Title Studies on Cryopreservation of Zebrafish (*Danio Rerio*) Oocytes Using Controlled Slow Cooling.

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STUDIES ON CRYOPRESERVATION OF ZEBRAFISH (*DANIO RERIO*) OOCYTES USING CONTROLLED SLOW COOLING

by

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ABSTRACT

Cryopreservation of fish germ cells has important applications in aquaculture, conservation of endangered species and human genomic studies. Although investigations on cryopreservation of fish sperm and embryos have been carried out extensively, cryopreservation of fish oocytes has not been studied systematically. The objective of the present study was to develop successful cryopreservation protocol for zebrafish oocytes at temperature of liquid nitrogen (-196°C), or if unachieved, to investigate the limiting factors associated with fish oocytes cryopreservation. In this study, the effects of cryoprotectants exposure and enzymatic treatments on oocytes survival were studied, and new viability tests for zebrafish oocytes were developed. The effects of controlled slow cooling with different cryoprotective agents, in different freezing media and at different cooling rates on cryosurvival of zebrafish (*D. rerio*) oocytes were investigated. Cryomicroscopic observations on zebrafish oocytes were also carried out.

Three reliable vital tests – trypan blue (TB) staining, ATP assay, and *in vitro* maturation followed by germinal vesicle breakdown observation (GVBD) were found suitable for assessment of oocytes viability. Vitellogenesis (stage III) was found to be the optimal developmental stage for cryopreservation. Methanol was found to be the best CPA for zebrafish oocytes. Combination of 4M methanol and 0.2M glucose in potassium chloride (KCl) buffer was found to be the optimal cryoprotective solution. Controlled slow cooling at 0.3°C/min rate, combined with seeding at -12.5°C and plunge to liquid nitrogen (LN) at -40°C were found to be the optimal conditions for cryopreservation of stage III oocytes. However, even with the optimal protocol, TB-assessed viability, i.e. the ratio of oocytes with intact plasma membrane after cooling to -196°C was 19.6±8%. Furthermore, GVBD experiments showed that none of the cryopreserved oocytes can be matured *in vitro*, and their ATP levels were decreased dramatically, indicating that successful cryopreservation of fish oocytes at liquid nitrogen temperature still remains elusive. Cryomicroscopic observations demonstrated, that the damages of oocytes are associated with intracellular ice formation (IIF). IIF occurred simultaneously with extracellular ice formation (EIF) in nearly 100% of the cases, and formation of lethal hexagonal type of ice was observed.

This study was the first systematic attempt to cryopreserve fish oocytes at liquid nitrogen temperature. The results provided will undoubtedly assist successful protocol design for cryopreservation of fish oocytes in the future.

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DECLARATION

I declare that this thesis is my own unaided work. It is being submitted for the degree of Doctor of Philosophy at the University of Bedfordshire. It has not been submited before for any degree or examination in any other University.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER 2. MATERIALS AND METHODS

CHAPTER 3. DEVELOPMENT OF SUITABLE VIABILITY ASSESSMENT METHODS FOR ZEBRAFISH (*DANIO RERIO*) OOCYTES

CHAPTER 6. FURTHER STUDIES ON CRYOPRESERVATION OF ZEBRAFISH OOCYTES USING CONTROLLED SLOW COOLING

Fig.7.7 a,b. The moment of EIF and IIF. Cooling rate 1°C/min. 4M Methanol + 0.2 Glucose in Hank's was used as the cryoprotective medium. Images were taken with 0.3 sec interval

Fig. 7.8 a-d. The moment of EIF and IIF. Cooling rate 2°C/min. 4M Methanol in Hank's was used as the cryoprotective medium. Images were taken with 0.3 sec interval.

Fig. 7.9 a-j. Gradual IIF. 4M Methanol in KCl buffer and a thin layer of solution was used. Cooling rate was 1°C/min. Images were taken with 0.3 sec interval.

Fig. 7.10 a, b. IIF by homogenous mechanism (volume-catalysed nucleation, VCN). 4M Methanol + 0.2M Glucose in KCl buffer as a cryoprotective medium. After 30 min incubation, all extracellular medium was substituted by silicone oil. Cooling rate was 130°C/min. Images were taken in 0.3 sec interval.

Fig. 7.11 a, b. IIF by homogenous mechanism (VCN). 4M Meth in KCl buffer was used as cryoprotective medium. After 30 min incubation, all extracellular medium was substituted by silicone oil. Cooling rate was 1°C/min. Images were taken in 0.3 sec interval.

Fig. 7.12 a-c. IIF by homogenous mechanism (VCN). CPA-free KCl buffer was used as cryoprotective medium. After 30 min incubation, all extracellular medium was substituted by silicone oil. Cooling rate was 1°C/min. Images were taken with 0.3 sec inerval. Fig. 7.12c shows the same oocyte after thawing.

Fig. 7.13 a-f. Different stages of cryopreservation cycle. Cryopreservation medium 4M Methanol + 0.2M Glucose in KCl buffer. Oocytes were frozen using the optimal cryopreservation protocol.

Fig. 7.14, a-f. Oocyte during the stage of thawing (130°C/min). 4M Methanol + 0.2M Glucose in KCl buffer as cryoprotective medium; after 30 min incubation extracellular medium is substituted by silicone oil.

Fig. 7.15. a,b,c. Formation of cracks in the ice at temperatures below -100°C. 4M methanol + 0.2M Glucose in Hank's was used as a freezing medium. "Plunge" stage (cooling rate 130°C/min).

Fig. 7.16 a-l. 4M Methanol + 0.2M Glucose in KCl buffer was used as a freezing medium. Oocytes were frozen using optimal cryopreservation protocol, thawing rate was 130°C/min.

LIST OF ABBREVIATIONS

ADH	Alcohol Dehydrogenase
ADP	Adenosine 5'-diphosphate
AFGPs	Anti-Freeze Glycoproteins
ATP	Adenosine 5'-triphosphate
BHA	Butylated Hydroxyanisole
BSA	Bovine Serum Albumin
cAMP	Cyclic Adenosine Monophosphate
cFDA	Carboxyfluoresceine diacetate
CPA	Cryoprotective agent = Cryoprotectant
DHP	17α-Hydroxy-20β-dihydroprogesterone
DMSO	Dimethyl Sulfoxide
DSC	Differential Scanning Calorimetry
EG	Ethylene Glycol
EIF	Extracellular Ice Formation
FCS	Foetal Calf Serum
GVBD	Germinal Vesicle Breakdown
IIF	Intracellular Ice Formation
MTT	Methyl Thiazole Tetrazolium = Thiazolyl Blue
MPF	Maturation Promoting Factor
N-Ac-Cys	N-Acetyl-Cysteine
NOEC	the No Observed Effect Concentration
PCR	Polymerase Chain Reaction
PEG	Polyethylene Glycol
PG	Propylene Glycol
PVF	Peri-Vitelline Fluid
PVP	Poly-Vinyl Pirrolidone

SCN	Surface Catalysed Nucleation
SDH	Succinate Dehydrogenase
TB	Trypan Blue
VCN	Volume Catalysed Nucleation
Vg	Vitellogenin

CHAPTER 1: INTRODUCTION

1.1 Aim of the research and significance

Cryopreservation of fish germ cells is an important measure in conservation of fish genetic material, which will have huge implications for aquaculture and protection of endangered fish species. Although fish sperm has been successfully cryopreserved (Lahnsteiner et al., 1997; Kopeika et al., 1997), the satisfactory protocol for cryopreservation of fish oocytes and embryos still needs to be developed.

Attempts to cryopreserve fish embryos have been taken since the 1950's and more than 20 species were studied (Harvey, 1983; Zhang et al., 1993a, b; Rana & McAndrew, 1995; Liu et al., 2001a; Liu et al., 2001b; Liu et al., 1998; Hagedorn et al., 1996; Hagedorn et al., 1997; Zhang et al., 1989; Robles et al., 2005); however, only a very limited success has been achieved. Cryopreservation of fish embryos is difficult, as embryos have a number of specific structural properties that compromise success: a) the large size of fish embryos, resulting in low surface-to-volume ratio, which slows down the rate of water and cryoprotectants diffusion during cryopreservation; b) the presence of complex membrane system results in low permeability of fish embryo, which does not allow sufficient cell dehydratation and cryoprotectant penetration, leading to intracellular ice formation during freezing which is lethal to the embryos; c) the high yolk content of fish embryos linked to their high chilling sensitivity (Liu, 2000).

Several alternative approaches have been applied to circumvent the problems of fish female germplasm cryopreservation, such as androgenesis, cryopreservation of blastomeres and primordial cells (Takeuchi et al., 2001; Yoshizaki et al., 2003). However, these methods are expensive and require highly skilled technologies, which may have serious limitations in routine commercial use. Moreover, androgenesis doesn't allow the inheritance of certain genetic factors, which can only be transferred by maternal oocyte cytoplasm, such as mitochondrial DNA and maternal mRNA which controls early stages of embryonic development.

Successful cryopreservation of fish oocytes may circumvent a number of difficulties associated with fish embryo cryopreservation (e.g. low permeability of embryo), and may offer an satisfactory alternative to

cryopreservation of embryos. Once achieved, it will contribute greatly both to wildlife protection programmes and also for aquaculture, as the creation of cryobanks of female reproductive cells will allow the preservation of fish genetic material for unlimited periods of time, with possibility of obtaining the viable progeny whenever necessary. Fish oocytes cryopreservation has not been studied systematically.

- The objective of the present study is to develop effective cryopreservation protocols for fish oocytes using zebrafish as a model, or if unachieved, to investigate the limiting factors associated with fish oocytes cryopreservation

1.2 Principles of cryobiology

Cryobiology (from Greek word *κρυος*, which means "frost" or "cold", and "biology") is a branch of biology that studies life at low temperatures and impact of low temperatures on biological material. Cryobiology deals with any temperature below the normal physiological range and includes such areas as hypothermia, hibernation, medical organ storage in ice, etc. However, in most of the cases cryobiology is associated with sub-zero temperatures and the effects of freezing and thawing on biological objects. Cryopreservation is the process of preserving and storing living systems in a viable condition at low temperatures. Usually, biological materials are stored in dry ice (-80°C), suspended into vapours of liquid nitrogen (below -130°C) or immersed into liquid nitrogen (-196°C), with the liquid nitrogen storage being the most widely used method. At these low (cryogenic) temperatures, any biological activity, including the biochemical reactions that would lead to cell ageing and death are effectively stopped (Mazur, 1984). However, cryopreservation itself is associated with cell damage and even death, which occurs during freezing to cryogenic temperatures or on rewarming to physiological temperatures.

1.2.1 Cell damage at hypothermic temperatures: chilling injury and cold shock

In cryobiology, sub-physiological temperatures above freezing are called hypothermic temperatures. The thermal injury to cells at hypothermic

conditions can be divided into two major types: chilling injury during slow cooling, and chilling injury at fast rates of cooling (cold shock) (Belous & Grischenko, 1994).

One of the main mechanisms of chilling (hypothermic) damage is the disturbance of homeostasis balance, resulted from different rates of cold suppression of enzymatic and non-enzymatic processes inside the cell. Enzymatically catalysed reactions (for example, pumping of K^+ ions into the cytoplasm and pumping of Na+ ions from the cytoplasm by membrane channel enzyme Na^+/K^+ -ATPase) at low temperatures are suppressed to a much higher extent than spontaneous processes, such as diffusion of Na^+ and K^+ iones through plasmatic membrane. This effect can be explained by analysing Arrhenius equation (which describes the dependence of energy-consuming processes on the temperature) and comparing it with equation for diffusion (which is similar to all dissipation laws). Therefore, lowering of temperature disturbs cellular homeostasis, which, in turn, negatively affects metabolism and structure of the cell. Also, hypothermic injury is associated with oxidative stress and lipid peroxidation which can cause apoptosis. There is also an increase of free intracellular iron (Fe^{++}) during cold exposure, which aggravates hypothermic effects (Muldrew & McGann, 1999; Willis, 1987).

Cold (temperature) shock is another type of damage of cells at hypothermic temperatures, which is cooling-rate dependent and occurs mainly at fast cooling rates. Main characteristics of cold shock include: 1) damage and death of cells occurs only at fast rates of cooling; 2) incubation in hypertonic solutions just before cooling amplifies cold shock 3) ultra-rapid cooling (vitrification) doesn't cause temperature shock (Gordienko & Pushkar, 1994). As a result of temperature shock cells lose intracellular K^+ and become permeable to substances to which they are normally impermeable (e.g., sucrose). Studies on animals and plants show that mitochondria of the cells become over-permeable and swollen after tissue samples are stored at low temperatures (+4°C) after fast cooling (Rauen et al., 1999). These facts pose the evidence that cold shock damages cell membranes. However, membrane-associated proteins and membrane phospholipids can not be found in solution surrounding the cells exposed to cold shock (Gordienko & Pushkar, 1994). This suggests that damage of membrane in this case is not related to depletion of these components from the membrane structure. There is also an

evidence of phase transitions of membrane lipids in cells exposed to cold shock (for instance, in human erythrocytes these transitions occur at 8-12°C) (Gordienko & Pushkar, 1994). At these narrow temperature intervals membrane properties change dramatically: rotation of spin markers introduced to membrane slow; a "kink" in Arrhenius plots of membrane permeability is seen and osmotic stability of the cells changes. Numerous folds form at temperatures of phase transition. These phenomena are a result of lateral separation of lipids and formation of vulnerable cholesterol-starvated membrane areas, which occurs during membrane transition from liquid-cristalline to gel phase (Watson & Morris, 1987). The composition of the plasma membrane and the percentage of the unsaturated fatty acid fraction significantly affects the fluidity of the membrane, phase transition temperatures, and as a result, susceptibility of cells to chilling (Zeron et al., 2002; Parks, 1997). The coagulation of cytoplasmic proteins and distortion of their sol-gel transients during rapid cooling also contributes to cold shock (Gordienko & Pushkar, 1994).

1.2.2 Freezing injury

Freezing injury of biological materials occurs at temperatures below freezing point. This kind of injury is closely associated with formation of ice crystals inside and outside of the cells.

