lead to numerous successful clinical applications of this technique within the field of assisted reproduction. As of today, all developmental stages of human embryos cultured in vitro have been successfully vitrified and warmed, with resulting offspring. Today, slow freezing technology still has the longest clinical track record, and greater 'comfort level' amongst embryologists. Nevertheless, vitrification with its increasing clinical application is showing a trend of greater consistency and better outcomes when compared to slow freezing technology. Therefore, when (not if) IVF programs overcome the fear of the 'unknown', and take on the challenge of the short learning curve with vitrification, then at that point vitrification will become the clinical standard for human embryo cryopreservation.

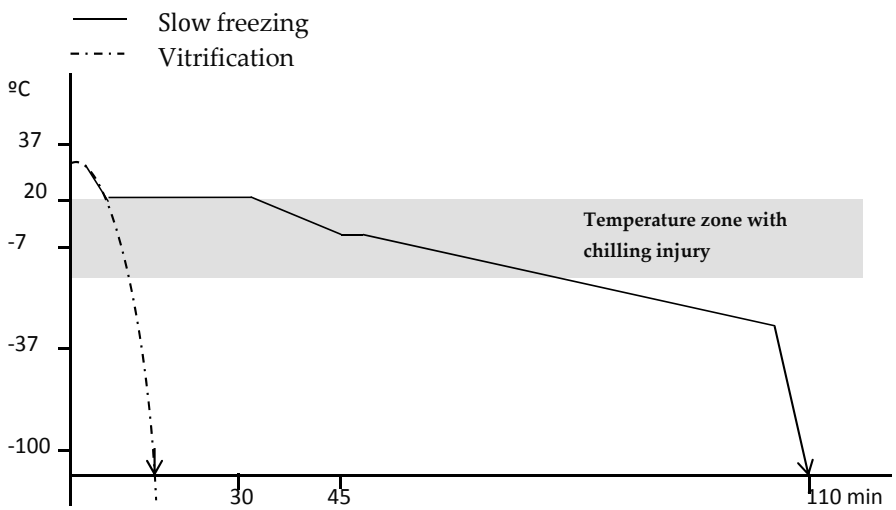

Figure 1. Cooling rates during slow freezing and vitrification. Note the relative duration of exposure of oocytes / embryos temperatures in the risk of chilling injury of each of the procedures [4].

Cryopreservation at low temperature slows or totally prevents unwanted physical and chemical change. The major disadvantage to using low temperature cryostorage is that it can lead to the crystallization of water, and thereby this approach can create new and unwanted physical and chemical events that may injure the cells that are being preserved. Although the results achieved by slow freezing in many cases seem quite successful [5, 6], ice crystal formation still renders traditional slow-freezing programs generally less consistent in their clinical outcomes. Another downside to the slow freezing approach is the time to complete such freezing procedures for human embryos, which can range from 1.5 to 5hrs. This is due to the fact that the slow rate of cooling attempts to maintain a very delicate balance between multiple factors that may result in cellular damage by ice crystallization and osmotic toxicity. Traditionally slow-freeze embryo cryopreservation has been a positive contributor to cumulative patient pregnancy rates, but ultimately the limitations of current slow-rate freezing methods in ART have become more evident in the shootout with vitrification-based cryostorage.

Vitrification is one of the more exciting developments in ART in recent years that attempts to avoid ice formation altogether during the cooling process by establishing a glassy or vitreous state rather than an ice crystalline state, wherein molecular translational motions are arrested without structural reorganisation of the liquid in which the reproductive cells are suspended. To achieve this glass-like solidification of living cells for cryostorage, high cooling rates in combination with high concentrations of cryoprotectants are used. A primary strategy for vitrifying cells and tissue is to increase the speed of thermal conductivity, while decreasing the concentration of the vitrificants to reduce their potential toxicity. There are two main ways to achieve the vitrification of water inside cells efficiently: a) to increase the cooling rate by using special carriers that allow very small volume sizes containing the cells to be very rapidly cooled; and b) to find materials with rapid heat transfer. However, one has to take into account that every cell seems to require its own optimal cooling rate, e.g., mature unfertilized oocytes are much more sensitive to chilling injury than any of the cell stages of the pre-implantation embryo. The earliest attempts using vitrification as an ice-free cryopreservation method for embryos were first reported in 1985 [7]. In 1993 successful vitrification of mouse embryos was demonstrated [8]. Furthermore, bovine oocytes and cleavage-stages were vitrified and warmed successfully a few years later [9]. In 1999 and 2000 successful pregnancies and deliveries after vitrification and warming of human oocytes were reported [10, 11]. Since that time, and because it seems to be that both entities appear to be especially chill-sensitive cells in ART, oocytes and blastocysts seem to receive a potentially significant boost in survival rates by avoiding ice crystallization using vitrification [12]. In general, vitrification solutions are aqueous cryoprotectant solutions that do not freeze when cooled at high cooling rates to very low temperature. Interest in vitrification has clearly risen as evinced by the almost exponential growth of scientific publications about vitrification. Vitrification is very simple, requires no expensive programmable freezing equipment, and relies especially on the placement of the embryo in a very small volume of vitrification medium (refered also as "minimal volume approach") that must be cooled at extreme rates not obtainable in traditional enclosed cryostorage devices such as straws and vials. The importance of the use of a small volume, also referred to "minimal volume approach" was described and published in 2005 [13, 14]. In general, the rate of cooling/warming and the concentration of the cryoprotectant required to achieve vitrification are in inversely related. In addition, recent publications have shown the dominance of warming rate over cooling rates in the survival of oocytes subjected to a vitrification procedure [15, 16].

During vitrification, by using a cooling rate in the range of 2,500 to 30,000°C/min or greater, water is transformed directly from the liquid phase to a glassy vitrified state. The physical definition of vitrification is the solidification of a solution at low temperature, not by icecrystallization but by extreme elevation in viscosity during cooling [17, 18]. Vitrification of the aqueous solution inside cells can be achieved by increasing the speed of temperature change, and by increasing the concentration of the cryoprotectant used. However, a major potential drawback of vitrification is the use of high concentration of cryoprotectant, and an unintentional negative impact of these cryoprotectants in turn can be their toxicity, which may affect the embryo and subsequent development in utero. It is therefore essential to achieve a fine balance between the speed of cooling and the concentration of the vitrifying cryoprotectants.

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This is necessitated by the practical limit for the rate of cooling, and the biological limit of tolerance of the cells for the concentration of toxic cryoprotectants being used to achieve the cryopreserved state. It is important to note that recently published papers [19, 20, 21, 22] have shown that the use of relatively high concentration of cryoprotectants such as 15% (vol/vol) ethylene glycol (EG) used in an equimolar mixture with dimethyl sulphoxide (DMSO) had no negative effect on the perinatal outcomes from blastocyst transfers following vitrification when compared with those from fresh blastocyst transfers.

Vitrification in principle is a simple technology, that is potentially faster to apply, and relatively inexpensive; furthermore, it is becoming clinically established, and is seemingly more reliable and consistent than conventional cryopreservation when carried out appropriately [23, 24].

Cryoprotectant agents are essential for the cryopreservation of cells. Basically two groups of cryoprotectants exist: 1) permeating (glycerol, ethylene glycol, dimethyl sulphoxide); and 2) nonpermeating (saccharides, protein, polymers) agents. The essential component of a vitrification solution is the permeating agent. These compounds are hydrophilic non-electrolytes with a strong dehydrating effect. Furthermore, these CPAs are able to depress the "freezing point" of the solution. Regarding the high concentration of cryoprotectant used for vitrification, and in view of the known biological and physiochemical effects of cryoprotectants, it is suggested that the toxicity of these agents is a key limiting factor in cryobiology. Not only does this toxicity prevent the use of fully protective levels of these additives, but it may also be manifested in the form of cryo-injury above and beyond that seen occurring due to classical causes of cell damage (osmotic toxicity and ice formation) during cryopreservation. In spite of this, the permeating CPA should be chosen firstly by their permeating property, and secondly on the basis of their potential toxicity. Because the permeating CPA is responsible for the toxicity (the key limiting factor in cryobiology), different cryoprotectants have been tested for their relative toxicity, and the results indicate that ethylene glycol (EG; MW 62.02) is the least toxic followed by glycerol. Additionally, these highly permeating cryoprotectants are also more likely to diffuse out of the cells rapidly and the cells regained their original volume more quickly upon warming, thus preventing osmotic injury. Therefore, the most common and accepted cryoprotectant for vitrification procedures is ethylene glycol (EG). Today EG is more commonly used in an equimolar mixture with DMSO. Often additives are added to the vitrification solution such as disaccharides. Disaccharides, for example sucrose, do not penetrate the cell membrane, but they help to draw out more water from cells by osmosis, and therefore lessen the exposure time of the cells to the toxic effects of the cryoprotectants. The non-permeating sucrose also acts as an osmotic buffer to reduce the osmotic shock that might otherwise result from the dilution of the cryoprotectant after cryostorage. In addition, permeating agents are able to compound with intracellular water and therefore water is very slowly removed from the cell. Hence the critical intracellular salt concentration is reached at a lower temperature. Removal of the cryoprotectant agent during warming can present a very real problem in terms of trying to reduce toxicity to the cells. Firstly, because of the toxicity of the vitrification solutions, quick dilution of them after warming is necessary; and secondly, during dilution water permeates more rapidly in to the cell than the cryoprotective additive diffuses out. As a consequence of the excess water inflow the cells are threatened by injury

from osmotic swelling. In this situation the non-permeating sucrose acts as an osmotic buffer to reduce the osmotic shock. During warming using a high extracellular concentration of sucrose (e.g., 1.0M) counterbalances the high concentration of the cryoprotectant agents in the cell, as it reduces the difference in osmolarity between the intra-and extracellular compartments. The high sucrose concentration cannot totally prevent the cell from swelling, but it can reduce the speed and magnitude of swelling [25, 26, 27].