1.2.2.1 Physicochemical processes during freezing

When the temperature of the system decreases, a number of physico-chemical processes occur. Decrease of temperature slows down the Brownian motion of molecules in the system. When the temperature reaches freezing point (which is determined by Raoult's law), ice nucleation may occur. In some cases nucleation and ice formation occur straight away at the freezing point; but more often *supercooling* takes place. Supercooling happens when a liquid is cooled below its freezing point without freezing. Supercooled state normally is promoted either by high viscosity of the solution (which makes difficult for ice crystals to form and expand), or by absence of centres of crystallisation in the solution. The supercooled state is not stable and any disturbance of physical conditions may cause nucleation of ice and crystallisation of solution. But even in the case that heterogeneous nucleators

are absent in the solution, when supercooling reaches critical level, homogeneous ice nucleation occurs. The germs of ice crystals appear in solution and new water molecules join their crystal lattice, leading to expansion of the ice crystals. More detailed description of processes of ice formation is given in Chapter 7, dedicated to cryomicroscopy (Sec. 7.1.2.1).
In the majority of the cases ice initiation occurs in the extracellular medium, but later ice crystals are likely to appear inside the cells as well. This phenomenon is called intracellular ice formation (IIF). In practice, IIF in the cell can occur through one of several mechanisms: homogeneous nucleation due to critical supercooling of cytoplasm (volume-catalysed nucleation (VCN)); or more likely – through heterogeneous nucleation by extracellular ice crystals, which can contact the cytoplasm through damaged plasmatic membrane - surface-catalyzed nucleation (SCN). More detailed the mechanisms of IIF are described in Sec. 7.1.4, "Cryomicroscopic observations of zebrafish oocytes".

When the ratio of ice phase increases, the ratio of unfrozen phase decreases with decreased temperature. At a certain point the unfrozen overconcentrated solution in "tortuous channels" becomes saturated, and salts start to precipitate from solution in the form of crystals. As a rule, this process shifts pH balance of the solution to the acid field. All damaging factors listed above and associated with exposure of cells to hyperosmotic media in the course of freezing, are known in cryobiology by generalised term "solution effects" (Pegg, 1987).

Finally, the temperature reaches the point where the liquid phase in a sample disappears: the whole mass of biological sample consists of crystalline ice, dehydrated protoplasm, crystals of salts and solidified cryoprotectants. This temperature is called eutectic point. The eutectic temperature is determined by freezing temperature of saturated solution of dissolved substances. For example, the eutectic temperature of sodium chloride (NaCl) solution is -21.8°C, which is close to -20.09°C, the freezing temperature of 5.4M (saturated) NaCl solution, theoretically calculated by Raoult's law. The eutectic temperature of cytoplasm of most of cell types is near -40°C, and is determined by the presence of inorganic salts (mainly NaCl), small organic molecules and colloids of biopolymers in the cytoplasm,

such as proteins. All known cryoprotectants have the property of lowering the eutectic temperature of solutions. For example, eutectic temperature of 77% methanol solution in water is -138°C. At the temperature range under the eutectic point the whole mass of the sample resides in solidified state, excluding tiny fraction of membrane-bound and macromolecule-bound (vicinal) water, which freezes at -150°C. (Belous & Grischenko, 1994).

Biological samples are normally plunged into liquid nitrogen (-196°C) from a chosen temperature, with a cooling rate of about 300°C/min (=5°C/sec) (Karlsson et al., 1996). After the plunge, events may develop in two different ways. First, intracellular ice formation occurs with deleterious consequences for the cells. However, if intracellular solution reached the adequate concentrations of CPAs, proteins and salts at the plunge temperature, another event takes place: the remaining internal liquid is converted into a glass state (vitrified) during the rapid temperature fall associated with plunge of the sample into LN. Therefore, formation of amorphous state takes plase even with non-vitrification cooling techniques (Mazur, 1984; Woods et al., 2003). After samples are plunged into LN, all metabolic, diffusional and transfer processes in the cells are "frozen" and the biological samples can be stored at its temperature (normally -196°C) over years or even decades without any further loss of quality.

1.2.2.2 Effect of freezing on biological sample

Lowering of temperature leads to dramatic decrease of rates of metabolic processes inside the cells and virtually complete cessation of metabolic reactions at the moment when all liquid phases inside the cells transfers to solid state. Crystallisation of water into ice phase and the resulting increase of concentration of salts in the solution also exposes the cells to highly hyperosmotic solutions. The combination of these effects and their consequences is known in cryobiology as "solution effects". Ice crystals, especially when they are formed inside the cells (IIF), cause dramatic damage to cell structures: mechanical puncture of cell membranes and destruction of cytoskeleton. Mechanical squeezing and squashing of the cells between growing ice fronts may also occur (Pegg, 1987). More detail on the effect of extracellular and intracellular ice crystals on living cells is described in Sec. 7.1.4, dedicated to cryomicroscopy. Other deleterious processes such as

cold-induced activation of membrane phospholipases resulting in lysis of membranes; leakage of lysosomal proteases into the cytoplasm; hypoxia due to cold-induced exit of oxygen from the solution and other effects also accompany the freezing process (Belous & Grischenko, 1994).

During cryopreservation, if the cooling rate is slow enough, there is enough time for substantial amount of unbound intracellular water to be driven by osmotic force to the extracellular medium, cells become sufficiently dehydrated (so volume of the cells decreases to 60-65% of initial cell volume) and intracellular ice formation doesn't occur until low temperatures, such as -40°C (Rall et al., 1983); or even if it does occur, the small-granular types of ice form, which are relatively harmless for the cell (Belous & Grischenko, 1994). Also, in such case there is a very substantial probability that cell protoplasm can solidify into a vitrified state, avoiding the IIF completely (Mazur, 1984). On the contrary, if the cooling rate is too fast, the formation of intracellular crystals of hexagonal ice occurs, which has lethal consequences for cells.

1.2.2.3 Damaging factors associated with controlled slow (equilibrium) and fast rates of cooling. Cell damage during thawing and post-thawing treatment

Slow cooling (equilibrium freezing) cryopreservation protocols are those protocols which involve cooling rates less than 10°C/min.

Slow cooling protocols enable sufficient level of cell dehydratation and therefore decrease the possibility of formation of intracellular ice. However, slow cooling is accompanied by various harmful effects, which may have negative impact on cell survival. Firstly, during slow cooling cells are exposed to hyperconcentrated salt and cryoprotectant solutions due to convertion of unbound water to ice crystals resulting in higher concentrations of salts and CPAs in unfrozen solution. Dehydratation of cells which occurs during slow cooling may cause denaturation and coagulation of cytoplasmic proteins, and also changes in cell membranes (lateral separation of membrane lipids and proteins; formation of protein clusters; increased rigidity and fragility of the phospholipid bilayer; occurrence of tangential tensions), that strongly affect permeability of membranes and may lead to their rupture. The leakage of lysosomal enzymes to cytoplasm; K^+ efflux from the cell (so-called 'Gardos

effect'); depolymerisation of cytoskeleton may also occur (Fuller, 1991). These processes, occurring at sub-zero temperatures, are often referred as solution effects and osmotic shock (Mazur & Schneider, 1986). For most of the cell types lethal osmolarity value is around 1800-2000 mOsm, and the critical shrinkage volume is 50-60% of initial cell volume (Meryman, 1970).

There is evidence that the major damage to plasma membranes occurs due to critical osmotic pressure gradient across it (Muldrew & McGann, 1990). Generally speaking, the cell membrane is the most vulnerable target for cryoinjury. Lateral separation of lipids, spiculation and vesiculation, which occur during chilling, make membranes brittle and cause loss of membrane phospholipids. Such membranes are particularly sensitive to osmotic shock. Membrane proteins, which are normally evenly spread on the membrane surface, accumulate together to form "caps", protein clusters. This makes membrane more heterogeneous and more sensitive to stressing factors. When such an impaired membrane is exposed under pressure, a concentration of tensions around heterogenities occurs. When mechanical tension reaches certain threshold, non-separative permeability and later the rupture of bilayer occurs. Dehydration of cells may also result in formation of abnormal disulfide (-S-S-) crosslinks between protein molecules, which impairs the function of cytoplasmic and membrane enzymes and channels (Levitt & Dear, 1970; Gordienko & Pushkar, 1994).

The effect of concentration levels of salts on cells has been experimentally determined. For example, 0.15 mol/L NaCl solution is isosmotic and ideal for most of cell types. During freezing, extracellular ice forms and the concentration of NaCl in unfrozen solution reaches 0.7 mol/L, most of the cells shrink to their minimal volume (50-60% of the initial volume) and lose ~65% of intracellular water. When NaCl concentration in extracellular solution reaches 0.8 mol/L, the osmotic damage of membranes begins, resulting in leakage of sodium and potassium ions through membrane channel proteins and cells start to lose their intracellular stock of potassium ions (Gardos effect). Incubation of cells with solutions of non-electrolites with similar osmotic pressure (about 1300 mOsm) causes similar effects. When concentrations of salts in unfrozen medium reaches 3 mol/L, lyotropic effects of electrolytes on membrane begin to manifest and cell membranes may simply dissolve (Muldrew & McGann, 1997).

When the concentration of salts in unfrozen phase reaches certain level, some salts start to precipitate, shifting the extracellular pH value to the acidic area. It has been proved, that pH value of biological samples significantly changes during the course of freezing, that may, in turn, substantially affect the properties of the cells and cause additional damage during cryopreservation. With the presence of phosphate buffer, the pH of the solution captured in so-called "tortuous channels" decreases only by 0.5-1 units, whilst without any buffer it decreases more markedly (by 1.5 -2.0 units). Addition of CPAs, such as DMSO or glycerol, to the solution also lessens the fluctuations of pH in the medium during freezing (Belous & Grischenko, 1994). On the contrary, *intracellular* pH of the frozen cells tends to increase due to hyperosmotic shrinkage of the cells and, therefore, hypertonic alkalinisation. pH shifts in the sample trigger a variety of physiological processes in the cell, mostly undesirable, such as dissociation of cytoskeleton, rigidification (and fragilisation) of plasma membranes, denaturation of cytoplasm proteins, convertion of cytoplasm from *sol* to *gel* phase, changes in structure of oocyte yolk and even distortion of a normal cell cycle (Belous & Grischenko, 1994; Humphreys et al., 1995; Dube & Eckberg, 1996; Fagotto & Maxfield, 1994).

Warming of frozen cell suspension during thawing leads to rapid decrease of extracellular solute concentrations due to melting of ice. Solutes normally penetrate through cell membranes much slower than water (Gordienko & Pushkar, 1994), hence the equilibration of osmotic pressure between the cells and a medium occurs mainly through water influx to cells. Spiculation and vesiculation of membrane which has occurred in a course of freezing resulted in decrease of membrane surface, and therefore membranes of thawed cells will experience additional mechanical tension (Grout, 1991).

In vitrified samples, during thawing there is always danger of recrystallisation and formation of intracellular ice, which will completely deplete the advantages of vitrification technique. Recrystallisation involves either nascence of new ice crystals from destabilized glassified liquid (in vitrified samples), or re-formation and growth of existing ice crystals. Therefore, for vitrified samples high thawing rates should be chosen, in order to minimize the risk of recrystallisation. But even at samples which are frozen following equilibrum slow-cooling protocol, which involves ice-formation, there

is a risk of recrystallisation and emergence of intracellular ice during thawing, especially at temperature range around -30°C. During such process, small-granular ice crystals (which are relatively safe for cells) may recrystallise into large crystals of hexagonal ice, which are lethal. Application of rapid warming rates helps to prevent this type of lesion as well (Pegg, 1987). So, fast thawing is also a common practice for cells cryopreserved by slow cooling.

When thawed cells are washed from CPAs and placed in normal, cryoprotectant-free cell medium, damage and lysis may also occur. Loss of cells on this stage is caused by so-called post-hypertonic lysis. In the course of freezing membrane of cell becomes damaged, and therefore with increased permeability for extracellular substances which normally stay outside the cell (especially to sodium ions). Post-hypertonic lysis occurs, when after thawing cells with damaged membrane and sodium-enrichened cytoplasm osmotically absorb excessive amount of water from the medium, that leads to their swelling and rupture (Muldrew & McGann, 1997). 'Gardos effect', i.e. the loss of potassium ions by cell through compromised plasmatic membrane, aggravates post-thawing injury (Gordienko & Pushkar, 1994). Slow step-wise washing of cells from cryoprotectant helps to soften effects of post-hypertonic lysis.

Even if the cells survived cryopreservation and all risk factors occurring during thawing (recrystallisation, CPAs toxicity, post-hypertonic shock, Gardos effect, etc.), there is still a probability, that numerous sub-lethal injuries, accumulated in the cell in the course of cryopreservation, will aggravate and lead to the death of the cell within the first hours (days) after freezing-thawing.

1.2.3 Cryoprotective agents

Cryoprotectants, or cryoprotective agents (CPAs) are chemicals that reduce the injuries of cells during freezing and thawing. There are about one hundred substances which have been applied as cryoprotective agents. Substances of many chemical classes have cryoprotective properties such as: alcohols (methanol, ethylene glycol, propylene glycol, glycerol, etc.), oxides (dimethyl sulfoxide, pyridine-oxide), amino acids (alanine, valine, proline, serine), carbohydrates (glucose and numerous mono- and disaccharides) and proteins (albumin, gelatine). Each of these groups of chemicals has different

mechanisms of cryoprotective action. There are two types of cryoprotectants: intracellular (penetrating) CPAs and extracellular (non-penetrating) CPAs.

1.2.3.1 History

Biologists have been probing the low-temperature limits of life since at least 1663 when Henry Power reported the survival of eels frozen outdoors or in a freezing bath with salt and ice in the laboratory (Sittig, 1963). In 18th century, French scientists Bufon and Gamaile were the first to report that the reason for cell cryo-death was the formation of ice crystals inside the cells, resulting in the rupture of cells (Belous & Grischenko, 1994). Major contribution to early low-temperature biology was also made by Spallanzani in the end of 18th century (Sittig, 1963), who investigated the phenomenon of reviving frozen or dried fishes, amphibia and invertebrates. In 1893 Swiss physicist Pictet stated reciprocal dependence of cryostability of alive organisms on the complexity of their organisation. He also came to conclusion that all chemical reactions completely stop at temperatures below -30°C. This is in agreement with modern concept of eutectic temperature, below which all cell components would be in solid state and therefore any chemical conversions are virtually impossible (Belous & Grischenko, 1994). In the beginning of 20th century, Maximov pointed out that protoplasm of cells of frozen organism experiences pressure from ice crystals which grow in the intercellular space. That, in turn, results in compression and coagulation of colloidal particles of protoplasm. In Maximov's view, the mortality of frozen cells is determined mostly not by the freezing temperature, but by the quantity of ice which formed inside the tissue and by degree of tissue dehydratation. Maximov also pointed out the cryoprotective properties of glycerol and sucrose for plant tissues (Diller, 1997; Gordienko & Pushkar, 1994). Luyet & Gehenio (1940) investigated the microstructure of ice crystals in details, and studied the influence of freezing rate and numerous solutes (salts, cryoprotectants, proteins, sugars, etc.) on size and shape of ice crystals. Moran (1935) discovered the phenomenon of cold-induced glassification of concentrated gelatine solutions (gels), and therefore set the basis for modern practice of vitrification of samples in highly concentrated solutions of cryoprotectants. Polge et al. (1949) reported that the addition of glycerol to cell suspension prevents their cryoinjury. Thus, glycerol was the first substance to be discovered as cryoprotective agent. In 1950s

Lovelock (1953) developed the first integral theory of cryoinjury. Based on observations of frozen erythrocytes, he concluded that the main reason for cell destruction is the lesion of the plasma membranes. He also noted the dependence of cryostability of cell membranes on their lipid-protein composition. In 1960s, Levitt proposed that cell injuries resulted from dehydratation, which occurs due to ice formation, are connected to the formation of pathological disulfide crosslinks between proteins (Levitt & Dear, 1970). Towards the end of 1960s, Meryman formulated the hypothesis of "minimal volume", i.e. the volume limit shrinkage below which is lethal for the cells. He also developed the basic concepts for the theory of cell osmotic damage during cryopreservation (Meryman, 1970). In 1970s, Mazur offered an integral "two-factor" hypothesis of cryoinjury, which postulates, that low-temperature damage of cells is caused, on one side, by the effects of hyperconcentrated salt solutions, and on another side – by formation of intracellular ice crystals. This theory is well-grounded, and explains and predicts the majority of observed cryobiological phenomenons (Mazur, 1984; Mazur et al., 1986).

1.2.3.2 Penetrating (intracellular) cryoprotectants

Intracellular (penetrating) cryoprotectants are those cryoprotective substances which penetrate inside the cell and conduct their protective action inside the cytoplasm and organelles.

The most widely-applied and studied cryoprotectants are DMSO, glycerol, propylene glycol and ethylene glycol. Oxymethylated derivatives of these CPAs are also widely used. These substances penetrate inside the cells and tissues, and act as ice-formation suppressors, membranotropic agents and osmotic buffers.

1.2.3.3 Non-penetrating (extracellular) cryoprotectants

Extracellular cryoprotectants are those cryoprotective agents, which do not penetrate inside the cells, but conduct their protective action from outside. Sugars, especially disaccharides, are often added to cryoprotective media in combination with intracellular CPAs, and conduct their protective action through osmotic dehydratation of cells, therefore lessening the risk of intracellular ice formation. Sugars such as sucrose, trehalose, lactose,

maltose, xylose, mannose and ribose are commonly used, as well as polyols of similar structure, such as sorbitol, ribitol, inositol, mannitol and erythrol. Another class of extracellular CPAs are various polymeric substanses. Polymers such as Poly-Ethyleneglycols (PEGs) and Poly-Propyleneglycols of different molecular weight, Poly-Vinyl Pirrolidone (PVP), HydroxyEthyl-Starch, variety of Ficolls and dextranes are used as extracellular cryoprotectants. Mechanism of action of polymeric compounds is mainly related to their membrane-protective effect. Molecules of these polymer substances form a sheath around plasmalemma, therefore stabilising it and promoting small-granular ice formation. In some cases "fortification effect" takes place, i.e. incrustation of polymer molecules into plasmalemma, and therefore its reinforcement (Belous & Grischenko, 1994). Also, it's reported, that polymers, e.g. PVP, act as "salt buffers", decreasing effective salt concentrations, alleviating "solution effects", and decrease the ratio of unbound water in the extracellular solution (Ashwood-Smith, 1987). In recent years, antifreeze glycoproteins (AFGPs) from the serum of polar fish species are also used as extracellular CPAs and ice-suppressors for various cell types (Feeney et al., 1974; Weng et al., 2000; Wu et al., 1998; Rubinsky et al., 1991). Hen egg yolk suspension is also often added to cryoprotective media, and is known for its properties to decrease the toxicity of CPAs and to protect membrane integrity, therefore improving cryosurvival of the cells (Isachenko & Nayudu, 1999).

Besides these cryoprotectants, there is also a large number of other substances, which are added to freezing medium in order to achieve better cryosurvival of frozen cells. While major cryoprotectants (DMSO, PG, EG, methanol, glycerol etc.) are used in high concentrations (0.5M - 2M or higher) to lower the freezing point of intracellular solution and decrease the amount of unbound water and suppressing intracellular ice formation, minor supplements are used in much lower concentrations and act by protecting vulnerable areas of the cell. These substances are: membrane-stabilising agents polyethylene glycol-400, polyethylene glycol-200, tocopherol, dexamethasone, acetyl salicylic acid, egg yolk suspension, glucose, proline, etc.; dehydrators sucrose and glucose, and antioxidants butylated hydroxyanizole (BHA), N-Acetyl-Cysteine, tocopherol, taurine, et cetera.

1.2.3.4 Mechanisms of protective action of cryoprotectants

Discussions in this section are focused on those commonly used penetrating cryoprotectants which are going to be used in the present study.

Methanol (CH_3OH, MW = 32; eutectic temperature = -138°C; eutectic concentration = 70%) is a cryoprotectant which has been successfully used for freezing for fish sperm (Lahnsteiner et al., 1997, Horvath et al., 2003), horse embryos (Bass et al., 2004), mouse embryos (Rall et al., 1984) and many other types of cells. It was also showed to be the best CPA in experiments with freezing of fish embryos (Liu et al., 1998; Chen & Tian, 2005), and in hypothermic storage of various cell types, including fish embryos (Ahammad et al., 1998). Methanol is well documented to be very permeant to embryos and oocytes of zebrafish and another species (Zhang et al., 2005; Walsh et al., 2004; Liu et al., 2003; Rall et al., 1984; Hagedorn et al., 1996; Czlonkowska et al., 1991). Methanol penetrates cells at a rate comparable to the rate of water transport (Walsh et al., 2004; Rall et al., 1984; Czlonkowska et al., 1991); and therefore, doesn't result in pronounced cell osmotic stresses during their incubation. Methanol has relatively low toxicity for fish at organism and cellular level; which is supported by numerous animal exposure experiments (Chemical Summary for Methanol prepared by Office of Pollution Prevention and Toxics U.S. Environmental Protection Agency, 1994; http://www.epa.gov/chemfact/s_methan.txt). Methanol is an effective CPA in controlled slow cooling experiments and vitrification techniques, although there are some reports indicating that methanol doesn't vitrify even at the highest concentrations possible (Ali & Shelton, 1993).

DMSO (CH_3SOCH_3; MW = 78; eutectic temperature = -66.5°C; eutectic concentration = 70%) is the most widely used CPA. It penetrates biological samples rapidly, and carries out its protective action colligatively, by lowering the freezing point of intracellular solution, decreasing the amount of unbound water, providing small-granular ice formation. The beneficial effect of DMSO includes its membranotropic action and properties to act as a "salt buffer". Due to its specific structure and amphiphylic nature, DMSO is able to form strong hydrogen binding with numerous substances such as water, solute salts, sugars, phospholipids and proteins, therefore increasing cell

cryostability. High affinity of DMSO to water molecules due to formation of hydrogen bonds influences the emergence and growth of ice crystals during freezing. The presence of DMSO in solution strongly promotes the formation of "safe", small-granular forms of ice. DMSO decreases effective amount of salts during freezing, therefore alleviating "solution effects". DMSO is also reported to be a membrane modifier and plastifier (Orvar et al., 2000; Yamamoto, 1989). It has been reported that cells with more fluid plasmalemmae survive freeze-thawing better than cells with rigid membranes (Graham et al., 2005; Zeron et al., 2002). DMSO fluidises plasma membranes during freezing, so that membrane becomes less brittle and less likely to be disrupted. Moreover, fluid state of the plasmalemma resulting from DMSO treatment means that any holes produced by ice crystals propagating through membranes can be resealed during thawing (Shier, 1998).

Ethylene glycol ($HOCH_2CH_2OH$; MW 62; eutectic temperature -63°C; eutectic concentration 76%) and propylene glycol ($HOCHCH_3CH_2OH$; MW 76; eutectic temperature -57°C; eutectic concentration 60%) are similar compounds, that differ only by presence of an additional methyl group in PG molecule. Solutions of these CPAs strongly promote small-granular crystallisation and amorphous solidification due to their high viscosity at low temperatures. They also lessen the quantity of unbound water within cells, therefore supressing intracellular ice formation. The advantage of Propylene glycol is its low cytotoxicity at cellular and organism level due to similarity to natural biochemical intermediates. The advantage of Ethylene glycol is its high penetration rate into cells of most types. Ethylene glycol is the main permeating CPA for cryopreservation of eggs of insects (Liu et al., 2003). Both Ethylene glycol and Propylene glycol are widely used for vitrification of mammalian oocytes and ovarian tissue. However, relatively high toxicity of Ethylene glycol to cells limits its application (Belous & Grischenko, 1994).

1.2.3.5 Mechanisms of toxicity of cryoprotectants

Although cryoprotectants are essential in successful cryopreservation of biological materials, all cryoprotective agents are damaging to cells themselves, and especially at high concentrations. Each CPA has its own mechanism of toxicity, but they all have certain similar effects on cell. Addition

and removal of CPAs causes inevitable osmotic stress to the biological sample and "strains" plasma membranes of the cells (Armitage, 1987; Gordienko & Pushkar, 1994). At high concentrations, CPAs cause denaturation of proteins, through distortion of their secondary and tertiary folding structure and therefore impairing the function of enzymes, structural proteins and channels of protoplasm. CPAs in high concentrations may also lead to disarrangement of membrane bilayers, therefore deteriorating the whole cell structure. There is a generic theory for toxicity of CPAs which indicates that the damaging effect of cryoprotectants at room temperatures results from the same properties of CPAs which promote their stabilizing effect at sub-zero temperatures. At low temperatures, CPAs molecules are being thermodynamically excluded from hydration shells of proteins, therefore stabilising the proteins, whilst at the room temperatures proteins are destabilised because the hydrophobic interaction between protein and CPAs molecules becomes substantial (Arakawa et al., 1990). The specific mechanisms of cytotoxicity of the main cryoprotectants used in this study will be described in details in Chapter 4, dedicated to CPAs toxicity (Sec. 4.1).

1.2.4 Controlled slow cooling as a method of cryopreservation

The procedure of controlled slow cooling of the biological sample to the required temperature at established cooling rate and according to specific cryo-profile has become possible due to availability of PC-controlled programmable coolers of new generation. Such devices allow to cool the sample according to strictly defined programme, by pumping the vapour of liquid nitrogen through the sample-containing chamber with defined intensity. The availability of controlled programmable cooler provides a valuable tool for the cryobiologist.

In controlled slow cooling, the crucial parameters which determine the success of cryopreservation are: cooling rate, ice-seeding temperature, liquid nitrogen plunging temperature, and thawing rate. The cooling rate is the rate at which samples are cooled. Factors affecting the choice of optimal cooling rate are discussed in detail in Section 1.2.4.1. Ice-seeding temperature is a temperature at which procedure of ice-seeding (i.e. deliberate initiation of ice nucleation) is carried out. The aim of ice-seeding is to avoid excessive supercooling of samples and therefore lessen the risk of IIF. Liquid nitrogen

plunge temperature is a temperature at which slow cooling of the samples in a programmable cooler is terminated and samples are plunged into liquid nitrogen (cooling rate at this stage reaches ~300°C/min) for long-term storage. The optimal plunge temperature should allow minimal (ideally <5%) risk of IIF in the cells (Mazur, 1984), and at the same time not to overexpose the sample to deleterious "solution effects". More in detail, the factors associated with ice-seeding and plunge temperature are discussed in Chapter 6.

1.2.4.1 Factors affecting the choice of cooling rate

The optimal cooling rate for equilibrum freezing for each cell type is determined by the "golden mean". "Golden mean" is the cooling rate which is slow enough to allow sufficient degree of cell dehydratation during freezing (which prevents intracellular ice formation), and also fast enough to avoid over-exposure of cells to toxic effects of CPAs and concentrated salt solutions (Muldrew & McGann, 1997). Optimal cooling rate is cell type specific and is determined by parameters such as the size of cells, their surface-to-volume ratio, permeability of their plasmatic membrane to water and cryoprotectant, degree of cell hydration; thermodynamic coefficient of homo- and heterogeneous ice nucleation, and the dependency of membrane permeability on temperature. Optimal freezing rate of cells is closely linked to the rates of water and cryoprotectant transport across cell membrane of that particular type of cells.

One of numerous methods of estimation of the optimal cooling rate was proposed by Indian researcher (Sreedhar, 2004). These calculations are based on the assumption that optimal cooling rate is the maximum cooling rate which leaves 5% of osmotically active water trapped inside the cell at -15°C (-15°C is the assumed temperature at which water transport ceases). Sreedhar's equations provide theoretically calculated values of optimal cooling rates for a given cell type.

The general trend in cryobiological practice is: the bigger the size of the cell, the smaller its surface-to-volume ratio, the lower its membrane permeability – the slower cooling rate should be used for its cryopreservation. Increase of CPA concentration in a sample normally lowers the value of optimal cooling rate (Mazur et al., 1970; Gordienko & Pushkar, 1994).

Optimal cooling rates of equilibrum freezing for vast majority of animal cell types are in the range of 0.1°C/min – 10°C/min. The optimal cooling speed largely depends on properties of cryopreserved cells. So, for human erythrocytes the optimal speed of freezing is about 3000°C/min; for cells from hamster kidney – 100°C/min; for the yeast cells – 10°C/min. For oocytes and embryos of most studied mammalian species optimal cooling rates are in the range of 0.3°C/min - 0.5°C/min (Bass et al., 2004; Picton et al., 2002; Visintin et al., 2000; Karlsson et al., 1996; Stachecki et al., 1998; Carroll et al., 1993; Crister et al., 1988; Lassalle et al., 1985; Czlonkowska et al., 1991; Czlonkowska et al., 1984). The cooling rates for cryopreservation of macroscopic samples of mammalian ovarian tissue are in the similar range: 0.3°C/min - 0.5°C/min (Yi et al., 2001; Shaw et al., 2000; Candy et al., 2000; Cox et al., 1996; Newton et al., 1996; Harp et al., 1994). Few examples of application of faster cooling rates (2°C/min) also can be found in the literature (Salle et al., 1999).