3. Sperm

Human spermatozoa can be successfully cryopreserved and utilized. Cryopreservation now plays an essential role in fertility preservation under the following scenarios:

- couples undergoing infertility treatment.
- cancer patients undergoing gonadotoxic chemotherapy or radiation.
- patients undergoing certain types of pelvic or testicular surgeries
- patients suffering from degenerative illnesses such as diabetes or multiple sclerosis; spinal cord disease or injury.
- men undergoing surgical sterilization such as vasectomy
- screening and quarantine of donor semen samples

Many advances in reproductive medicine in the past five decades have made cryopreservation of human spermatozoa an invaluable tool for the clinical management of infertility and sperm banking. The advent of in vitro fertilization (IVF) and intracytoplasmic sperm injection (ICSI) with microsurgical sperm handling techniques along with advances in female gamete acquisition have resulted in an increased demand for the cryopreservation of semen and tissue samples, often containing a very limited number of spermatozoa. Sperm cryopreservation also makes it possible for cancer patients to preserve their fertility prior to gonadotoxic chemotherapy or radiation. Applications of sperm banking are not limited to cancer patients but extend to patients undergoing certain types of pelvic or testicular surgeries; those who suffer from degenerative illnesses such as diabetes or multiple sclerosis; spinal cord disease or injury; and persons in occupations where a significant risk of gonadotoxicity prevails. Sperm cryopreservation is also available to men undergoing surgical sterilization such as vasectomy, in the event that children may be desired in the future. Another use for semen cryopreservation is to allow donor semen samples to be quarantined while appropriate screening is performed to prevent the transmission of infectious pathogens during therapeutic donor insemination (TDI) [28].

In cases of severe male infertility, single or lesbian women, the use of donor sperm is the only approach to address fertility issues [29, 30]. Advances in sperm cryopreservation have created opportunities for many families to achieve pregnancies through therapeutic donor insemination or IVF with donor sperm.

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At present, some 30,000 births per year worldwide are attributable to frozen donor sperm inseminations [31].

Although major improvements have been made in sperm cryopreservation, there are many unresolved technical issues. Since freezing protocols differ between types of cells, the ideal conditions for human sperm freezing and thawing need to be perfected. To add more complexity, samples with abnormal semen parameters, such as severe oligospermia or high seminal fluid viscosity, often require unique cryopreservation conditions. For example, the particular cryoprotectants can affect cooling rates. In addition, storage temperature can significantly influence cryopreservation outcome. Liquid nitrogen (LN2) can offer long-term survival of spermatozoa due to essentially absent metabolic activity, such as chemical reactions, genetic modification or aging of cells [32]. A conventional slow freezing protocol has been in use for many years and very little has changed in terms of methodology and reagents. While freezing aims to preserve cells it can also easily destroy them if certain precautionary steps are not taken into consideration. During cryopreservation cells and tissue undergo dramatic transformation in chemical and physical characteristics as the temperature drops from+37 to-196°C. The cells can lose up to 95% of their intracellular water. The concentration of solutes increases considerably, triggering the possibility of osmotic shock. Moreover, potential intracellular ice crystallization and mechanical deformation by extracellular ice may cause significant injury leading to cell death. Furthermore, if cells survive freezing, they might sustain additional damage during the thawing process due to osmotic shock, uncontrollable swelling and ice re-crystallization [33].

Remarkably, the first reference of empirical sperm freezing dates as far back as the late 16th century, but it was only with the discovery in 1937 by Bernstein and Petropavlovski that glycerol can aid spermatozoa in surviving long term freezing, that sperm cryopreservation became practical. Expansion of artificial insemination for the dairy industry led to further important research in the field of cryobiology [34]. Shortly after these practices were initiated with animals, the first pregnancies were reported in humans after insemination with frozen spermatozoa.

The next milestone was the discovery of the possibility to store human spermatozoa in LN2at-196°C, resulting in superior recovery rates compared to storage at higher temperatures between-20 and-75°C. After the era of empirical freezing; cryobiology matured to its fundamental stage, focusing on the biophysical and biochemical principals of cryopreservation, further advancing the field [35]. A comprehensive review of the historical background of sperm freezing was recently published and is recommended for readers looking for more details [36].

3.1. Cryopreservation of epididymal and testicular spermatozoa

Couples with male factor infertility represent 30 to 40% of the infertile population. Azoospermia accounts for 10% of cases of confirmed male infertility, and often requires surgical retrieval of spermatozoa. Since the introduction of ICSI, many cases of severe male infertility can now be successfully treated. Cryopreservation of surgically retrieved spermatozoa is a valuable component in the effective management of male infertility, reducing the necessity of repeat surgeries. Diagnostic sperm retrieval prior to IVF has several benefits including the possibility

of freezing spermatozoa for future use, or if none are retrieved, initiation of the IVF stimulation cycle can be postponed or avoided. Testicular spermatozoa have been utilized to achieve pregnancy in couples with severe male factor infertility, with reported pregnancy rates similar to ejaculated spermatozoa, according to a meta-analysis study [37]. In the case of obstructive azoospermia, recovery of spermatozoa by aspirations varies from 45 to 97% [38, 39]. In cases of non obstructive azoospermia recovery depends on the degree of testicular pathology and varies from 0 to 64% [40, 41]. A second or third surgery can increase the chance of complications including hematomas, inflammation, testicular devascularization, fibrosis and permanent testicular damage [42]. To avoid this, if pregnancy is not achieved during the first ICSI attempt, a repeat of the surgical procedure would not be required if a portion of the surgical specimen has been banked. Cryopreservation of surgically retrieved spermatozoa can also aid the coordination of oocyte retrieval and avoids the pressure of having the urologist available on the day of the ICSI procedure. Usually the number of spermatozoa obtained during a surgical procedure is limited, and in the case of testicular sperm they may not be fully matured. In the future, if no mature spermatozoa are recovered, spermatogonial stem cells or early germs cells could potentially be matured in vitro and used for fertility treatments [43].

There are significant technical challenges for successful cryopreservation of testicular tissue due to its complex structure and intracellular interactions. Different cells of testicular tissue will have dissimilar responses to cryopreservation and require different concentration of CPAs. Freezing larger pieces of tissue is not advisable as it would increase resistance of heat transfer and penetration of CPAs leading to variation in cooling rates within different parts of the tissue. In addition, seminiferous tubules capture liquid and increase chances of ice formation [33]. To avoid these difficulties, cryopreservation of smaller tissue fragments or mincing tissue prior to freezing has been advocated [44].

3.2. Cryopreservation in oncological patients

Quite often in clinical practice, the long term effects of cancer therapy on a patients' ability to have children in the future is not adequately addressed [45]. While the priority is to eliminate the cancer and save their life, fertility preservation especially among adolescent or young adults to ensure the potential of procreation with their own gametes after treatment, needs to be considered. Impaired spermatogenesis has been demonstrated before treatment in some patients with malignancies, depending on their location (eg. testicular cancer) or type (eg.Hodgkin's lymphoma) [46]. Current treatment options such as surgery, chemotherapy and/or radiation can impair spermatogenesis and sexual function and lead to temporary or permanent infertility [47].

The scale of negative effects of cancer treatment on spermatogenesis depend on the specific gonadotoxicity of administered chemotherapeutic agents, number of chemotherapy treatment cycles, radiotherapy field location and dosage, type and stage of the cancer, and age of the patient. Considering combination cancer therapy, uncertainty in individual esponse to treatment and the large number of confounding variables, it becomes very challenging to assess the risk of iatrogenic infertility in many patients. The ability of cancer survivors to have their own biological offspring is very important for many oncology patients, especially at

http://dx.doi.org/10.5772/58746

younger ages [48]. Advances in early diagnostic investigation and treatments have led to increasing numbers of young cancer survivors.

Cryopreservation of semen has changed the reproductive prospects for young patients diagnosed with cancer. Unfortunately, banking services continue to be underutilized since cancer patients and their families are not always informed about the potential fertility risks associated with cancer treatments, or the availability of banking. According to some surveys, less than 20% of patients undergoing chemotherapy or radiation treatment are informed about the adverse effects of such treatment on spermatogenesis or are offered sperm banking for fertility preservation. Cancer patients are usually under huge physiological and time pressure to make cryopreservation decisions while dealing with a life threatening situation. To complicate matters, some young patients are unable to produce semen samples by masturbation. In such cases, PVS or electro-ejaculation under general anaesthetic might be required. Surgical retrieval of testicular tissue may be an option for prepubertal boys who are not capable of producing mature sperm. Testicular tissue cryopreservation has been reported in boys with cryptorchidism to preserve fertility [49]. Cryopreserved testicular tissues can be autografted to restore reproductive functions; however recurrence of neoplastic process is a concern in oncology patients and such procedures are still considered to be experimental [43]. A multidisciplinary team approach is important to ensure that patients have the opportunity to preserve their fertility potential if they elect to do so.