For cryopreservation of fish embryos, slow cooling at such rates as 0.07°C/min, 0.3°C/min and 0.5°C/min were applied (Zhang et al., 1989; Zhang et al., 1993; Rana & McAndrew, 1995), as well as vitrification techniques (Robles et al., 2005; Chen & Tian, 2005).

1.2.4.2 Ice seeding

Ice seeding is a procedure of deliberate initiation of ice nucleation commonly practiced in cryobiology to avoid excessive supercooling of samples and therefore lessen the risk of intracellular ice formation. Ice seeding is commonly conducted by touching the sample holders by forceps which have been previously held in liquid nitrogen. This causes the local cold spot on the wall of the container, which leads to ice nucleation in adjacent solution and spreading of ice front throughover the sample.

The ice seeding temperatures, which effectively prevent IIF are advantageous for cells, meanwhile the ice seeding temperatures, which are too low and hence promote IIF because of excessive degree of supercooling, are damaging for cells.

Different ice seeding temperatures are used by cryobiologists. It is common to use ice seeding temperature of -7°C for 1.5 molar concentration of cryoprotectants (Stachecki & Willadsen, 2000; Stachecki et al., 1998; Picton

et al., 2002; Yi et al., 2001; Salle B et al., 1999; Lassalle et al., 1985; Carroll et al., 1993), although different seeding temperatures such as -5°C (Crister et al., 1988); -8°C (Candy et al., 2000); -9°C (Newton et al., 1996) for 1.5M concentration of CPAs were also used. For 2M concentration of CPAs seeding at -7.5°C is common, although seeding temperatures so high as -6°C (Bass et al., 2004) and even -5.5°C (Zhang et al., 1989) have been used. For 3M concentration of methanol, seeding temperature -10°C has been successfully applied (Czlonkowska et al., 1991) for freezing of sheep embryos.

More in details the problems which are associated with choice of ice-seeding temperature will be described in Chapter 6, Section 6.1.4.

1.2.4.3 Liquid nitrogen plunge

Plunge temperature is the temperature at which slow cooling of the samples in a programmable cooler terminates, and samples are plunged into liquid nitrogen for long-term storage. When samples are being plunged into liquid nitrogen, the temperature of the sample decreases very rapidly (200-300°C/min) until it stabilises and equilibrates with the temperature of liquid nitrogen (-196°C). Plunge temperatures used in cryopreservation practice are normally below the eutectic temperature of water-salt-cryoprotectant system (-20°C - -40°C). This means that at plunge temperature almost all water inside and outside the cells is in the solid (ice) phase; excluding small fraction of membrane-bound (vicinal) water, which freezes at approx. -150°C (Belous & Grischenko, 1994). After the samples are equilibrated with the temperature of liquid nitrogen at -196°C, all biochemical and physical processes in the biological material – metabolic reactions, diffusional processes, dissipation of energy, membrane movements and motions of proteins and nucleic acids, etc. - are virtually stopped (frozen), and samples can be stored in such state over years or even decades practically without any further loss of viability.

1.2.4.4 Thawing

Thawing is an important stage of cryopreservation cycle, as during thawing many processes may occur, which may negatively affect the viability of preserved cells.

Slow thawing is usually conducted in a programmable cooler, or less often by exposing the samples to the air at room temperature. Fast thawing is normally conducted through immersing the samples into a water bath with a set temperature (usually 20°C – 38°C). Some practitioners also use high-frequency electromagnetic fields (i.e. special "microwave ovens") for thawing samples, however "microwave thawing" was sometimes considered inadequate, as it creates dramatic differentials of temperatures throughout the sample, and also the "islets" of high temperatures, which can also damage the cells (Robinson et al., 2002). Thawing of cells in water or air also has certain limitations, including uneven warming of the samples and the lack of controls over the process.

Most of cryobiologists considered the fast thawing as more advantageous, as it lessens the risk of recrystallisation and formation of large ice crystals inside the sample. Fast thawing also shortens the time of exposure of cells to CPA solutions at above-eutectic temperatures (Gordienko & Pushkar, 1994). However, there is an opinion that with application of slow cooling rates, the effect of thawing rate on viability of the sample is not essential. The study by Abbeel et al. (1994) showed that the choice of thawing rate should be determined by the temperature at which cryopreserved samples were plunged into liquid nitrogen, and also by the nature of the cryoprotectant used (Abbeel et al., 1994).

1.2.4.5 Removal of cryoprotectant and post-thaw handling

After the samples are thawed and warmed to normal (physiological) temperatures, it is necessary to remove the cryoprotectants. This normally means washing cells in cryoprotectant-free cell medium. During these procedures, osmotic stress is induced for the cells due to drastic transient of osmotic pressure across the plasmatic membrane. In order to ameliorate this undesired effect, step-wise dilution of cryoprotectants is often used (Wessel & Ball, 2004). When one-step equilibration is used, the cells are placed straight

into CPA-free medium until virtually all CPA gets diffusionally removed from the cells.

After cryoprotectant removal thawed cells are often incubated in specially designed media, supplemented with raised concentrations of nutritional substances and membrane-stabilisers, such as sugars, in order to enhance the recovery of the cells from damages associated with freeze-thawing they undergone (Belous & Grischenko, 1994). After that thawed cells can be used for clinical, practical or research purposes.

1.3 Cryopreservation of oocytes and embryos of other aquatic species

Cryopreservation of fish gametes has been studied extensively in the last three decades. The successful cryopreservation of spermatozoa from many species including salmonid, cyprinid, silurid and acipenseridae fishes is well documented (Rana & Gilmour, 1996; Maisse, 1996; Lahnsteiner et al., 1997). In the last 25 years attempts to cryopreserve fish embryos have been conducted on about 20 fish species (Harvey, 1983; Zhang et al, 1993). Although a few embryos have been shown to survive for a short period of time after cooling to liquid nitrogen, these results could not be repeated, and successful cryopreservation of fish embryos still remains elusive (Zhang et al., 1989; Robles et al., 2005; Chen & Tian, 2005; Kopeika et al., 2006). Cryopreservation of fish oocytes has not been studied systematically. Next few sections review the experience on cryopreservation of aquatic gametes more in detail.

1.3.1 Cryopreservation of fish embryos

There are few reported cases of successful cryopreservation of fish embryos. Unfortunately, none of them can be repeated. Zhang et al. (1989) cryopreserved the embryos of common carp (*Cyprinus carpio*) using controlled slow cooling (cooling rate -0.07°C/min; 2M DMSO as cryoprotectant), 18.8% (3 of 16 embryos) survived after thawing. There is also a recent report on relatively successful vitrification of Japanese flounder (*Paralichthys olivaceus*) embryos in a concentrated mixture of CPAs: 20% propylene glycol with 13% methanol. Hatching rate of 5% was reported in this study (Chen & Tian, 2005). Kopeika et al. (2006) reported 3-hour post-thaw survival and movement of a single zebrafish embryo, microinjected with

sucrose to 0.5M concentration and cooled at 0.3°C/min to -25°C. The most remarkable point in this report is that the embryo, which survived for 3 hours after thawing, had undergone total intracellular crystallisation. In another recent study, winter flounder (*Pseudopleuronectes americanus*) embryos were vitrified by direct plunge into the liquid nitrogen, using a complex mixture of cryoprotectants containing 5M DMSO, 1M EG, 2M methanol and 10% sucrose. Although no hatching was achieved, some of the thawed embryos (0.92% of total number of frozen embryos) have shown the development and movement inside the chorion during up to 5 days after freeze-thawing (Robles et al., 2005).

Other key studies on fish embryo cryopreservation are listed below. In the report of Rana and McAndrew (1995) zebrafish heartbeat stage embryos were frozen in 1M DMSO, methanol, EG or glycerol after 1 hour equilibration using slow cooling (-0.1, -0.5, -1°C/min to -10, -15, -20, -25, -30°C). The best results were obtained with DMSO (12% hatching rate at -25°C; with cooling rate of 0.5°C/min). Similar results were also obtained with rosy barb embryos, which gave 19% hatching rate under the same conditions.

Intensive studies on cryopreservation of zebrafish embryos have also been carried out in our laboratory and by other groups (Zhang et al., 1993a; Zhang et al., 1993b; Liu et al., 2001a; Liu et al., 2001b; Liu et al., 1998; Hagedorn et al., 1996; Hagedorn et al., 1997) using both controlled slow cooling and vitrification. Best results were obtained with 2M methanol, which allowed 8% embryos survival at -25°C. Howewer, no embryos have survived cooling to -30°C (Zhang et al., 1993a).

1.3.2 Cryopreservation of oocytes and embryos of aquatic invertebrates

Cryopreservation of eggs of other aquatic species is associated with similar difficulties, as the cryopreservation of fish oocytes. Studies on cryopreservation have been carried out with the eggs of aquatic invertebrates, including shrimp, crab, rotifer, polychaetae, oyster, abalone, starfish, sand dollar and sea urchin. Cryopreservation has been conducted with sperm as well as oocytes, embryos and larvae. Majority of studies have been conducted with comercially important species of oyster and shrimp. Successful spermatozoa cryopreservation has been reported for more than 30 species of

aquatic invertebratae (Gwo, 2000). A limited success has been reported on the cryopreservation of eggs and embryos of aquatic species including the oocytes and embryos of Pacific oyster (Lin & Chao, 2000; Smith et al., 2001; Tervit et al., 2005), larvae of Eastern oyster (Paniagua-Chavez et al., 1998), embryos of hard clam (Chao et al., 1997), larvae of sea urchin (Naidenko & Koltsova, 1998), embryos of rotifer (Toledo & Kurokura, 1990) and the juveniles of marine polychaetae (Olive & Wang, 1997). Starfish (*Nardoa variolata*) immature oocytes don't survive cryopreservation by controlled slow cooling up to date, but vitrification of these cells has brought some limited success, with 51% of frozen-thawed cells which have been able to complete maturation, and several eggs which were able to be fertilized (Kaseoglu et al., 2001; Hamaratoglu et al., 2005). With commercially important Echinodermate sea urchin (*Evechinus chloroticus*), although sperm and larvae have been cryopreserved efficiently, the successful cryopreservation of oocytes and fertilized eggs still remains elusive (Adams, 2003).

Successful cryopreservation of molluscs oocytes remained elusive until recently (Grout et al., 1992). Major breakthrough in this field was made in the beginning of our century by New Zealand group of researchers: Pacific oyster (*Crassostrea gigas*) eggs have been cryopreserved at liquid nitrogen temperature with up to 25% post-thaw fertilization rate (Smith et al., 2001). The achieved success was even enhanced in more recent studies, where up to 50% of oyster oocytes survival was reached with slow (0.3°C/min) cooling and 10% ethylene glycol as cryoprotectant (Tervit et al., 2005).

1.4 Use of zebrafish (*Danio rerio*) as a model system

1.4.1 General information on zebrafish; breeding and handling.

Zebrafish (*Danio rerio,* or *Brachydanio rerio*) is a tropical Cypriniform, representative of the family *Cyprinidae*. It is a freshwater teleost species, originally found in slow streams and rice paddies of the East India and Byrma (Axelrod & Schultz, 1955). The fish rarely exceeds 4 - 5 cm in length and has a cylindrical body with 7-9 dark blue horizontal strips on silver, which run into the caudal and anal fins, and an olive green back. Males are slimmer than females and possess a golden sheen, while females are more silverly and their abdomen is bigger, particularly prior to spawning. The fish are capable of

withstanding wide ranges of temperature (15.5 – 43.3°C) and pH (6.6 – 9.2) (Axelrod & Schultz, 1955).

Zebrafish have a number of qualities (including uncomplicated maintenance, short generation time, high fecundity, all-year round reproduction, transparency of eggs, etc.), making them a convenient model species for studies on fish oocytes (Westerfield, 2000; Kimmel et al., 1995).

1.4.2 Development of zebrafish oocytes

The development of the zebrafish oocytes is divided into five stages based on morphological criteria and on physiological and biochemical events (Selman et al., 1993).

Stage I After several mitotic divisions, oogonia (early germ cells in the fish ovary) become early oocytes which contain chromatin-nucleolus and perinucleolus.

Stage IA: pre-follicle phase of primary growth (chromatin-nucleolus stage). During this stage oocytes progress through the early stages of prophase 1 of meiosis and arrest in diplotene, thus, "freezing" before completion of the 1-st meiotic division.

Zebrafish oocytes in the primary growth stage have diameter 20-140 μm. The first stage of oocyte development involves the development of basic cellular structures such as enlargement of the nucleus and appearance of multiple nucleoli and other subcellular organelles. There is a substantial amount of protein synthesis in the developing oocyte which is referred to as "endogenous vitellogenesis". Oocytes are located on the periphery of the ovarian lamellae, either isolated or more often, forming cysts (nests). Transmission electron microscopy reveals the presence of a large nucleus in a central position surrounded by a rim of cytoplasm, where electron-dense material, clusters of mitochondria and Golgi complexes are found. Chromatin, initially of a delicately granular appearance, progressively becomes condensed in several variably sized nucleoli.

At this stage, follicle cells are present, but do not completely circumscribe individual oocytes, and the zona radiata is not distinct.

Stage IB: follicle phase of primary growth (perinucleolus stage)

Oocytes at this stage don't exceed 140 μm in diameter. A single layer of prefollicle cells initially surrounds the entire nest of early meiotic oocytes in the zebrafish ovary. At this stage, as the cells in the oogonia nest cellularize, a sheath of squamous follicle cells surrounds individual oocytes that are in the pachytene stage of the first meiotic prophase. The cells within the zebrafish follicle are in intimate contact during the growth period; long processes – macrovilli - extend from the follicle cells towards the oocyte and microvilli originating from the oocyte penetrate deep into the spaces between adjacent follicle cells. Follicle cells undergo dynamic changes in morphology. They increase gradually in height to become more cuboidal and then adjacent cells separate from each other to form intercellular spaces. Later, two cell layers, the theca and granulosa cells can be easily distinguished in a follicle and support further growth. The theca and granulosa cells are responsible for production of reproductive steroid hormones that regulate successive stages of reproduction. Inner, granulosa layer is composed of cells with flattened morphology and showing cellular extensions-macrovilli that extend towards the oocyte surface. The space between the oocyte and granulosa layer is progressively occupied by growing zona radiata, composed from a material that appears to be synthesized both by the oocyte and liver. The cytoplasmic extensions of the granulosa cells pass through the pores of zona radiata. At the end of stage I, there is a well defined oocyte encased in its follicle.

Stage II (cortical-alveolar stage). Zebrafish oocytes of this stage have a diameter 140-340 of μm. The major event at this stage is the appearance of cortical alveoli on the periphery of the oocyte – the organelles, which will play an important role at fertilization: the contents of cortical alveoli will be released into perivitelline space after fertilization to form perivitelline fluid and prevent multiple sperm entry. As stage II proceeds, cortical alveoli continue to accumulate and increase in size. Later, at vitellogenesis, growing yolk mass will displace them towards the periphery cortex. In the later phase, the ooplasm gradually loses its basophilia, while the space appears between the

granulosa layer and the oocyte surface (Matova & Cooley, 2001; Bardakci et al., 2000).

Stage III (vitellogenesis). Vitellogenesis is the synthesis and uptake of egg yolk proteins from the plasma. The diameter of vitellogenic oocytes ranges from 340 to 690 μm. Vitellogenesis regulation involves the interaction of the anterior pituitary in the brain, the follicle cells, the liver and the eggs. The anterior pituitary of the fish produces hormones known as gonadotropins and releases these hormones into the circulation. The gonadotropins directly stimulate the theca and granulosa cells to produce estrogen which travels in the blood to stimulate the liver to produce vitellogenin, which is the precursor to the egg yolk proteins. Vitellogenin is secreted into the blood and is taken up by the oocyte through pinocytosis, mediated by specific receptors. The receptor-mediated uptake of vitellogenin is developmentally regulated, Ca^{++}-dependent, and the oocytes must reach a certain size before they are able to sequester vitellogenin. Vitellogenin, after it is adsorbed by oocyte, is accumulated in the yolk platelets (yolk bodies), where it is further processed into smaller yolk proteins (lipovitellin, phosvitin, β-component) by enzyme cathepsin D (Hartling & Kunkel, 1999). Lipovitellin and phosvitin are stored in yolk bodies in crystallised form until they are used as a nutrition by developing larva. The enzymes of same cathepsine family conduct full proteolysis of yolk proteins later in embryogenesis, during consumption of the yolk by the growing embryo. Vitellogenesis is the longest phase of oocyte development and requires a great amount of nutrient input. Yolk vesicles first appear in the cytoplasm surrounding the nucleus and gradually increase in number and size, while moving towards the periphery. A membrane delimits yolk vesicles. Apart from yolk bodies, many oil droplets also appear in ooplasm. As vitellogenesis proceeds, most of oocyte cytoplasm become occupied by yolk bodies, which afterwards in the course of stage IV (maturation) will be fused with each other to form yolk mass. At the beginning of vitellogenesis stage zona radiata surrounding oocyte becomes more distinct. During vitellogenesis the ooplasm continues to lose its basophilia (Wallace & Selman, 1981; Wallace & Jared, 1976; Campbell & Jalabert, 1979; Riggio et al., 2003; Ayzenshtadt, 1984).

Stage IV (maturation). Maturating zebrafish oocytes have a diameter larger than 690 μm. Maturation is triggered by the steroid hormone progesterone. Calcium signalling also takes place; the oocytes incubated in calcium-absent medium fail to pass maturation (Smith & Ecker, 1969). It is the final stage of oocyte growth, resulting in stage V oocyte – mature egg. In zebrafish, maturation phase is very short and normally takes up to 4 hours. During this stage, the nucleus of the egg migrates from the center of the egg to the periphery and the second division of meiosis resumes but pauses again before completion. The membrane surrounding the nucleus disappears in a process referred to as germinal vesicle breakdown (GVBD). Certain uptake of water by oocyte occurs during maturation, although in zebrafish this process is much less pronounced than in fish of seawater species. In zebrafish, hydration results in increase of volume of oocytes by 10-15%. The process of oocyte hydration is driven by cleavage of yolk proteins (mainly Vitellogenin-I) into free amino acids, which increases osmolarity of oocyte cytoplasm and creates force to drive the water influx into the egg. Swelling of oocytes during maturation can often be seen in females as the belly becomes even more distended and firm. During the oocyte maturation the oil droplets are concentrated around the nucleus and, as may be observed with light microscopy, they then coalesce into one big oil drop that migrates towards the animal pole, together with the nucleus. The yolk granules progressively fuse to form a continuous mass of fluid yolk. As a consequence, the ooplasm becomes displaced to a peripheral rim surrounding the yolk mass. The yolk becomes clear and translucent as hydration is completed.

In the late maturation phase, the oocyte is called a 'hydrated oocyte'. Large irregular vacuoles and small oval vesicles may be observed in this cytoplasm. These vesicles, the 'cortical alveoli' have a size and consistency lower than, and an appearance lighter, than yolk vesicles. Oocyte maturation also leads to changes in activity of a number of enzymes, particularly of carbohydrate metabolism. Carbohydrate metabolism changes in direction favoring glycogenolysis and cessation of gluconeogenesis (Klyachko et al., 1995).

Maturation results in **stage V, mature egg** (700-750 μm in diameter), which is ready to be spawned into the fresh water and fertilised. In

appearance the mature egg is homogeneous, finely granular, and weakly basophilic. The nuclear contents are diffuse in the ooplasm. The ooplasm, restricted to a narrow rim, lies beside the zona radiata at the oocyte periphery. When maturation is complete, the oocytes are ovulated from the follicle teguments. Firstly, the zona radiata becomes more compact during its transformation into the chorion. It decreases in thickness. The pore canals, crossing the zona radiata, reach a diameter of approximately 0.48 µm and transverse striations are no longer evident. Just prior to oocyte ovulation, follicle cells become flat, most cytoplasmic projections disappear, and contacts between follicle cells and the oocyte are broken. As processes extending from follicular cells towards oocyte (macrovilli) are withdrawn, nothing attaches the egg to its surrounding follicular sheath. The entire follicular epithelium is then "pulled away" from the egg, and the oocyte is now situated in the ovarian lumen and surrounded only by its own plasmalemma and by zona radiata (chorion). The process of ovulation is regulated by prostaglandins. Ovulation should occur shortly after maturation, otherwise the eggs will become overripe. After an oocyte is released into the lumen of the ovary, the cells of theca and granulosa layer remain in the ovarian stroma as the postovulatory follicle (Garcia-Diaz et al., 2002; Wallace & Selman, 1981; Selman et al., 1993; Bidwell, 1992).

1.4.2.1 Structure of stage III (vitellogenic) zebrafish oocyte

Follicle. Ovarian follicle consists of a developing oocyte and two surrounding it cellular layers: outer – theca, and inner – granulosa. The follicle has nutritional, regulatory and protective function for growing egg. Follicle cells mediate the transport of many substances, including vitellogenin, from the blood to the oocyte. The follicle increases the selectivity of intake of substances from blood plasma to oocyte, and concentrates proteins around the oocyte. In some species of fish the *in vitro* vitellogenesis can process in the defolliculated oocytes, and in other species it can not. The follicle is also a key source of steroid hormones required for oocyte maturation. Some zebrafish follicle cells seem to have distinct functions; for example, a single specialized cell, called micropylar cell, participates in the formation of the micropyle, the sperm entry point in the zona radiata.

The inner follicle layer, granulosa, consists of steroidogenic cells which extend their extensions - macrovilli towards the surface of oocyte. Microvilli originating from the oocyte penetrate deep into the spaces between adjacent follicle cells. While follicle cells withdraw their processes prior to ovulation, microvilli remain on the surface of the ovulated zebrafish egg. Outer, thecal layer, consists of fibroblasts, collagen fibers, blood capillaries, and special steroidogen cells. Theca is covered by the surface epithelium (Ayzenshtadt, 1984; Detlaf, 1977).

Oocyte. Zona radiata (vitelline envelope) is the layer covering the oocyte, which continues to surround oocyte after its ovulation from follicle. After fertilization of oocyte zona radiata is called chorion. Its thickness is 1.74 μm. Zona radiata is traversed by numerous pores and canals, through which the microvilli extend. Zona radiata is acellular layer. It is composed of three layers of different thickness and density. An external layer, which appears clear and homogeneous, is rich in polysaccharides. An intermediate layer is composed of fibrillar proteins. An internal layer of the zona radiata has a thickness of 0.61 μm, is electron-dense and compact, and has a multilaminar organization. It is composed mainly of tyrosine-rich proteins. The material of zona radiata is partially synthesized by the growing oocyte itself, and partially – by the liver, which produces zona radiata proteins in response to hormonal stimuli. The structure of zona radiata provides optimal diffusive gas exchange, selective transport of materials to the oocyte, effective protection against fungi and bacteria, and mechanical protection for the oocyte. In general, zona radiata (chorion) is a porous acellular membrane easily permeable for water, electrolytes and most of cryoprotectants, but impermeable for macromolecular substances.

Micropyle. The micropylar canal forms through the wall of zona radiata. The sperm cell will penetrate chorion to the oocyte surface through this canal.

Microvilli. These oocyte cytoplasmic projections go through pores in zona radiata, and closely contact with follicle cells. The macrovilli from

granulosa cells are situated in the same channels as microvilly and adjacent with them. The structure of microvilli is upheld by the cytoskeleton of actine filaments. They create close connections between the oocyte and the follicle layer, which have nutritional and regulatory function. It is also considered, that microvilli are needed to enlarge the egg surface, improve substance exchange between the follicle and oocyte, and, finally, provide a reservoir of plasma membrane material to be used during the embryo cleavage after fertilization.

Oolemma (vitelline membrane). There is an evidence, that membranes of fish oocytes contain high percentage of unsaturated and poly-unsaturated fatty acids (Aras et al., 2003), that may have a substantial effect on its microviscosity and cryostability. As it was shown on amphibian eggs, the oolemma of vitellogenic follicular oocytes is permeable mainly for K^+, and non-permeable for Na^+. This selectivity is regulated by calcium ions, as in absence of Ca^{++} in the medium the oolemma acquires permeability for Na^+ ions (Ecker & Smith, 1971; Maeno, 1959). The oolemma is impermeable for Cl^- anions. This property is important for building up an electrical potential across the oolemma, and in the future development of oocyte - for signalling purposes. When sperm cell penetrates the mature oocyte, the oolemma becomes permeable for Cl^- ions, which leads to the development of an action potential and triggers a chain reaction of further processes (Maeno, 1959). The vitellogenin receptors are inserted into the oocyte plasma membrane.

Cytoplasm. The cytoplasm of fish oocytes (ooplasm) possesses such organelles as rough endoplasmic reticulum, Golgi complexes, ribosomes, lysosomes, yolk vesicles, cortical alveoli and mitochondria. Most of the water in zebrafish oocytes (up to 75%) is thought to be unbound and osmotically active, which is supported by data on zebrafish embryos (Liu, PhD Thes., 2000). However, in oocytes of other species (for example, in Salmonid fishes) the amount of bound water may be up to 90% of total oocyte water content (Rana & McAndrew, 1995). The cytoplasm of fish oocytes is richer in Na^+ ions and contains less K^+ ions than the cytoplasm of most of cell types (Morrill, 1965). When the oocyte grows, its cytoplasm accumulates large quantities of mRNA; so protein synthesis can be continued even after maturation, when the

chromatin transfers to the "compact", inactive state and transcription becomes impossible (Smith & Ecker, 1969). For the same reason the large stores of ribosomes are accumulated in the vitellogenic egg, in order to provide sufficient level of protein synthesis even at metaphase II of meiosis, when transcription is virtually impossible. Also limited evidence exists, that some ribosomes and mRNA transcripts may penetrate into the developing egg from maternal organism by pinocytosis, and thus influence oocyte growth and subsequently the development of the embryo (Brooks et al., 1997). The ooplasm of fish oocytes contains large amount of glycogen (Ayzenshtadt, 1984; Terner, 1968), which is accumulated as an additional to yolk nutritional source. Some of the free glucose is also present (Terner, 1968).

Structure and organisation of **mitochondria** in the egg differs from the structure and organisation of mitochondria in somatic cells. In oocytes, mitochondria form large, fused and branched network of channels, referred as "mitochondrial cloud", "Balbiani body", or even as "yolk nucleus". The mitochondria are connected by so-called "nuage", the substance which consists of ribonucleoproteins and proteins. Some mitochondria in oocytes may sequester yolk proteins and therefore be transformed into a kind of yolk platelets.

The **nucleus** of oocyte (also called the germinal vesicle) is large and eccentrically placed. Zebrafish, as all Cypriniform fishes, is ancestrally tetraploid (Klyachko et al., 1995), therefore the nucleus of oocyte contains doubled amount of genetic material. The germinal vesicle is rich in sulfhydryl-group containing proteins (Smith & Ecker, 1969). In karyoplasm of fish oocyte concentrations of sodium and potassium ions are 2-3 folds higher than in cytoplasm (Ayzenshtadt, 1984).

Cortical alveoli (also known as: cortical granules, cortical vesicles, yolk vesicles or vacuoles, carbohydrate yolk, endogenous yolk) are the vesicles which origin from Golgi complex. The contents of cortical alveoli, "hydrophyllic colloid", is made of proteins, lectine and mucopolysaccharides. Enzymes β-D-glucuronidase, trypsin-like protease, β-1,3-gluconase, acid

phosphatase and other are present in cortical alveoli. The cortical alveoli fuse with plasmalemma at fertilization, to release their contents in the space between plasmalemma and zona radiata, and therefore to form perivitelline fluid (PVF) (Wessel et al., 2001; Parks, 1997; Ayzenshtadt, 1984; Nosek, 1984).

Annulate lamellae is the tight multi-layer pack of lamellae, connected with rough endoplasmic reticulum. They are intensely saturated with electrone-dense pores. The exact function of annulate lamellae is unclear. There is evidence, that at certain stages of oocyte growth, annulate lamellae can become transformed to Golgi apparatus, which in turn produces the cortical alveoli.