The posthumous use of semen is an entirely separate and complex ethico-legal subject. The ethical and legal aspects of posthumous assisted reproduction have been recently addressed by the European Society of Human Reproduction and Embryology Task Force on Ethics and Law [50].

4. Oocytes

The cryopreservation of human oocytes constitutes an important step forward in Assisted Reproductive Technology (ART) despite the fact that for more than 2 decades oocyte cryopreservation has long been the focus of unsuccessful efforts to perfect its clinical application. More recently, vitrification as an alternative to traditional slow freezing protocols has been shown to provide high degrees of success in vitrified metaphase-II human oocytes. Although oocyte cryopreservation historically has low efficiency mainly because of low rates of survival, fertilization, and cleavage, data on ~ 2000 "frozen oocyte" babies born worldwide since 1986 exists. The question arises as to what makes oocytes so unique compared to embryos, besides differences in cell size and membrane permeability? Oocytes have a low volume-to surface ratio; hence they are less efficient at taking up cryoprotectant and at loosing water. Other differences to be considered are a) that the maternal DNA is held suspended in the cytoplasm on the meiotic spindle & not within the protective confines of the nuclear membrane, therefore damage in the DNA and microtubules could explain the limited success of oocytes, b) the oocyte is arrested in a state primed for activation, and c) the changes in its environment can cause parthenogenetic activation. What are the applications then for oocyte cryopreservation

in the US? One application would be to preserve fertility in women with malignant/premalignant conditions who would have to undergo treatment that might negatively impact their future ability to have children (50,000 per year <40 yr old), also in women who may want to delay childbearing ('clock-tickers') because of their careers, partnership status or psychological/ emotional reasons. A very interesting approach is donor oocyte banking, which makes the donor-recipient cycle more convenient by facilitating the "egg donation" and allows quarantining of the oocytes, which provides a unique advantage in economy as well as feasibility. Other applications are if a male is unable to produce a semen sample on the day of egg retrieval and or it could also eliminate ethical/moral questions of producing extra embryos. Overall, oocyte cryostorage offers an opportunity to reduce number of embryos generated per IVF cycle, and therefore lessening the pressure on the patient to increase the number of fresh embryos transferred. In addition, while also reducing embryo cryostorage it has the benefit of helping women "retain ownership" of their ability to be genetic parents at a time of their choosing, a time of greater convenience & health. The live born babies from cryopreserved oocytes have shown no apparent increase in congenital anomalies. Although 13 years later after the first slow-freeze birth, the number of reported babies born as a result of vitrified oocytes is now approaching that of slow-frozen oocytes without any increasing risk in congenital abnormalities [51]. Vitrification of oocytes does not appear to increase risks of abnormal imprinting or disturbances in spindle formation or chromosome segregation [52]. It has the greatest potential for successful oocyte cryopreservation and with its increased clinical application is showing a trend to greater consistency and better outcomes (similar to outcomes between fresh or warmed oocytes). Vitrification of oocytes, when applied to properly screened patients, will be a useful technology in reproductive medicine practice and will constitute a major step forward in ART.

Fortunately to date, no significant increase in abnormalities has been reported from these cryostored oocyte pregnancies [53], regardless of the historical concerns that cryopreservation of mature oocytes might disrupt the meiotic spindle and thus increase the potential for aneuploidy in the embryos arising from such eggs. These concerns have mostly been allayed by publications that show no abnormal or stray chromosomes from previously frozen oocytes [54], and FISH comparison of embryos from fresh and thawed oocytes show no increase in anomalies [55]. There also appears to be adequate recovery of the meiotic spindle post-cryopreservation whether using conventional or vitrification technology [56, 57, 58]. The scientific literature on oocyte cryopreservation grows daily it seems. Most reports focus on clinical pregnancy rates [59, 60], and as such while this data is helpful to increase our confidence in the technology, it does little to research new directions for oocyte cryopreservation.

5. Embryos

In 1983, Trounson and Mohr [61] announced the first pregnancy from a previously frozen human embryo obtained from in vitro fertilization (IVF). The first live birth after embryo cryopreservation was reported in 1984 in Australia, and the first in the United States followed in 1986. Since that time, cryostorage and subsequent use of human embryos has

http://dx.doi.org/10.5772/58746

become standard practice in assisted reproductive technology (ART) and is now involved in a significant proportion of all infertility treatments. In fact, the 2002 National Summary of Fertility Centers Report (NSFCR) determined that 97% of the 391 American centers reporting to the Society for Assisted Reproductive Technology (SART) offer cryopreservation [62]. Hoffman et al. [63] surveyed all SART-reporting clinics in the United States with regard to their cryopreservation practices and also found that virtually all of the 340 responding clinics freeze embryos and store them on site, accounting for over 400,000 frozen embryos as of 2002 [64].

Advances in assisted reproductive technologies have expanded procreative options for many people experiencing infertility. With the evolution of in vitro fertilization (IVF), more embryos often result from each cycle of ovarian stimulation than can safely be returned to a woman's uterus for implantation [65]. To reduce multiple gestations and their morbidity, avoid embryo destruction, improve cost effectiveness, and preserve future options for infertile couples, embryo cryopreservation (freezing) has developed as a routine practice in most IVF clinics [66]. Once embryos are frozen, they may be used for future pregnancy attempts, donated to another couple, designated for stem cell or other research, or discarded.

Cryopreservation allows the transfer of a limited number of embryos back to the uterus and the storage of the remaining embryos for future use, thus maximizing the cumulative effectiveness of an in vitro fertilization (IVF) cycle [67]. In addition, cryopreservation makes feasible the postponement of embryo transfer (ET) in a future cycle, thus decreasing the incidence of ovarian hyperstimulation syndrome in high-risk patients, while it maintains the probability of pregnancy [68].

Cryopreservation of embryos also has an enormous potential in Preimplantacion Diagnosis programmes (PGD). Therefore, not only does it allow us to conserve those normal embryos not transferred, but can also benefit hyporesponsive patients thanks to the accumulation of embryos in cycles [69]. An extension of embryo freezing is the embryo donation program, by which, following the course of Article 11 b of the current legislation (Law 14/2006, May 26), many couples that are not subsidiaries of Assisted Reproduction Techniques, may resort to these thanks to the anonymous donation of surplus embryos from IVF [69]. The embryonic stage in which we will perform cryopreservation is key in obtaining acceptable results, not only in embryonic survival rates, but in implantation and birth rates too. Besides a correct embryo selection of the best quality embryos is crucial for success in the cryopreserved embryo transfer Programme [69]

Conventional cryopreservation of pronuclear zygotes (2PN) is well established in countries such as Germany where freezing of later stage human embryos is by law or by ethical reasons not allowed. The time to complete the conventional protocol to cryopreserved zygotes is 98min. In Germany the clinical pregnancy outcomes arising from the frozen/thawed 2PN cycles is about 18%, with an implantation of around 10% per embryo transferred. The time to complete vitrification of zygotes requires approximately 12min. Recently successful vitrification of 2PN with high survival (~ 90%), cleavage rates on day-2 (>80%), and blastocyst formation of 31% and pregnancies were reported [70, 71, 72, 73]. Zygote vitrification implemented as a clinical setting can provide a clinical pregnancy rate of close to 30%, with an implantation rate of 17%

[73]. The pronuclear stage appears well-able to withstand the vitrification and warming conditions, which is probably due to the significant membrane permeability changes that occur post-fertilization; such changes to the oolemma may also make it more stable and able to cope with the vagaries of the cold-shock and striking osmotic fluctuations that occur during the vitrification process.

Activation of the embryonic genome occurs after the 8-cell stage (3 days post oocyte retrieval) is reached [74]. If the activation does not occur, the embryo will not survive further. Therefore, the improvement of human IVF outcomes requires identification of embryos that will progress beyond the 8-cell stage. Blastocyst culture (5 days post oocyte retrieval) allows for the transfer of embryos that clearly have an activated embryonic genome. This requires that the elimination of embryos in extended culture from day 3 to day 5 should depend solely on their inherited survival potential and not be a consequence of an adverse effect exerted by the sequential media used for culture beyond day 3. Additional advantages in cryopreserving at the blastocyst stage are: 1) At this stage a lower numbers of embryos can be transferred in fresh cycles, resulting in less high order multiple pregnancies, 2) The same is true for cryopreserved blastocysts showing higher pregnancy rates and implantation per thawed embryo transferred, 3) Approximately 120 hours (day five) into development the healthy human embryo should be at the blastocyst stage comprised of some 50 to 150 cells, of which about 20 to 30% make up the inner cell mass (ICM), the remainder making up the trophectoderm (TE), 4) the higher cell number allows better compensation for cryo-injuries, which results in greater viability and faster recovery, 5) the cytoplasmatic volume of the cells is lower, thus the surface-volume ratio is higher, and that in turn makes the penetration of the cryoprotectant faster, and 6) on average fewer embryos per patient were frozen-stored, but each one when thawed has a greater potential for implantation [75].

6. Cryopreservation of ovarian tissue

Cryopreservation of ovarian tissue is of interest to women who want fertility preservation beyond the natural limit, or whose reproductive potential is threatened by cancer therapy, for example in hematologic malignancies or breast cancer. It can be performed on prepubertal girls at risk for premature ovarian failure, and this procedure is as feasible and safe as comparable operative procedures in children.

At birth, the ovaries contain the lifetime complement of primary oocytes which are arrested in the prophase stage of meiosis 1 and are surrounded by a single-layered epithelium to form the primordial follicles. Ovarian cortex presents several advantages when compared with isolated oocytes:

- It contains the important pool of growing follicles.
- It does not necessitate the in vitro maturation/in vitro fertilization /embryo culture steps if it is associated with grafting.
- No previous ovarian stimulation is necessary.

http://dx.doi.org/10.5772/58746

Consequently cryopreservation of ovarian cortex is an alternative to cryopreservation of isolated oocytes or embryos. It could be used as an emergency preservation and as infertility therapy method for valuable animals. Ovarian cortex cryopreservation has been developed in human in order to preserve fertility in young women submitted to gonadotoxic therapy [76, 77]. In human newborns were obtained after orthotopic autograft of frozen-thawed ovarian cortices [78].

It is obvious that, to achieve successful cryopreservation of ovarian tissue, it is essential to maintain the functional status of the whole mixture of different cell types: oocytes, granulosa cells, epithelial cells, fibroblasts… This represents a major difficulty, because the optimum kinetic of cooling is different for each cell type. Oocytes are large cells, with a low surface to volume ratio, surrounded by zona pellucida. Immediately adjacent to the oocyte are corona radiata cells that have long cytoplasmic extensions which penetrate the zona pellucida, ending in oocyte membrane. These processes and gap junctions are important in the metabolic cooperation between the oocyte and surrounding layers of granulosa cells, which form the cumulus-oocyte complex during the growth phase. Consequently, at the opposite to cryopreservation of isolated cells, a cryopreservation protocol for a tissue represents a compromise between the requirements of the different constitutive cells.

The early work on ovarian tissue cryopreservation was performed in animal studies: rabbit [79] and rat [80, 81]. The earliest positive results were obtained when glycerol (15%) plus serum were used as cryoprotective agents (CPAs) for cryopreservation of rabbit granulosa cells, via a slow cooling protocol [79]. An equilibration period was necessary to achieve CPA penetration into the tissue. For this reason small samples were recommended. A rapid rewarming by plunging the samples into a water bath at 40°C was the most effective procedure [80]. Normal offspring were obtained from mice with orthotopic ovarian grafts of tissue that had been frozen and stored at-79°C [82]. Vitrification of ovarian tissue was also investigated. Nevertheless, Isachenko et al suggested that in human, low freezing protocols were more promising than vitrification protocols [83].

This technique has also been developed in rabbit [84], mouse [85], rat [86], ewe [87, 88], cow [89]. Vanessa Neto and her group [90] have obtained newborn rabbits after autografting of cryopreserved ovarian cortex. Also, their team developed this technique in cat [90] and dog [91].

Several techniques have been applied to ovarian cortex cryopreservation: slow freezing, vitrification. Simultaneously to ovarian tissue cryopreservation, numerous researches have been conducted about ovarian tissue grafting: orthotopic, heterotopic, auto-, allo-and heterografting [92].

The most common cryopreservation method is the slow freezing procedure, consisting of an initial slow, controlled-rate cooling to subzero temperatures followed by rapid cooling as the sample is plunged into liquid nitrogen for storage (−196°C). At such a low temperature, biological activity is effectively stopped, and the cells functional status may be preserved for centuries. However, several physical stresses damage the cells at these low temperatures. Intracellular ice formation is one the largest contributors to cell death; therefore, freezing

protocols use a combination of dehydration, freezing point depression, supercooling, and intracellular vitrification in an attempt to avoid cell damage.

Currently used ovarian cortex cryopreservation protocols have been direct, or slight modifications of the methods developed for isolated oocytes and embryos. There were primarily developed by trial and error adjustments of cooling and warming rates, and choice of CPA and CPA concentrations. However, because there are a large number of protocol variables potentially affecting cell viability, an exhaustive experimental search for the optimal combination of these parameters has long been considered to be prohibitively expensive in terms of time and resources.

6.1. Chemical and physical parameters affecting equilibration and freezing processes of ovarian tissue in mammalian species

The result of a cryopreservation process is influenced by several chemophysical parameters affecting directly or not the functions and the integrity of the ovarian cells along the freezing process, from the equilibration to the thawing. Among these parameters, the method of equilibration, the freezing rate, the composition of the freezing solution and notably the nature of the permeating CPAs and the non-permeating CPAs, the concentration of each CPA, the use of serum, or the rate of thawing may be investigated to know the relative influence of each of them and the induced cell injuries.

In general, we can expect coupled flows of water and CPAs when CPAs are added, during freezing, thawing and when CPAs are removed from the cells, resulting in a series of anisosmotic conditions. During freezing, the cells dehydrate and shrink and remain shrunken during storage, but return to their isosmotic volume upon thawing. Finally, the cells are subjected to potentially lethal swelling upon CPA dilution and removal. During the controlled slow cooling extracellular ice formation is induced (seeding) at a temperature just below the solutions' freezing point, and then the cooling continues at a given rate in the presence of a growing extracellular ice phase, which raises the extracellular solute concentration in the unfrozen fraction and results in water being removed from the cell via exosmosis.

Permeating CPAs, such as glycerol, dimethyl sulfoxide, ethylene glycol or propylene glycol are typically included in the cryoprotective medium, to protect the cells against injury from the high concentrations of electrolytes that develop as water is removed from the solution as ice. During the equilibration step the inner cell water is partly replaced by the permeating CPAs. However, the CPAs can be damaging to the cells, especially when it is used at high concentrations. The toxicity can be reduced by decreasing the time or the temperature of the equilibration step [93]. But equilibration at low temperatures requires increasing the exposition time to freezing solution. Furthermore, the CPAs may have dramatic osmotic effects upon the cells during their addition and their removal.

Consequently, the use of several steps of increasing concentrations of CPAs during the equilibration allows reducing the osmotic gradient. The cells exposed to such permeating CPAs undergo initial dehydration, followed by rehydration, and potential gross swelling upon removal. This osmotic shock may generate membrane damages by mechanical means and

predisposition of the cell to injuries during the other steps of cryopreservation, or even cell death [94]. These kinds of damages could be reduced by using cells surfactant such as serum. During the freezing step, the follicular preservation depends on the nature and the concentration of the CPAs.

Control of the cooling and warming rates is also crucial, as the freezing/thawing rates and the temperature of seeding also influence the ice properties. If cells are cooled too rapidly during the controlled slow cooling process, water does not exit the cells fast enough to maintain equilibrium and, therefore, the oocytes and other ovarian cells freeze intracellularly, resulting in death in most cases. If cooling is too slow, the long duration can cause 'solution effects' injury resulting from the high concentration of extra-and intracellular solutes, probably due to the effects of the solutes on the cellular membrane or through osmotic dehydration. During warming the small intracellular ice crystals might subsequently undergo recrystallization, forming bigger ice crystals that rupture the cell membrane, thus leading to fatal damage. Finally, the thawing and the removal of the CPA depend on the temperature and on the presence of non-permeating CPA limiting the osmotic swelling during rinsing.

7. Contamination in cryopreservation

One of the first thing we must learn is to differentiate their respective importance are the concepts of contamination and cross contamination of samples. The first relates to the contamination of the sample by freezing or by direct contact with the cooling solution and the second refers to the contamination of the sample within the common container which is in contact with all cryopreserved samples, some samples may be contaminated or the liquid nitrogen (LN2) might be contaminated producing a possible cross-contamination. The potential for disease transmission and pathogen survival through contaminated LN2 has been proposed by many authors [95-97], and the evidence of contamination in human patients has been described for different pathogens [98-104]. It has to be stated that none of the reported infections after insemination or ET in humans and domestic animals can be clearly attributed to the applied cryopreservation and storage procedure but the use of safe cryopreservation protocol is very important to avoid human cell contamination or cross contamination in common LN2 tanks.

Although cryopreservation had a boom in the mid 70's and early 80's with the opening of the first sperm banks in America and Europe, it was not until the mid-80's when we saw the need for biological samples cryopreserved in quarantine and the lack of screening leads to infection of several recipients that had been inseminated with semen samples from donors HIV+those unaware of their disease [105]. In these cases it was found that samples stored in the same containers with frozen HIV+samples were not contaminated, otherwise in 1995, six patients undergoing cytotoxic treatments hermetic problems developed an outbreak of acute hepatitis B after undergoing an autologous cryopreserved material that had been stored in the same cryogenic container as other patients infected with hepatitis B [106].