The **yolk** occupies most of oocyte volume, resulting in its large size. For instance, in amphibian oocytes (which are similar to fish oocytes) yolk occupies 50% of egg volume and makes up 80% of total egg protein. Yolk is accumulated in the course of vitellogenesis in order to provide nutrition for the developing embryo: energy in form of lipids and carbohydrates, and structural material such as membranes and ribosomes (Thomas, 1968). Yolk consists of **yolk platelets (bodies, vesicles)** and oil globules. Vitellogenin (Vg) is a glycolipophosphoprotein, which is produced by the liver in response to estrogen stimulation, then released into the bloodstream, taken up by developing oocytes by receptor-mediated pinocytosis, and chemically modified in the process of yolk formation. There are two forms of vitellogenin in *Danio rerio:* major Vg (149kDa) and minor Vg (176kDa). In ooplasm Vg is enzymatically cleaved into two major yolk proteins, lipovitellin and phosvitin, and to the third yolk protein, β-component. Cathepsin D enzyme, contained in lysosomes, is involved in the cleavage of vitellogenin into the lipovitellin and phosvitin. Lipovitellin is rich in lipid and poor in phosphorus and phosvitin is rich in phosphorus and poor in lipid. Phosvitin is a protein with a very long repetitive sequence of phosphorylated serine residues (Hiramatsu et al., 2000). Yolk vesicles form from the maturing endosomes, which have fused with lysosomes; while receptors are recycled back to the oocyte surface until vitellogenesis is completed. The contents of most yolk bodies is not homogeneous and usually displays the presence of one or several rectilinear

masses, immersed into the superficial matrix. These bodies are the crystals of proteins lipovitellin and phosvitin. Apart from lipovitellin and phosvitin, another proteins were detected in yolk granules (especially those rich in tyrosine, tryptophan, cystine, arginine, lysine and cysteine); and also such substances as carbohydrates, non-polar lipids, phospholipids and cholesterol. In yolk bodies lipids are mainly bound to lipovitellin. Yolk has strong buffer properties in a broad pH range, with pK_a ~6.6. This may be due to presence of highly phosphorylated protein phosvitin. pH of yolk platelets in amphibia and fish is acidic (around 5.6). Low pH in yolk vesicles might be required to build and maintain the crystal lattice of the yolk. Apart from yolk proteins, such enzymes as cathepsin D, acid phosphatase, Na^+/H^+- exchanger are found in yolk bodies.

Also, yolk vesicles contain very high concentrations of sodium ions: 35-50% of total sodium in oocytes is retained in yolk. In yolk vesicles concentration of Na^+ is 100mM; meanwhile in the rest of ooplasm it is only 6-14mM. Calcium was also detected in yolk granules. Pools of sodium and other ions in the yolk may be needed to cover ionic needs of developing embryos, which will exist in low-ionic environment (fresh water) (Fagotto & Maxfield, 1994, Sarasquete et al., 2002). Yolk of fish oocytes contains molecules of linear DNA. Its origin and function is still unclear. Probably, DNA is absorbed by oocyte from plasma during pinocytosis, together with vitellogenin (Ayzenshtadt, 1984). Also, the yolk mass has some mRNA deposited in it, which encodes growth factors and receptors, regulating the processes of embryo development. Thyroid hormones of maternal origin are also stored in egg yolk and may have significant effects on fish embryo development. (Brooks et al., 1997; Debnath et al., 1998; Garcia et al., 2001; Hartling et al., 1997).

1.4.2.2 Structure of stage IV (maturing) zebrafish oocyte

During this stage of oocyte growth a number of structural changes occur, which result in the formation of the mature egg. During this stage, the nucleus of the egg (germinal vesicle) migrates from the center of the egg to the periphery and then the nuclear membrane disappears, as the second division of meiosis resumes. This process is called germinal vesicle

breakdown (GVBD). Now there is a polarized distribution of yolk, ribosomes, mitochondria over the egg. The relatively yolk-free region of the oocyte is known and the animal hemisphere and the yolk-rich region is known as the vegetal hemisphere. The establishment of the animal-vegetal axis (the imaginary line connecting the two poles of each hemisphere) defines the anterior-posterior axis of the fish embryo, the first specification of the body plan.

Certain hydration of cytoplasm takes place, although in zebrafish and other freshwater species this phenomenon is much less pronounced than in oocytes of marine fish. Hydration is thought to be caused by partial degradation of yolk proteins into free amino acids, which causes the increase of osmolarity of the ooplasm and hence the uptake of water (Finn et al., 2002). In amphibian oocytes, there is evidence that before maturation and ovulation the egg uptakes sodium, chloride and calcium ions (Morrill, 1965). Similar processes are likely to occur in fish oocytes. These changes should provide the sufficient internal stock of ions for the embryo, which will develop in hypoionic environment. The oil droplets of the yolk coalesce into a single oil drop. A similar process happens with the yolk bodies, yolk granules progressively fuse and form a continuous mass of fluid yolk. As a result, the yolk becomes clear and translucent.

The assembly and rearrangement of cytoskeleton and meiotic spindle contractile proteins consumes large quantities of energy. This is reflected in changes of morphology: during reactivated meiosis, the mitochondria migrate to the peri-nuclear region and form a dense ring-like aggregate that envelops the spindle. This probably serves to concentrate them for localized activities that require high levels of ATP, such as actin polymerisation and chromosome migration (Boulekbache et al., 1989). Significant changes occur in oolemma: during maturation, it progressively loses its receptors and channel proteins, and as a result, the egg becomes much less permeable for water and solutes (Ecker & Smith, 1971; Pennequin et al., 1975; Smith & Ecker, 1969; Naora et al., 1962). However, oolemma remains permeable for such ions as Na^+ and K^+ (Maeno, 1959). The electrical potential of oolemma also changes during maturation (Morrill, 1965). Obviously, these changes in the properties of plasmatic membrane are required to prepare the oocyte for transfer from the

ovarian lumen (with physiological salts and solutes concentrations) to a hyposmotic environment – fresh water.

1.4.2.3 Structure of stage V zebrafish oocyte (mature egg)

The size of zebrafish full-grown ovulated eggs is approx. 0.7 mm. In appearance the mature egg is homogeneous, finely granular, and weakly basophilic. The nuclear contents is diffuse in the ooplasm. Condensed chromosomes and cytoskeletal structures of the meiotic spindle are observed. After GVBD the protein synthesis in oocyte is strongly activated (Ecker & Smith, 1971). The plasmatic membrane, as it was mentioned above, is low-permeable for water and dissolved substances. The ooplasm, restricted to a narrow rim, lies beside the zona radiata at the oocyte periphery. The structure of egg cortex, i.e. 3-dimensional network of endoplasmatic reticulum tubules and cytoskeletal elements, provides successive response of an oocyte to fertilization by sperm cell: calcium mobilization from endoplasmatic reticulum and triggered by it initiation of numerous intracellular processes, leading to embryo development (Sardet et al., 2001).

In the mature oocyte the energy production through anaerobic glycolysis prevails over mitochondrial breathing until fertilization. Normally, the mature oocyte carries very high energy charge value, which is required to provide energy for embryo development after fertilization. For instance, the energy charge of mature carp oocyte reaches 0.84-0.86 (ECharge = ([ATP] + 1/2[ADP]) / ([ATP] + [ADP] + [AMP]) (Boulekbache et al., 1989). After fertilization of the mature egg, a small amount of perivitelline fluid is formed between the chorion and oocyte. As the result of water activation and fertilization of the fish egg, the contents of cortical alveoli are released into the space between oolemma and zona radiata. Osmotic uptake of water from environment occurs, and hence the perivitelline fluid forms. Its function is to prevent polyspermia and to form an microenvironment for developing embryo. (Selman et al., 1993).

1.4.3 Regulation of oocyte development

Oogenesis in zebrafish is regulated hormonally. The hypothalamus of female fish, in response to environmental external stimuli (duration of lightday, water temperature, nutrition quantity and quality, availability of males),

produces gonadotropin-releasing factor, which stimulates the pituitary gland of the fish to synthesize hormone gonadotropin. Gonadotropin triggers the production of steroid hormones in the ovary. The synthesis of 17-β-estrogen by follicle granulosa cells is induced through upregulation of intracellular messenger activin. 17-β-estrogen then triggers a number of physiological processes in fish, including synthesis of vitellogenin and "chorion proteins" in the liver the synthesis of receptors for vitellogenin in the oocyte, and uptake of yolk protein by the oocyte. Finally, at the end of vitellogenesis, 17-β-estrogen induces synthesis of another steroid hormone, DHP (17α-Hydroxy-20β-dihydroprogesterone). In this process such intermediate messengers as maturation promoting factor (MPF), cyclin B, polypeptide cdc2 and other are involved. DHP triggers the maturation of oocyte and numerous processes which accompany it : migration and breakdown of germinal vesicle, formation of meiotic apparatus, changes in oolemma properties, transformation of yolk, and other intracellular events which result in mature egg. The effect of DHP is non-genomic, but membrane-bound, and is associated with such secondary messengers as calcium and inositol phosphates.

1.5 Difficulties and complications associated with cryopreservation of fish oocytes

One of the most important features of fish oocyte, which makes this cell type unique, is its exceptionally large size (Selman et al., 1993). Fish eggs (>1 mm in diameter) are thousand times larger than, for example, human eggs, which are only ~100 μm in diameter (Brooks et al., 1997). The large size of fish oocytes, resulting in low surface-to-volume ratio, makes the diffusion of CPAs and water throughout the cytoplasm difficult. Moreover, single lesion in plasmatic membrane may lead to the death of the oocyte. Fish oocytes, especially mature ones, have reduced membrane permeability to water and solutes, including cryoprotectants, when compared to other cell types (Ecker & Smith, 1971; Pennequin et al., 1975; Smith & Ecker, 1969; Zhang et al., 2005). Mature fish oocytes are highly hydrated, although in zebrafish and other freshwater species it is less pronounced than in marine fish eggs (Wallace, 1981). Water makes up approx. 60% of vitellogenic zebrafish oocyte mass, and oocytes increase volume even more (by 10-15%) during maturation (Selman et al., 1993).

Large fish oocytes (stages III, IV, V) contain large amount of highly specific intracellular material - yolk (Wallace & Selman, 1981), which has been proven to be sensitive to osmotic shock and freezing. For mammalian oocytes, it has been proven that the presence of yolk mass in the cell creates difficulties during freezing and partial removal of yolk benefits their cryopreservation (Nagashima et al., 1994; Otoi et al., 1997). The problems are associated with yolk lipids. It is well-documented, that tissues which are rich in non-polar lipids such as human adipose tissue are extremely difficult to cryopreserve (Gempt et al., 2005). Therefore, presence of massive amount of lipids in the yolk of fish oocytes (McFarlane & Norton, 1999) may substantially complicate their cryopreservation. Several studies reported significant changes in oocyte yolk structure after freezing-thawing, that result in coalescence of yolk granules into one mass and yolk becoming translucent (Nagashima et al., 1994; Lubzens et al., 2005). Cryopreservation of mature (stage V) zebrafish oocytes may be more complicated. At this stage, the nuclear envelope is absent and chromatin is being arrested in metaphase II of meiosis (Wallace & Selman, 1981), this makes the genetic material of mature egg highly vulnerable to such effects as exposure to cryoprotectants and to cooling due to cold-induced reversible depolymerisation of meiotic spindle (Agca, 2000; Isachenko et al., 2000; Zenzes et al., 2001; Parks, 1997). Genome of fish eggs was repeatedly reported to be sensitive to cold shock (Valenti, 1975).

Mature oocytes are more susceptible to the effects of ageing and overripening than immature ones, and to the structural and functional abnormalities associated with these processes (Boulekbache et al., 1989). Cryopreservation of immature oocytes has disadvantages when compared to mature eggs: adult female fishes need to be sacrificed in order to obtain stage III oocytes; and after thawing the immature eggs need to be incubated and undergo maturation *in vitro* before artificial fertilisation can take place.

However, a number of studies have indicated that immature oocytes of aquatic species, such as amphibians and teleost fishes, are much more permeable to water and solutes than mature eggs (Ecker & Smith, 1971; Pennequin et al., 1975; Smith & Ecker, 1969), including direct data on zebrafish stage III and stage V oocytes (Seki et al., 2005; Zhang et al., 2005); and also less hydrated than mature ones (Wallace & Selman, 1981) which poses additional benefits for their cryopreservation. Mature zebrafish oocytes

were found to have extremely low permeability to such CPAs, as glycerol, EG and PG (Seki et al., 2005), whereas the permeability of immature oocytes to these substances was much higher. It is assumed, that such low permeability to water and solutes is developed in mature eggs in order to withstand the impact of strongly hypotonic environment, which fresh water (where they are spawned to) represents (Seki et al, 2005). In amphibian eggs it was shown, that in mature oocytes intracellular ice formation is more likely to occur, and occurs at higher temperatures, than in oocytes of earlier stages (Kleinhans et al., 2005). Moreover, mature eggs are fairly sensitive to the conditions of the environment and may easily develop spontaneous cortical reaction even in absence of sperm cell, due to influence of factors such as physical contact, hypotonicity of the medium, temperature change and the presence of certain compounds (Plachinta et al., 2004; Isayeva et al., 2004; Zhang et al., 2005; Runft et al., 2002). The spontaneous cortical reaction is undesirable, because the oocytes which underwent it can not be fertilised anymore and, therefore, are lost for reproductive purposes.

Supposed high chilling sensitivity of zebrafish oocytes must also be taken into account (Isayeva et al., 2004). The data on one-cell zebrafish embryos showed that incubation of embryos at 0°C for only 10 minutes resulted in 50% loss of their hatching rate, and 30 min incubation at this temperature resulted in total death of embryos (Liu, 2000). It's worth pointing that ice formation was not involved.

1.6 Intended approaches in the present study

Studies in the following areas were undertaken in this investigation:

- Development of suitable vital tests for zebrafish oocytes.
  Studies on fish oocytes cryopreservation are impossible without the availability of reliable vital tests for assessment of the viability of fish oocytes. There has been no reliable tests for evaluation of the viability of unfertilised fish oocytes, although one method using cFDA has been suggested (Pearl & Arav, 2000).

- Studies on toxicity of cryoprotectants to zebrafish oocytes.

  Cryoprotectants, used to protect biological material from damaging effect of low temperatures, can be toxic to cells themselves. Hence, it is important to determine the impact of cryoprotectants on oocytes as a first step in freezing protocol design.

- Studies on controlled slow cooling of zebrafish oocytes.

  In the present investigation, studies will be focused on cryopreservation of zebrafish oocytes using controlled slow cooling. The effect of varying of such cooling parameters, as choice of CPA, choice of cooling rate and final cooling temperature on cryosurvival of zebrafish oocytes will be studied.

- Studies on effect of additional cryoprotective supplements to a freezing medium. In this part of the study, in addition to cryoprotective supplements, the effect of freezing medium, seeding temperature, plunge temperature and pH on cryosurvival of zebrafish oocytes will also be studied.