7.1. Cells and tissue contamination

In the field of assisted reproduction, although it hasn't been detected any contamination in the cryopreservation of gametes and embryos, the probability and the occurrence is low, the risk is not zero so it is recommended to follow the rules in biosecurity manuals for both the physical and chemical risk as well as the risk of contamination and cross contamination of samples.

The case in 1985 where there was infection with hepatitis B in the cryopreserved samples [106] the infection was due to an error in packaging and storage of samples. With time a deterioration of the bags containing infectious material causing the infection of the LN2 and other samples was observed. Further studies have shown that the storage of samples is decisive. There is evidence that frozen samples in hermetically sealed straws are not contaminated even if they are in contaminated containers with contaminated LN2 and LN2 does not contaminate infective biological samples that were frozen in a sealed container [107,108] During the cryopreservation, biological samples go through many processes before being cryopreserved. In the case of IVF cells are subjected to a phase of procurement, fertilization, development, transfer and finally cryopreservation. This represents an approximate 6-day process in which many factors can affect the contamination of the sample at the end of the process. We can find contamination or cross-contamination in the following cases [109]:

- Handling contaminated biological samples (semen, follicular fluid, tissue, etc.). Without precautions to avoid contamination outside the base plate to be used for conservation (cryotube, straw, etc.). It is very important to disinfect and clean the container before filling it with LN2 [110]. In this regard to ensure an adequate level of biosafety a study is needed of infectious diseases transmissible from any patient or donor who wants to freeze any samples. According to Castilla [111] the clinic policy for a donor with infectious diseases is radically different to that of a patient with any of these diseases wanting to freeze biological material for autologous use. In the first situation, the biological material at hand will not freeze. In the second, the biological material should be frozen but with measures that we discuss later. Screenings for infectious diseases that normally must be submitted are: To analyse serological studies for syphilis, hepatitis and HIV. To analyse the clinical studies infective clinical phases: toxoplasmosis, rubella, herpes virus, cytomegalovirus (CMV), Neisseria gonorrhoeae and Chlamydia trachomatis. These tests are required for donors of semen every 6 months. As the risk of disease transmission during storage in LN2 is mainly viral. Interestingly, the American Society of Fertilisation [112], ESHRE [113], British Andrology Society (BSA) [114] and the Spanish Association of Tissue Banks (AEBT) [115] also recommend serologic screening for CMV, not just clinical. The presence of CMV in semen has been associated with active disease (anti-CMV IgM+or recent seroconversion anti-CMV IgG+). Similarly, these companies recommend performing serologic tests for HTLV-I and HTLV-II. But although it is clearly demonstrated the transmission of human papilloma virus by using LN2 cryotherapy and has been shown IUI transmission of herpes simplex virus (HSV) [116], none of the scientific associations mentioned above recommend a culture for detection or serological studies of HPV donors or patients with infection who are going to freeze biological material because the analysis to detect these deceases are not very sensitive. As rubella serologic screening of donors, its low prevalence in this population

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means that serological tests have a low positive predictive value, making it unadvisable. Finally, we believe a patient who needs to freeze some reproductive biological material should have at least one serology for HIV, hepatitis B and C. This proposal is consistent with the recommendations of the AEBT for cryopreservation of semen [115].

- Use of contaminated culture media. In these cases the degree of cross-contamination would reach very high levels having an impact on many patients. Although the preparation of embryo culture media and sperm extenders from specific ingredients are avoided in human clinics, it continues to be a common practice in animal ART [117]. Nevertheless, many ingredients of embryo culture media and sperm extenders act as stabilizers for many micro-organisms at freezing temperatures (milk, serum or serum albumin, sucrose, sorbitol and other sugars). Unfortunately, the most common cryoprotectors (CPs) in applied oocyte cryopreservation and embryo (glycerol, DMSO, ethylene glycol, propylene glycol, methanol etc.) are toxic for cells. Also bacteria and viruses efficiently protect from cryoinjuries, eg Concentrations of DMSO as low as 5% enveloped viruses defend against the trauma of freezing [118]. The Fact That microorganisms survive in association with germplasm is not only important from the potential of disease transmission by embryo transfer to recipients, but also in approaches to the storage of samples for testing and health certification of embryos or international movement. On the other hand we must also bear in mind that all culture media containing antibiotics to prevent or limit survival of microorganisms.

- Conservation of contaminated material or straws cryotubes closed or sealed badly flawed causing the breakdown of the frozen straw, leaving the contaminated sample directly exposed to the LN2 tank risking contaminating the other samples. Closed systems can be sealed in many ways (thermal sealer, ultrasound sealer, radiofrequency sealer, polyvinyl alcohol powders, and solid caps). Given the sealing time and the temperature reached does not affect the cryopreserved sample, we have to ensure that the seal is airtight and that the device is built of resistant material to low temperatures of LN2 (Ionomeric resins, quartz glass capillary, Polyvinyl chloride, Polyethylene glycol tetralato, etc).

- Using contaminated LN2 during the freezing process. In this case we have proposed some solutions that we will see later.

- Poor source management of LN2 from our supplier contaminating commercial LN2 that comes to our lab in the process of manufacture or transportation and filling ourcontainers.

- For transportation of contaminated material in containers. Storage containers should be emptied and cleaned periodically due to the risk of lost straws or small particles of contaminated material that falls to the bottom of a large container [119,120]. Most of the companies of LN2 containers provide cleaning protocols. The main problem is the cleaning of transport cylinders called "dry" because the material that absorbs the LN2 in these bottles is difficult to sterilize. Bielanski [121] describes a method of disinfection of commercial dry shippers with two different types of a LN absorbent. Based on the results presented, it appears that solutions of sodium hypochlorite and ethylene oxide are equally useful for the disinfection of dry shippers constructed with a hydrophobic LN absorbent. In contrast, for dry shippers

without a hydrophobic LN absorbent it is advisable to use gas only for decontamination in order sterilization to avoid their damage by liquid disinfectants.

- The air in the room. If the air that reaches the lab comes from another area that could be contaminated and there isn't a good filter. Some laboratories do not have filtration systems or positive pressure to prevent air contamination.
- Operators. If they are infected then that can lead to contamination by contact or peeling during processing of samples or the handling of cryogenic tanks. Staff must meet certain health and hygiene conditions: negative serology for HIV, HCV, HBV and vaccination against hepatitis B and other viral diseases for which there is a vaccine available. We must also have a detailed description of their jobs, tasks and responsibilities. In addition the centre must provide the worker training in freezing techniques for updating and improving procedures.
- Use of open devices. In recent times there is much talk of closed or open system and the possibility of contamination, so many countries have banned open systems and the trend is to ban the high risk of sample contamination. In a closed or semi-closed device the nitrogen of common container is never in contact with biological material frozen on the inside so cross-contamination cannot produced. In the open system, the biological material is in contact with the common nitrogen so contamination from the sample is very easy if the LN2 is contaminated or contamination of LN2 if the sample is contaminated. The latest study done by Criado and his group [122] showed 45% of contamination in an open device (Cryotop) Vs 0% of contamination in a semi-close device (Ultravit) equal and using a contaminated laboratory LN2.

7.2. Cooling solution contamination

The cooling solution plays a significant role in avoiding contamination of biological samples. It means that we will freeze the sample and we will deposit it for a long storage until thawed and used. Normally the LN2 cooling solution is the most widely used in cryopreservation and survival of pathogens at high temperatures (-196 ° C) has already been proven by many studies [195-97,121,122] cases also involved in seeing cross-contamination of human papillomavirus [108,123].

The need for better cooling rates to avoid formation of crystals in cryopreservation has resulted in the discovery and use of new cooling solutions (slush, slurry, etc.). So there are more components to consider when contamination is to be avoided. Using these new cooling solutions gives a lower temperature than the LN2 temperature and much faster transmission. The Slush nitrogen is obtained by a vacuum pump (Telstar TOP-3; Telstar S.A., Terrassa, Spain) that solidifies part of the LN2 in a few minutes. On return to normal atmospheric pressure, the nitrogen collapses, and the subcooled LN2 has solid particles in it commonly referred to as ''slush'' [124].The advantage of Slush nitrogen lies not only in the temperature difference with respect to LN2 (-196°C Vs-210°C) but also in the reduction of the Leiden frost effect, which is the formation of a layer of vapor around the sample when immersed in the cryogenic liquid from room temperature decreasing the cooling rate [125,126]. It has not yet been demonstrated

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the survival or non survival of pathogens in this cooling solution of 15-20 ° C difference in LN2, this is obtained by vacuum pressure, which can lead to rupture of the cell wall of pathogens to balance internal and external pressure of these in the process of forming Slush. The 'Slurry' nitrogen is a mix of LN2 with different particles for example copper powder. At present investigations are being carried out as an alternative to LN2 to increase the cooling rate because with this cooling solution the thermal conduction is increased. Likewise, experiments are ongoing with various solutions to increase the thermal conduction and the cooling rate.

Sample tank	Identified microbial contamination			Years of storage	Total no. of stored samples
	Liquid nitrogen	Semen	Embryos		
Research					
Laboratory tanks					
1	*Staphylococcus auriculans*	Nd	Nd	20	580
2	*Bacillus licheniformis, Bacillus* spp.	*Stenotrophomonas maltophilia, Staphylococcus sciuri*	Nd	15	840
3	CDC group IVc-2, *Alcaligenes faecalis*	*Proteus vulgaris*	Nd	8	460
4	*Brevundimonas vesicularis*	*E. coli*	Nd	15	1350
5	*Stenotrophomonas maltophilia*	—	Nd	12	650
6	*Staphylococcus capitis*, unidentified Gram-negative rod, *Comamonas acidovorans*	*Morganella morganii, Gemella morbillorum, Stenotrophomonas maltophilia, Citrobacter koseri*	*Bacillus subtilis, Ochrobactrum anthropi, Staphylococcus epidermidis*	15	1200
7	*Stenotrophomonas maltophilia, Comamonas testosteroni*	*Stenotrophomonas maltophilia, Citrobacter koseri*	*Stenotrophomonas maltophilia, Bacillus* spp., *Pseudomonas fluorescens, Acinetobacter lwoffi*	18	1480
8	*Bacillus pumilus, Ralstonia pickettii*	—	*Stenotrophomonas maltophilia, Bacillus* spp., unidentified Gram-negative rod	10	960
Commercial tanks					
9	Nd	Nd	—	10	58 456
10	Nd	*Aspergillus* spp.	—	30	128 430
11	*Aspergillus* spp.	*Corynebacterium xerosis, Bacillus sphaericus*	—	30	24 962
12	Nd	*Stenotrophomonas maltophilia*	—	35	150 000
13	Nd	*Stenotrophomonas maltophilia*	—	15	280 864
14	*Aspergillus* spp.	*Photobacterium damsela*	—	15	260 912
15	Nd	*Bacillus sphericus, Corynebacterium* spp., *Staphylococcus sciuri*	—	12	262 642
16	*Stenotrophomonas maltophilia*	*Ralstonia pickettii*	—	12	404 955

Nd, not detected; —, not available for testing.

Table 1. Microbial contamination of embryos and semen during storage in liquid nitrogen (adapted from Bielanski *et al.*, 2003)

These cooling solutions "alternatives" are only used at the time of freezing the sample and once frozen, it passes to the general container that is filled with LN2, although these solutions where they freeze cool samples have to be sterile we have to ensure that the general LN2 container does not have contact with the frozen sample in order to not contaminate the sample and the LN2 if the sample is positive for any pathogen. Retrospective studies in which commercial LN2 cryotanks were examined after 35 continuous years of service revealed various bacterial and fungal contaminations in the LN2 detritus [117]. Many of the identified bacteria isolated in these studies were ubiquitous environmental micro-organisms and were rare opportunistic pathogens of low significance in producing disease in humans or animals (Table I). It should be acknowledged that some of the isolates may have been derived from laboratory contamination during semen and embryo processing for cryopreservation rather than genuinely being present within the sample. In agreement with Bielansky and Vajta the risk of contamination by human pathogens seems to be rather low. Components of the standard LN2 production system comprise a compressor, a cryogenerator and containers. From a practical point of view,

the complete sterilization and maintenance of sterility in such a robust system might be a very demanding task, if possible at all. Accordingly, some ubiquitous bacterial agents can be expected in any commercially produced LN2. Nevertheless, it is an 'in and out' system and only air-borne contaminants are supposed to enter it (LN2 compressor) via air used for LN2 production. As they are not air-borne, it is unlikely that viral agents of human concern such as HIV, hepatitis and herpes viruses would enter the LN2 production system.

Microbiological contamination of embryos and semen during storage in LN2 [117]

One of the biggest discussions recently in the world of cryopreservation focuses on the importance of the sterility of LN2. As shown in Table I and in total agreement with Bielansky and Vajta and many other authors the commercial LN2 reaching our lab is not contaminated enough to cause any infection to freeze biological material. The major problem is common containers where the samples are deposited with a LN2 stored for months, years or even decades in contact with many samples, which, many clinics do not empty and do not disinfect, so it is in common containers where we can find the highest risk of contamination and cross contamination.

As a possible solution to minimize the risk of freezing biological material some systems have been proposed where we sterilize the LN2 and where we ensure that the sample is not in contact with LN2 with the use of semi-close devices or devices that are the only ones that guarantee a hermetic sealing of the device and avoid any risk of breakage of the solder thus ensuring the aseptic samples. The fact that LN2 can be quickly and safely sterilized could encourage the clinical application of human cell/tissue vitrification, both with open carriers and with closed systems. The problem is that if this device is an open device and is passed to the general container where all the other cryopreserved samples there is a huge risk of cross contamination, so it has not helped.

- LN2 Filtration: One of the solutions that have been developed is the filtration of LN2. Air Liquid has marketed CERALIN a liquid filtration system through LN2 ceramic filters. The CERALIN ON LINE consists of two elements of liquid filtration connected in series and inserted into a section of vacuum transfer line. The ceramic membrane is made from multiple layers formed into a multi-channel element. It is housed in a vacuum insulated pipe, itself installed close to the end-use point. The filter minimizes the pressure drop and avoids the vaporization of the LN2. Thus it avoids nitrogen losses. Several sizes are available, depending on the nitrogen flow. The efficiency of this equipment was investigated and proved in laboratory. The filter is located downstream of the nitrogen vessel. During operation, LN2 flows through the filter and over the ceramic membrane. The result is high-purity LN2 with a bacteria count of less than 1 CFU/L gas. Additionally, the large filtration area of the membrane and low level of contamination of LN2 means it is likely to be several decades before filter saturation.

- UV Sterilization: This method is based on emitting the minimum dose on UV radiation necessary to kill micro-organisms that can survive at the boiling point of nitrogen (-196°C) and which is irradiated in a temperature-controlled regimen, within a short time interval, before the LN2 completely evaporates. The extremely radiation-resistant bacterium

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Deinococcus radiodurans is inactivated (>-4log) by administe-ring 400.000 μWs/cm² per each sterilization cycle. An adequate amount of UV radiation deactivates the growth of all kinds of micro-organisms, from viruses like Hepatitis (which require an 8.000 UV dose) to fungi like Aspergillus Niger (330.000 UV dose) [127]. At CRYO 2011 Dr. Parmegiani spoke about a new dispositive of UV sterilization of the common containers with cells or tissues inside but the scientific community thinks that is too dangerous biological samples exposed to UV rays without any protection. Although his group is proposing special canisters "not transparent" I think they have to do many more tests to rule out damage to the samples because the common view is confirmed that UV light is harmful, even if used just overnight decreased embryo developmental rates.

- LN2 Steam: As an alternative to hermetical storage in LN2, cryostorage contamination might be avoided by storing the carrier containing the vitrified oocytes in LN2 vapour [128, 129]. However, Grout and Morris [130] maintain that storage in the vapour phase of LN2 still carries a risk of sample contamination. Storage of semen in LN2 vapours was discarded early in the development of sperm cryopreservation techniques and it was found that long-term viability of sperm was reduced compared with LN2 storage [131,132]. However, recent experiments with new materials have succeeded in developing the technique with acceptable results for both semen and embryos [133,134] and in our last experiment we demonstrated 0% of contamination in vapor nitrogen in an experimental contaminated laboratory LN2 (non published). The drawback of the generalization of this form of storage is the need for careful monitoring of temperature in different parts of the container, which makes the marketing of these containers type [134] more difficult.

- Before entering discussions regarding the sterility in LN2 used for vitrification, we should debate the use of communal containers, which is where cross-contamination can be found, as there is a possibility that the "contaminated cells" could come into contact with each other, and where a number of viruses and bacteria are found, which would never be found in the commercial LN2.

7.3. Contamination in transport

To carry out a safe transportation of biological material we should clearly distinguish a number of concepts [111].

1. Infectious substances: those that contain viable microorganisms (bacteria, virus, prions, parasite, fungus) or bacterial toxins that are known or believed to cause disease in animals or humans.

2. Diagnostic specimens: human or animal materials (body fluids, blood, tissue, tissue fluids, etc.). Obtained for diagnostic or investigational [135].

Most often transported biological reproductive materials are cryopreserved semen donor and follicular fluid when the laboratory is separated from the follicular puncture site. In both cases, we consider the recommendations to follow are those of diagnostic specimens. There are several documents related to the transport of biological material, such as the Universal Postal

Union (UPU), the International Aviation Organization (ICAO) and International Air Transport Association (IATA) [136-138].