- Studies on ice nucleation patterns of zebrafish oocytes using the cryomicroscope.

  In order to be able to reveal the ice formation patterns in zebrafish oocytes, and temperatures of homo- and heterogeneous ice nucleation, cryomicroscopic studies will be carried out.

- Additional studies on the effectiveness of the optimal cryopreservation protocol using ATP test.

  ATP content in the cell is a trustworthy parameter for evaluation of viability of the oocytes, as ATP is the vitally important intracellular energy carrier, and therefore shortage of ATP indicates depletion of the vital potential of the cell.

CHAPTER 2. MATERIALS AND METHODS

2.1 Introduction

Five main areas of study were covered by the project: development of suitable viability assessment assays for zebrafish oocytes; toxic effect of cryoprotectants on zebrafish oocytes; controlled slow cooling of zebrafish oocytes; addition of cryoprotective supplements for cryopreservation of zebrafish oocytes and further studies on oocytes viability/quality after controlled slow cooling, including cryomicroscope observations and determination of cell ATP levels of cryopreserved oocytes. All experiments were carried out in laboratories at Luton Institute of Research in Applied Natural Sciences (LIRANS), University of Bedfordshire, UK.

2.2 General methods

2.2.1 Maintenance of zebrafish

2.2.1.1 General information

Adult zebrafish (2-5 month old) were obtained from Aquascope Ltd. (Birmingham, UK) and the MRC 'Human Genome Mapping Project' laboratory (Hinxton, UK). As many of these fish originated from sources in the Far East and their cultural history is not always available, one of the considerations that needs to be taken into account is their taxonomy/genetic makeup and its implications in cryobiological studies.

The genetic diversity of experimental stock may lead to some variation of structural and biochemical properties of oocytes obtained from different fish which, in turn, could contribute to inter-individual differences between replicates, i.e. to the variance within groups. Such individual differences was illustrated by the results obtained by Billard et al. (2004), who demonstrated a wide range of inter-specimen variation in ATP levels in cryopreserved sturgeon sperm. Lipid composition of cell membranes, including the ratios of

saturated and unsaturated acyl chains in phospholipids, the nature of phospholipids polar groups, and cholesterol content of membranes, are determined both genetically and by nutrition (Horrobin, 1995). As lipid composition of membranes can affect their cryo-stability (Graham et al., 2005; Zeron et al., 2002), any genetic diversity of the experimental material could contribute to an increased variation. More discussions can be found in the Introduction, Sections 1.2.2.3, and 1.2.3.4.

However, as supported by the results of genomic studies (PCR amplification) of Meyer et al. (1993) on mitochondrial ribosomal RNA, the genetic material of the *Danio* genus is quite conservative, and the sequences of selected genes did not vary between wild-type *Danio rerio,* and its aquarium breeding morph, the 'leopard' danio (*Danio rerio frankei*). This suggests that genetic deviations within and between different stocks of wild-type striped *Danio rerio* are small and negligible for the purposes of non-genomic research.

The use of fish from external suppliers is common practice for non-genetic studies within the zebrafish community. However, the lack of information of the genetic composition of the fish used is acknowledged and the possible implications recognised.

Zebrafish were kept in 40-litre filtered and aerated tanks in a temperature-controlled room at the Research Centre, University of Luton. Up to thirty fish were kept in each tank. A light / dark cycle of 14/10 hours was used. Aged tap water or deionised water with added sea salts (0.25 g/L) was used for maintenance of adult fish. Tank water was changed every 5-7 days. Filtration of the tank water was carried out using an electric air-pump to pump air through an upturned funnel that was surrounded by filter floss (King British®, England) in a plastic beaker (1L) immersed in the fish tank. The funnel and floss were held in position by a layer of smooth gravel. Water was pulled through the gravel and floss by the suction effect generated by the rising air bubbles, and debris (fish excrements, food remains, etc.) were

retained by the filter floss. Additional information for fish maintenance is given in Table 2.1.

Table 2.1. General information for zebrafish care

Room temperature	26 ± 1°C
Water temperature	28 ± 2°C
Water pH	7.6 - 8.0
Water DO	80 ± 5%
Water hardness express as $CaCO_3$	150-200 mg/L

2.2.1.2 Feeding

Fish were fed twice a day: in the morning with 'Tetramin' (Tetra, Germany) flake fish food (ingredients: processed fish and fish derivatives, cereals, yeast, vegetable protein extracts, molluscs and crustacerans, oils and fats, algae, various sugars, EEC permitted colorants and preservative), and in the afternoon with adult brine shrimp (*Artemia salini*). Brine shrimps were specially prepared in sea-water-filled, aerated hatcheries (52.5 g of sea salts in 1.5 litres of deionised water). During weekends and holidays, automatic fish feeders (Fish Mate F14 Aquarium Fish Feeder) were used.

2.2.1.3 Collection of oocytes: stages III, IV and V

To obtain stage III oocytes (0.5 - 0.6 mm in diameter) and stage IV oocytes (>0.6 mm in diameter), zebrafish were anaesthetised in a lethal concentration of tricaine (0.6 mg/ml for 15 min) and then decapitated. The bellies were dissected, the ovaries were removed and placed into oocyte medium (Hank's solution or KCl buffer). The composition of Hank's solution was: 0.137M NaCl, 5.4 mM KCl, 0.25mM Na_2HPO_4, 0.44mM KH_2PO_4, 1.3mM $CaCl_2$, 1mM $MgSO_4$, 4.2mM $NaHCO_3$; pH 7.4. The composition of the KCl buffer was: 55 mM KCl; 55 mM K acetate; 1 mM $MgCl_2$; 2 mM $CaCl_2$; 10 mM HEPES; pH adjusted to 7.4 by 1M KOH. Ovarian cumulus was then gently disaggregated by fine steel forceps, and stage III and IV oocytes were separated. In order to obtain unfertilized stage V (mature) zebrafish oocytes,

adult female fish was separated for up to 5 days in a separate tank or a large beaker. Spawning females were placed into tricaine solution (0.2 mg/ml for 5 min), they were then taken out and their surface was dried carefully with filter paper. Gentle massage of the fish's belly was then carried out, and stage V oocytes were squeezed out and collected in a Petri dish with 0.5-1 mL of Hank's solution. The squeezed females were then placed into a separate beaker for several days to recover before releasing back to the system.

Oocytes of stages III, IV and V can be easily distinguished from each other by visual observation (Fig.2.1). Stage III (vitellogenic) oocytes (0.5 - 0.65 mm); are opaque; slightly yellowish in colour. Stage IV (maturating) oocytes (>0.65 mm) are easily separated from cumulus mass and their colour is slightly darker than that of stage III oocytes. Stage V (mature) oocytes (~0.75 mm) are transparent and colourless.

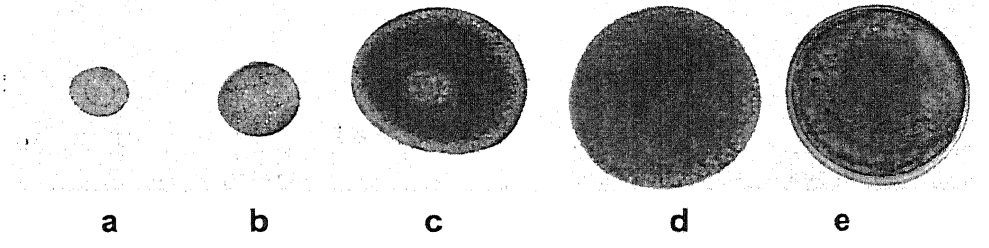


Fig. 2.1 Developmental stages of zebrafish oocytes

a) primary growth stage (stage I)

b) early cortical alveolar stage (stage II)

c) vitellogenesis (stage III)

d) maturation (stage IV)

e) mature egg (stage V)

2.2.2 Chemicals

Information on the chemicals used in present study is given in Table 1 of Appendix A. Fresh aqueous solutions were prepared in deionised water shortly before their use. If necessary, solutions were stored in a fridge (+4°C) or freezer (-20°C).

2.3 Development of suitable viability assessment assays for zebrafish oocytes. Enzymatic treatments of zebrafish oocytes

In order to develop viability assessment methods for fish oocytes, a number of fluorescent dyes, namely: ethidium bromide, propidium iodide, calcein blue, carboxyfluoresceine, rhodamine-123; widely-used vital stain trypan blue and mitochondrial stain thiazolyl blue (MTT) were tested for their suitability for zebrafish oocytes along with *in vitro* maturation assay. 20-30 oocytes were used for each observation. Stage III oocytes were used, and for MTT test – stage III and stage V oocytes.

2.3.1 Obtaining of negative control

To obtain dead oocytes, the samples were immersed into 99% methanol for 10 min at room temperature (22°C), after which oocytes were rinsed thoroughly several times with Hank's solution. Such treatment would effectively kill the oocytes, as methanol at such a high concentration denaturates all cellular proteins, including enzymes, and destroys their tertiary structure (Leong, 2000).

2.3.2 Ethidium bromide assay

The structure of ethidium bromide is shown in Fig. 2.2. 1 mg/ml stock solution of ethidium bromide was made up in ethanol and kept refrigerated. Working solution was prepared by dilution of stock solution to 10 μg/ml in Hank's medium. For staining, oocytes were incubated in working solution for 30 min at 22°C. Leica DMIL inverted fluorescent microscope and N2.1 filter was used for observations. Cells stained red were presumed dead.

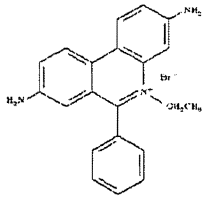


Fig. 2.2. Ethidium bromide structural formula

2.3.3 Propidium iodide assay

The structure of propidium iodide is shown in Fig. 2.3. To carry out the assay, 1 mg/ml stock solution of propidium iodide was made up in ethanol and kept refrigerated. Working solution was prepared by dilution of stock solution to 10 μg/ml in Hank's medium. For staining, oocytes were incubated in working solution for 30 min at 22°C. Leica DMIL inverted fluorescent microscope and N2.1 filter was used for observations. Cells stained red were presumed dead.

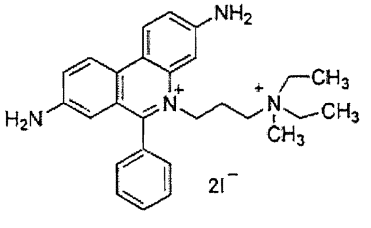


Fig.2.3. Propidium iodide structural formula

2.3.4 Calcein blue assay

The structure of calcein blue is shown in Fig. 2.4. 2 µM solution of Calcein blue in Hank's medium was prepared. For staining, 0.5 mL of calcein solution was added to the equal volume of the sample. Observation is carried out after 5 min of incubation. Leica DMIL inverted fluorescent microscope and I3 filter were used for observations. Green-fluorescent cells were presumed viable. About

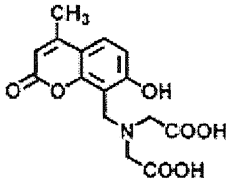


Fig. 2.4. Calcein blue structural formula

2.3.5 cFDA assay

The structure of carboxyfluoresceine is shown in Fig. 2.5. Oocytes were immersed into 1mL of 1 µM/L cFDA in Hank's medium. Microscopic observation followed immediately. Leica DMIL inverted fluorescent microscope and I3 filter was used for observations. Fluorescent green cells were presumed viable.

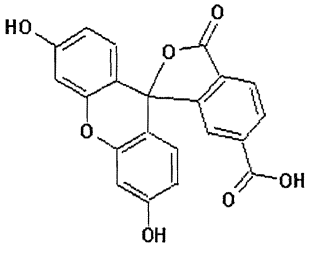


Fig. 2.5. Carboxyfluoresceine structural formula

2.3.6 Rhodamine 123 assay

The structure of rhodamine 123 is shown in Fig. 2.6. 30 µg/ml working solution of rhodamine 123 was made up in Hank's medium and kept refrigerated. Oocytes were incubated in Rhodamine 123 for 30 min at 28°C; they were then washed twice with Hank's. Leica DMIL inverted fluorescent microscope and N2.1 filter was used for observations. Viable oocytes are fluorescent yellow-green.

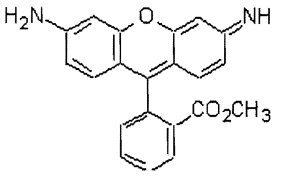


Fig. 2.6. Rhodamine 123 structural formula

2.3.7 Thiazolyl blue (MTT) assay

The structure of thiazolyl blue is shown in Fig.2.8. For MTT test, oocytes were incubated in 2 mL of 0.5mg/ml MTT solution for 1.5 hours at 28°C. Oocytes viability was then checked visually and microscopically.

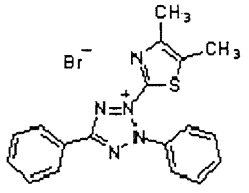


Fig. 2.7. Thiazolyl blue (MTT) structural formula

2.3.8 Trypan blue (TB) assay

The structure of trypan blue is shown in Fig. 2.7. For the TB test, oocytes were incubated in 1 mL 0.2% trypan blue solution at room temperature for 5 min and then washed with Hank's medium. Stained cells were considered non-viable, and unstained cells - viable.

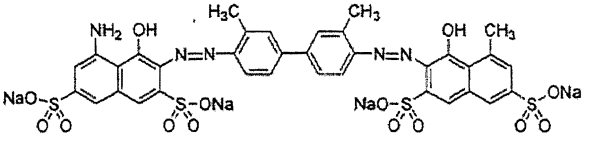


Fig. 2.8. Trypan blue structural formula

2.3.9 *In vitro* maturation and germinal vesicle observation (GVBD) assay

For GVBD assay, vitellogenic oocytes were incubated in 10 mL of 60% L-15 (Leibovitz) medium supplemented with 1 mg/ml DHP (17α-Hydroxy-20β-dihydroprogesterone) and 0.1 mg/ml gentamicin for up to 24 hours at 28°C (Selman et al., 1994). The germinal vesicle breakdown in oocyte after completion of *in vitro* maturation can be observed visually through changes in cytoplasm, which loses its opacity and becomes translucent and slightly yellowish. Cells that failed to complete maturation and germinal vesicle breakdown remain opaque. GVBD assay is not applicable for stage IV and V oocytes as they have already commenced or completed their maturation and germinal vesicle breakdown *in vivo*.