At European level, all documents related to transport are based on the recommendations of the Committee of Experts of the United Nations Dangerous Goods (UN) [139]. There is also a european agreement on international transport of dangerous goods by road (ADR), approved by RD 2115/9838 [140]. We will describe some aspects of the mentioned regulations on the transport of diagnostic specimens. The basic system consists of packaging:

1. Primary container, watertight, leak proof, labeled and contains the sample. This container should be wrapped in absorbent material. In terms of labeling, according to AEBT, if it is a semen sample from a donor, must contain an alphanumeric code that identifies the donor and the sample number of the donor. On the other hand, if the sample is for autologous use may be noted also the surname of the patient [115].
2. Secondary container, sealed, leak-proof and protects the primary container. You can place multiple primary containers wrapped in a secondary container. This should be sufficient absorbent material used to protect all primary containers and avoid collisions between them.
3. Outer shipping container: the secondary container is placed in a shipping package that protects the secondary container and its contents from outside elements, such as physical damage and water. The data forms, letters and other identifying information of the sample should be placed taped outside the secondary container. The label for submitted materials consists of:
    - **a.** Basic triple packaging.
    - **b.** Does not require signs from United Nations (UN).
    - **c.** No substances require pictogram or declaration from the sender.
    - **d.** Biological material for clinical use" must be indicated.
    - **e.** Tag address:
        - Name, address of destination, as detailed as possible, and phone number.
        - Name, address, telephone number and contact person at the semen bank.
    - **f.** The documents included with the storage conditions and special instructions for shipping. One of the special considerations that we must have in mind when transporting a sample of semen is not breaking the cold chain, so you must use a container or LN2 as well as avoiding the possible use of dry ice.
    - **g.** Permission for import / export and declaration.
    - **h.** Label orientation.
    - **i.** Date and time of departure of Semen Bank [115].

The requirements to be met for local transport are as follows:

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1. Sealed and resistant containers.
2. Threaded tubes upright (rack, tray...).
3. Use of resistant boxes and perfect closure.
4. Secured box in the transport vehicle.
5. Appropriate Labeling.
6. Have the forms with necessary details.
7. Vehicle with kit (gloves, absorbent material, disinfectant, waste container, etc.).

You must ensure perfect coordination of transport between the sender, carrier and recipient to ensure delivery. Thus, each party involved should carry out its part perfectly and appropriately. So stand out from other actions that the sender must ensure the proper identification, packaging, labeling and documentation according to established biosafety guidelines in the "Recommendations of the Committee of Experts of the United Nations Transport of Dangerous Goods" transporting must be kept in appropriate conditions (temperature, light...) the material from which the sender receives it until it is delivered to your destination and have the appropriate licenses to perform this type of transport, and finally, the recipient must confirm with national authorities that the material can be legally imported.

According to AEBT [115], the possibility of returning a material that hasn't been used should be avoided, as a rule, the return of the semen that has been provided by the Bank, as it will only accept the return of the displayed when you meet the following 3 conditions:

1. The sample wasn't thawed.
2. You can demonstrate the integrity of the packaging (the seals are intact).
3. The temperature of the sample was maintained throughout the transport.

8. New techniques for in-vitro fertilization patients: Ultravitrification

Today the differences between Slow freezing and Vitrification are known worldwide. We all know that slow freezing is characterized by a prolonged cooling curve and the use of low concentrations of cryo-protectors generally ''non-toxic'' for the cells (1–2 M) with cell injury due to ice formation [141] and that Vitrification is characterized by the rapid procedure and the use of a high concentration of cryo-protectors (4-6M) to prevent cell damage that is toxic to most mammalian cells [7,142-149].Thus, vitrification with a semi-close devices have a better cooling rate without cross-contamination or novel cyopreservation techniques are needed that allow rapid cooling to achieve vitrification in the absence of high concentration of CPA or if is possible without CPA.

The requirements and relationships for conditions to achieve satisfactory vitrification in the area of mammalian ART are well displayed in the equation of Yavin and Arav [151]

$$\text{Probability of vitrification} = \frac{\text{Cooling and warming rates} \times \text{Viscosity (CPA concentration)}}{\text{Volume}}$$

Probability of vitrification by Yavin and Arav [151]

The main points to be gathered from this relationship are that the smaller volume of the vitrification solution in which the cellular material is placed for the vitrification process, the faster cooling and warming rate that can be achieved and the lower concentration of CPAs needed reducing the detrimental effect of the inherent toxicity of CPAs and increasing the overall success of the procedure.

What would happen if We could vitrify without CPA's or with a low concentration of CPA's? What would happen if We could combine the advantages of Slow freezing and Vitrification and vitrify with low concentrations of CPA's with a secure and free contamination device? That is Kinetic vitrification (Ultra-vitrification). Perfecting the techniques of Vitrification has been achieved a morphological survival rate comparable to normal Vitrification protocol [123] or a 59.1% of blastulation rate in mouse embryo [151] with Kinetic vitrification and concentrations of CPA's typical of Slow freezing.

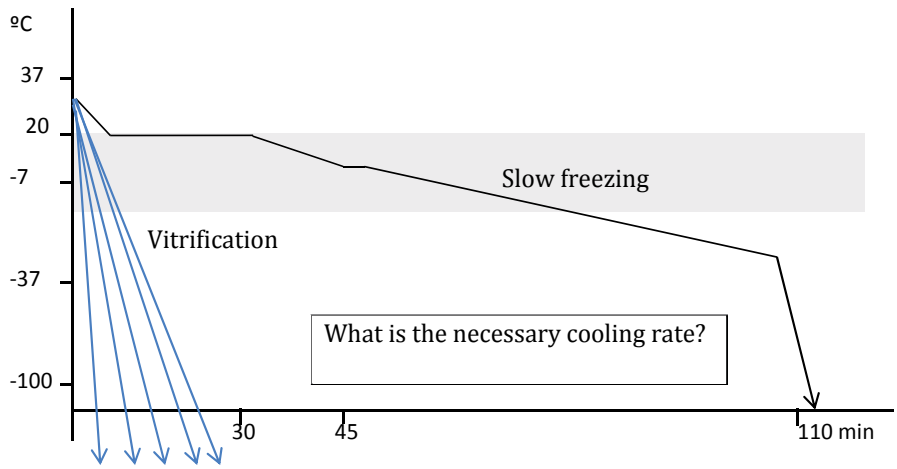

Figure 2. Necessary cooling rate to have a good probability of vitrification

Previous studies have tried to achieve high cooling rates for cell vitrification. However, none of them utilized low CPA concentrations (1.5-2 M). In 1985, Rall and Fahy successfully vitrified mouse embryos in 6.5 M cryoprotectant cocktail solution [7]. In that case the method consisted in a 0.25 ml straw container plunged into LN2; the cooling rate was 2.500 °C/min. When this container was plunged into Slush nitrogen, the cooling rate increased up to 4000 °C/min [152]. The use of OPS (instead of the 0.25 ml straw) in LN2 increases this cooling rate up to 5.300 °C/min [152] and to 10.000–20.000 °C/min if plunged in Slush nitrogen [152,153]. Similar cooling rates were achieved in the case of a Cryoloop quenched in Slush nitrogen [154]. The use of

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electron microscope copper grids has also been investigated, but the cooling rates were in the same order of magnitude that the afore mentioned works: 11.000–14.000 °C/min in the case of plunging the grid in LN2 [155] and 24.000–30.000 °C/min if plunged in Slush nitrogen [126, 155]. From Boutron's theory, none of these approaches reaches the critical cooling rate to achieve vitrification with low concentration of CPA (1.5-2M). It´s impossible to use open devices with Slush nitrogen as the cell is on the outside and there is a possibility of detaching from the device.

Adjusting to Yavin and Arav formula the Ultra-vitrification technique arose achieving a cooling rate above 250.000 °C/min and of 90.000 °C/min in thawing. This rate is one order of magnitude higher than the highest cooling rate achieved in different strategies (electron microscope copper grids in Slush nitrogen [126,155], whilst keeping all the advantages of a straw-like form for the container and being in the range of the necessary cooling rate to achieve vitrification. To have this increase in the cooling rate a few changes were made to the normal vitrification process:

Slush Nitrogen

As a cooling agent this technique uses Slush nitrogen, much colder than LN2 (-196°c Vs-210°C) and with the property of avoiding the Leiderfrost Effect. When something is submerged in LN2, bubbles rise to the surface through the device, varying the thermal conductivity from the outside into the inside of the device. This does not happen with Slush nitrogen. Slush nitrogen is achieved with a vacuum pump in 5 to 10 minutes and it remains slush for a further 5 – 10 minutes before returning to liquid.

It was shown for oocytes and embryos that increasing the cooling rate would improve survival rates by up to 37% [156]

Figure 3. Slush Nitrogen

Quartz Micro-capillary

Another determining factor to achieve a high cooling rate is the device used. To increase the thermal conductivity and minimize the volume, this technique has used a quartz microcapillary. This has a 0.2-0.3 diameter allowing to ultra-vitrify 0.1-0.2 μl with a 0.01mm wall, a lot thinner than any other device (0.075 mm in OPS). Another important characteristic is the material it is made from: Quartz. The thermal conductivity of quartz glass is a lot higher than that of plastic of which other devices are made of. This converts it in one of the materials that best conducts the temperature. [157]

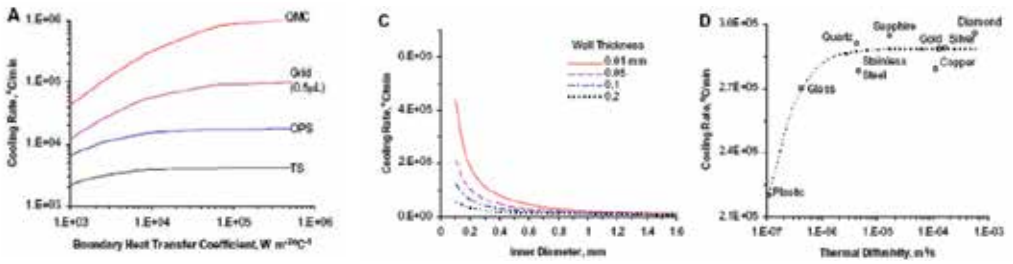

Figure 4. Xiaoming He et al [157]

In a thermal performance of quartz capillaries for vitrification done by Risco and his group [158] a commercially available version of the OPS (MTG Medical Technological Vertriebs, GmbH) was used. The thermal conductivity of these PVC straws was 0.19 W m_1 K_1. The inner diameter is 0.800 mm and the thickness of its wall is 0.075 mm (Fig. 1a). The QC used (The Charles Supper Company, Inc.) have an inner diameter of 0.180 mm and a wall thickness of 0.010 mm. These geometrical improvements (4.44 times smaller in diameter and 7.50 times thinner) translate not only into a faster heat transfer, but also into a 20 times reduction in volume of the contained solution (for a given height). This is beneficial because the thermal conductivity of the quartz glass is 1.3 W m_1 K_1, that is almost one order of magnitude higher than that of PVC.

A clear heat release peak is present during cooling as well as melting during rewarming. (b) Thermal history for QC when filled with a 1.5M propane-1,2-diol and 0.3M sucrose cryoprotectant solution quenched in LN2 and then thawed in a water bath at 37°C. Crystallization of water is not obvious during cooling, but melting is shown during rewarming. (c) Thermal history for OPS when filled with a 1.5M propane-1,2-diol and 0.3 M sucrose cryoprotectant solution quenched in Slush nitrogen and then thawed in a water bath at 37°C. In this case, crystallization during cooling and melting during rewarming was not recorded. However, visual inspection reveals the presence of ice. (d) Thermal history for QC when filled with a 1.5Mpropane-1,2-diol and 0.3 Msucrose cryoprotectant solution quenched in Slush nitrogen and then thawed in a water bath at 37°C. The sample keeps its transparency over all the cooling–rewarming cycle, an indication of the capability of this approach to vitrify the studied solution. All these changes have allowed us to maintain a concentration of cryoprotectors typical of slow freezing, 2 M PrOH+0.5 M sucrose, obtaining a morphological survival rate of

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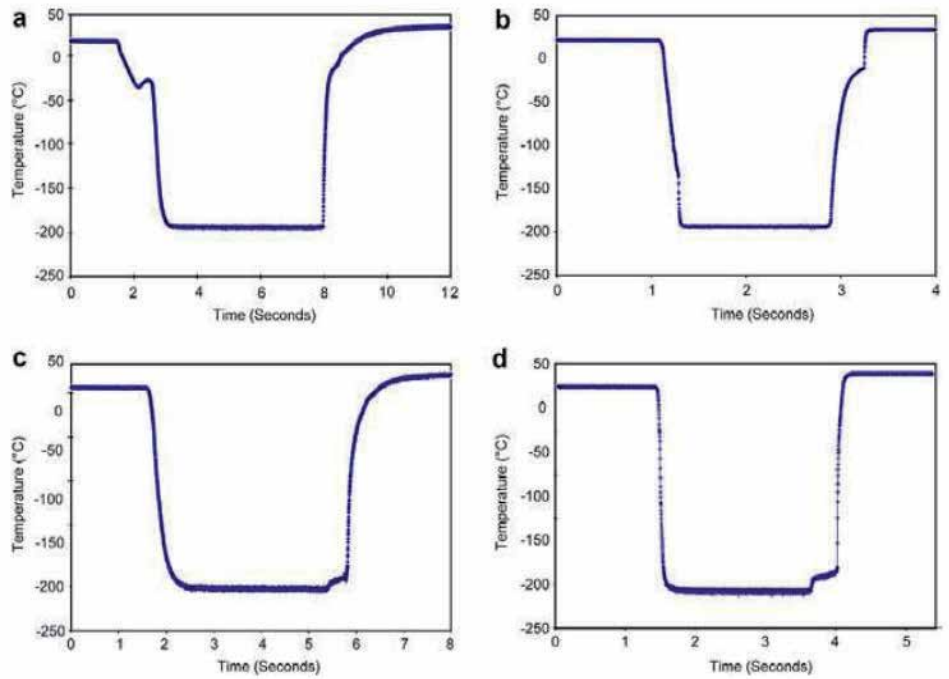

Figure 5. a) Thermal history for the OPS when filled with a 1.5M propane-1,2-diol and 0.3M sucrose cryoprotectant solution quenched in LN2 and then thawed in a water bath at 37°C.

92 % in human oocytes [124]. Dr. Ho-Joon Lee et al [151] tested this new technique on mouse ooctyes and they saw that using Ultra-vitrification with low concentrations of cryoprotectors improved the fertilization rate and above the blastulation rate. Only the use of Ultravit device in this technique ensures the non contamination of the sample or cross-contamination in communal containers.

%	Slow freezing [159]	Vitrification [159]	Ultravitrification mourine oocytes [151]	Ultravitrification human oocytes [124]
Surv. rate	61	91.8	92.5	92
Fert. Rate	61.3	67.9	75	?
Blast. rate	12	33.1	59.1	?

Table 2. Comparison between slow Freezing, Vitrification and Ultravitrification [162,152,124]

This comparison demonstrates the use of low concentration of cryoprotectant in the Ultravitrification protocol favours the morphological survival (92%) and increases the blastulation rate (59.1%). Thus confirming the hypothesis that cryoprotectants are toxic to the biological

sample and if we could find a vitrification protocol that would allow us to vitrify without cryoprotectant, we would achieve a better embryo development and a greater chance of pregnancy in the case of freezing eggs or embryos. A lot more studying is needed regarding this new technique but a priori the results indicate that we can hopefully lower the concentration of the cryoprotectants decreasing the toxicity in cells.

9. Conclusion

Cryopreservation has always been a fundamental tool in assisted reproduction but increasingly assumed a more important role because it serves not only to optimize ART treatments but thanks to the possibility of both cells and tissues cryopreserved successfully, we can offer the possibility reproductive future of cancer patients.

Cryopreservation takes many utilities; the most traditional approach is optimization of IVF treatments. In this sense it has been used in the cryopreservation of sperm for sperm banks or uses it to treatment when there is a problem to get it on the day of egg retrieval. The sperm freeze has been doing for decades due to the relative ease of the technique and the good results are achieved, therefore it is more widespread use of sperm cryopreservation in cancer patients or patients who are going to be vasectomized getting preserve their fertility for the future.

From the revolutionary emergence in the late 70's in vitro fertilization (IVF), the possibility of storing surplus embryos from IVF programs was a need and offered an excellent alternative Cryobiology. Other situations where cryopreservation is very useful are reported when an embryo transfer is dangerous for the health of the patient for risk of ovarian hyperstimulation (OHSS).

Another key point in recent years is gaining more importance is the freezing of oocytes. Because of its large size and the amount of water that contains inside, the oocyte has always presented difficulties for cryopreservation. The need for better cooling rates to avoid formation of crystals in cryopreservation has resulted in the discovery and use of new cooling solutions (slush, slurry, etc.). These new cooling solutions and the new vitrification media with little or no cryoprotectant has become possible that cell cryopreservation excellent results after thawing.

Up to date, the studies that have evaluated the health of children born through the procedure of egg thawing have reflected that there is no increase in the incidence of congenital abnormalities. And it remits us to prestigious investigations such as that published by Noyes in 2009 [160] with nearly 900 babies born through freezing and thawing of oocytes, in which it reflected that these children were completely normal. More recently Levi Setti in 2013 [161] published another study in which it stated that, after studying 954 pregnancies, there is clear evidence that the children born by this technique have the same probability of malformation and the same complications during a pregnancy and delivery as children born by other assisted reproduction techniques in which fresh eggs have been used.

Therefore, up-to-date no published investigation makes us believe that oocyte vitrification is dangerous for the children that will be born as a benefit of the technique. Although 13 years

later after the first slow-freeze birth, the number of reported babies born as a result of vitrified oocytes is now approaching that of slow-frozen oocytes without any increasing risk in congenital abnormalities [160]. Vitrification of oocytes does not appear to increase risks of abnormal imprinting or disturbances in spindle formation or chromosome segregation [162]. It has the greatest potential for successful oocyte cryopreservation and with its increased clinical application is showing a trend to greater consistency and better outcomes (similar to outcomes between fresh or warmed oocytes). Vitrification of oocytes, when applied to properly screened patients, will be a useful technology in reproductive medicine practice and will constitute a major step forward in ART. Today these results and the constant research to continue advancing to achieve even further improvements in the cryopreservation protocols have allowed the creation of egg banks around the world like OVOBANK that provide patients do not have to travel to find donors in cities where there isn´t a high variability of egg donors with similars results to those treatments with fresh eggs.

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Edited by Hideaki Yamashiro

Cryopreservation of germ cells has potential applications not only for production of next generations of animals but also for general reproductive biology including several field of biology. The present book Recent Advances in Cryopreservation is written by scientists from 5 countries in the field of somatic cells, fish, livestock and humans. In the near future, frontiers in cryopreservation will conceptualise new cryobiological ideas and technologies. We hope the book will be interesting to wide audience.

ISBN 978-953-51-5396-2

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