2.3.10 Enzymatic treatments of zebrafish oocytes

2.3.10.1 Enzymatic disaggregation of cumulus

Oocytes obtained from fish ovary are primarily integrated in cumulus (Fig.2.9). To obtain oocytes at different developmental stages it is necessary to disaggregate cumulus. Enzymatic disaggregation of cumulus was tested as an alternative to mechanical maceration as it is time consuming. It was also presumed that enzymatic disaggregation would be a more gentle method than mechanical disaggregation. For disaggregation of cumulus, collagenase-hyaluronidase solution was used to treat oocytes, a method which has been previously used with amphibian and fish oocytes (Choe & Sackin, 1997; Campbell & Jalabert, 1979).

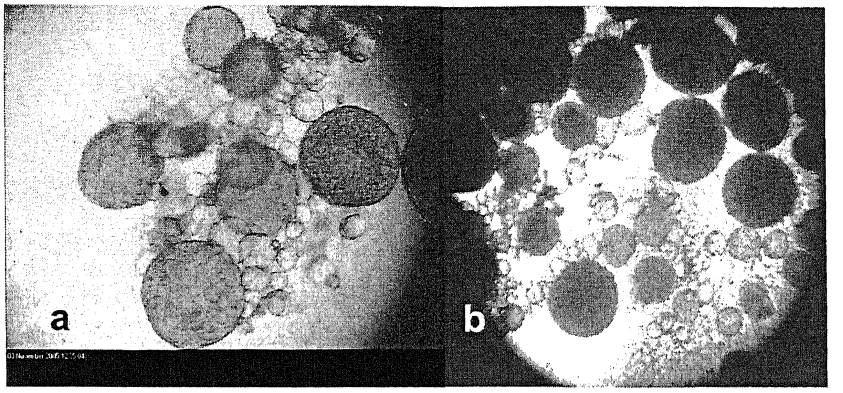


Fig. 2.9. Cumulus mass, obtained from zebrafish ovary, containing oocytes of different developmental stages : (a) stages I and II; (b) stages I,II, III.

Solution of hyaluronidase 4% + collagenase type II 4% was made up in Hank's medium and stored frozen at -20°C. Before experimental use, the enzymatic solution was thawed and diluted twice with Hank's medium, to a final concentration of hyaluronidase 2% and collagenase type II 2%. Solution of bovine serum albumin (BSA) (10%) was made up in Hank's medium and stored frozen at -20°C. For the experiment with BSA-protected disaggregation, enzymatic solution was thawed and mixed with an equal volume of BSA solution, to final concentration of hyaluronidase 2%, collagenase type II 2% and BSA 5%. For enzymatic disaggregation, ovarian cumulus was immersed into collagenase-hyaluronidase solution, and incubated at 25°C for 15 min or 30 min, oocytes were then rinsed in 10% fetal calf serum (FCS) in order to stop enzymatic reaction. For BSA-protected enzymatic disaggregation, ovarian cumulus was immersed into BSA-collagenase -hyaluronidase solution, and incubated at 25°C for 15 min or 30 min, oocytes were then rinsed in 10% FCS. After washing in Hank's medium, viability of oocytes was assessed using TB test and GVBD test. Large numbers of oocytes (50 and more) pooled from different female fishes were used for each treatment.

2.3.10.2. Enzymatic defolliculation

Stage III (vitellogenic) zebrafish oocytes are surrounded by a follicle layer - a structure which has nutritional, regulatory and protective function for growing eggs (Fig. 2.10). After disaggregation of cumulus mass, either by mechanical or enzymatic methods, the vitellogenic oocyte still remains inside the follicle. The follicle layer may decrease the permeability of oocyte to various substances, including cryoprotectants.

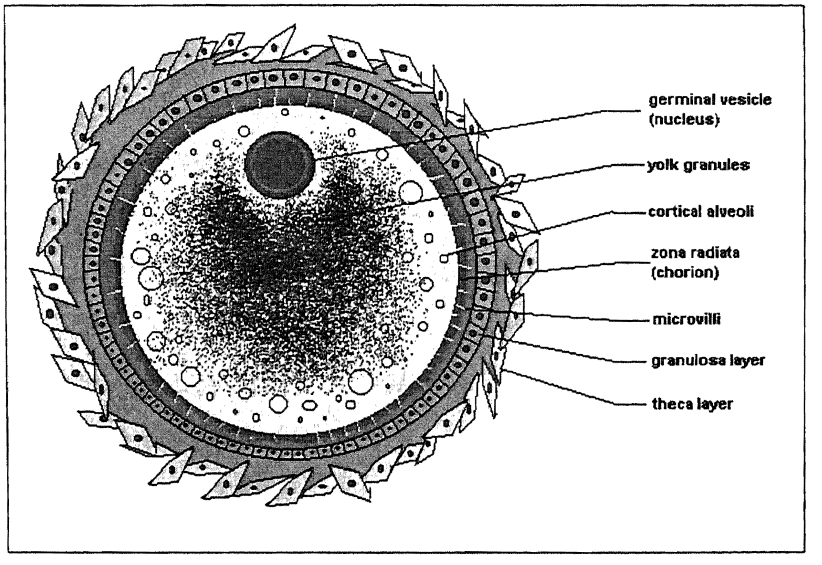


Fig. 2.10. The structure of the late vitellogenic zebrafish follicle

To carry out enzymatic defolliculation, 4% trypsin solution was made up in Hanks' medium. This solution was stored frozen at –20°C and thawed immediately before experiments. It was then diluted with Hanks' medium to 2% concentration. Ovarian cumulus, slightly macerated with forceps, was immersed into the enzyme solution for up to 10 min, it was then washed with 10% fetal calf serum several times in order to stop the enzymatic reaction. Oocytes were then rinsed few times in Hank's medium. The evaluation of morphological integrity of oocytes after treatments was carried out by microscopic observation. The integrity of oocyte plasmatic membrane was evaluated following 5 min incubation in 0.2% trypan blue.

2.4 Toxicity of cryoprotectants to zebrafish oocytes

Cryoprotectants (cryoprotective agents, CPAs) can be toxic to cells. Hence, it is important to determine the impact of cryoprotectants on oocytes as a first step in freezing protocol design. Ideally the cryoprotectant should have low toxicity and be able to permeate the oocyte plasma membrane. Four penetrating cryoprotectants have been selected for toxicity studies, namely methanol, dimethyl sulfoxide (DMSO), propylene glycol (PG) and ethylene glycol (EG). The toxicity of two sugars, sucrose and glucose, was also studied.

2.4.1 Exposure of zebrafish oocytes to different CPAs

Oocytes were held in full-strength Hank's solution at 22°C immediately after collection. Solutions of CPAs were made up in Hank's at a range of concentrations: 0.25M, 0.5M, 1M, 2M, 3M and 4M. The highest concentration of CPAs used in the experiments was 4M except for MTT test on stage III oocytes and for TB test on stage IV oocytes, where the highest concentration used was 2M. The reasons for this were that after initial experiments, it became apparent that MTT test was not very sensitive and stage IV oocytes (a short transient stage between stage III and stage V) were not convenient for use in cryopreservation, therefore, 3M and 4M concentrations were not used in the subsequent studies.

None of the fluorescent dyes, mentioned in Section 2.3 (Ethidium bromide, Propidium iodide, Calcein blue, cFDA, Rhodamine 123) were used in this and following parts of study, as previous experimentation has proved their inefficiency.

For CPA toxicity studies, 15-40 oocytes were put into each well of the 6-well cell culture plates. The excess of Hank's solution was removed and 2ml CPA solution was added. Oocytes were then incubated in CPA solutions for 30min at room temperature. Control oocytes were incubated in Hank's solution under the same conditions. After incubation in CPAs, oocytes were washed twice with Hank's solution and viability tests were conducted. The No Observed Effect Concentrations (NOECs) were then determined.

2.4.2. CPA exposure of oocytes at different developmental stages

Zebrafish oocytes of three different developmental stages were used for toxicity studies, namely stage III (vitellogenic), stage IV (maturating) and stage V (mature) oocytes. Oocytes of each of stage were obtained as described in Section 2.2.1.3; and were exposed to different concentrations of CPAs as described in section 2.4.1. Oocytes viability was then assessed using three vital tests: TB, MTT and GVBD. For stage IV and V oocytes, MTT and GVBD tests were not applicable and only TB test was used.

2.4.3. Studies on toxicity of supplements and KCl medium

While some of the supplements used in this part of the investigation (such as egg yolk suspension, aspirine, N-Ac-Cysteine or poloxamer-188) are known to be non toxic to cells, other additives may have some toxic effect on zebrafish oocytes. For example, PEG-400 may lead to disruption of membrane structure and cause chromosome abnormalities (Belous & Grischenko, 1994; Biondi et al., 2002). Incubation of oocytes in the medium with high potassium content (i.e. in KCl buffer) may lead to depletion of their membrane electrical potential and cause a number of other undesirable changes (Maeno, 1959). Toxicities of polyethylene glycol-400 (PEG) together with toxicity of KCl medium to zebrafish oocytes were therefore studied.

To study the toxicity of KCl buffer, oocytes were extracted from female fish either in Hank's medium or in KCl buffer and kept in these solutions for 30 min at 22°C. Oocytes viability was then assessed using TB staining or GVBD test.

As methanol was found to be the most effective cryoprotectant of all CPAs tested, the separate experiments on toxicity of methanol solutions in KCl buffer were also carried out. Stage III zebrafish oocytes were separated in KCl buffer, and kept in solutions of 2M Methanol or 4M Methanol in KCl buffer for 30 min at 22°C. Oocytes were then washed twice with KCl buffer, and their viability was assessed using TB test and GVBD test. The results obtained

from these studies were compared with the results obtained from other toxicity studies (section 2.4.1). For experiments on toxicity of PEG-400, stage III zebrafish oocytes were incubated in 5% solution of PEG-400 for 30 min and 60 min, and then their viability was assessed either with TB test, or GVBD test.

2.5 Cryopreservation of zebrafish oocytes using controlled slow cooling

After cryoprotectant toxicity studies, investigations were carried out on cryopreservation of zebrafish oocytes using controlled slow cooling. For each replica, 100-200 stage III oocytes from the same female were used .

2.5.1 Use of different final freezing temperatures

Full-strength Hank's solution was used as a working medium for all procedures. Solutions of CPAs (2M methanol, 2M DMSO, 2M PG, 1M EG) were made up in Hank's medium. Stage III oocytes were used for all cooling experiments. Oocytes were incubated in cryoprotectants solutions for 30 min at room temperature (22°C). Control oocytes were incubated in Hank's solution under the same conditions. Oocytes were then loaded into 0.5 mL plastic straws and put into a programmable cooler (Planer KRYO10 Series II). About 100-200 vitellogenic oocytes from each fish were used, and each straw contained about 30 oocytes. Four or more females were used for each treatment. The following cooling protocols were used in the experiments: for 2M CPAs cooling rate was 2°C/min from 20°C to -7.5°C; samples were seeded manually and held at -7.5°C for 5 min ; the straws were then cooled to -10°C, -15°C, -20°C or -25°C at rate 1°C/min, 0.5°C/min, 0.3°C/min and 0.1°C/min (Fig. 2.11).

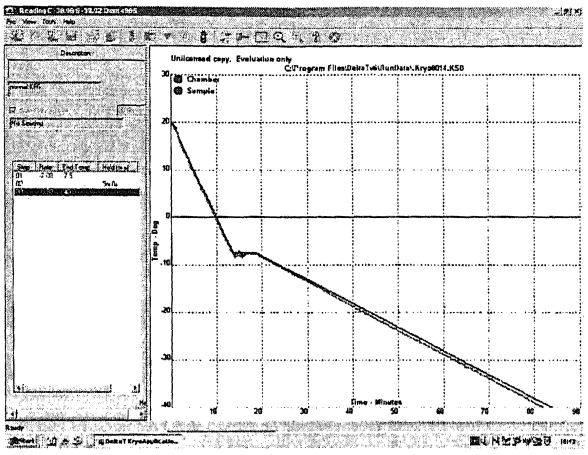


Fig. 2.11. A working screen of DeltaT software, which controls a programmable cooler from an IBM- compatible personal computer. Right window graphically depicts one of the cooling profiles used in our experiments.

For 1M CPA, cooling rate was 2°C/min from 20°C to -5°C ; samples were seeded manually and held at -5°C for 5 min; straws were then cooled to the same final temperatures at 1°C/min, 0.5°C/min and 0.3°C/min. Oocytes incubated in cryoprotectant-free Hank's medium were used as controls. Thawing was carried out by immersing straws into a 27°C water bath for 20 seconds. After thawing oocytes were washed in Hank's medium twice (CPA-containing medium was removed, pure Hank's solution was added and stirred), and viability was evaluated using TB staining or GVBD test. In the case of methanol, because of its low toxicity, higher concentrations of this cryoprotectant, 3M and 4M, were also used. For 3M methanol the seeding temperature was -10°C, and for 4M methanol the seeding temperature was -12.5°C. Post-seeding cooling rate was -0.5°C/min for both concentrations; and the final freezing temperatures were -10°C, -15°C, -20°C, -25°C, -30°C and -40°C.

2.5.2 Use of different CPAs

Four CPAs were used in these experiments: propylene glycol (PG), ethylene glycol (EG), dimethyl sulfoxide (DMSO) and methanol. No Observed Effect Concentrations (NOEC) for 30 min incubation at 22°C, which had been

previously determined in toxicity experiments (see section 2.4.1) were used for each CPA (1M for EG, 2M for PG, 2M for DMSO, and 2M, 3M and 4M for methanol.

2.5.3 Use of different cooling rates

Four different post-seeding cooling rates were used: 1°C/min, 0.5°C/min, 0.3°C/min and 0.1°C/min. 0.3°C/min and 0.1°C/min cooling rates were only used with 2M methanol, because after initial experiments it became apparent that methanol was the best CPA, and therefore it was used in subsequent studies.

2.5.4. Use of different viability assessment methods

In the majority of the cases, TB test was used for assessment of viability of cryopreserved oocytes. It's worth noticing, that TB test assesses the integrity of plasma membrane, not the physiological status of the whole cell. The cells rejected by TB test (stained blue) are lethally damaged and non-viable; but if the cell is not stained by TB, it can be live, or dead but with intact plasma membrane. GVBD observation was also used in some experiments to validate the results obtained with TB test as GVBD test is more sensitive than TB test. GVBD test was applied for following treatments: freezing with 2M PG, 2M DMSO, 2M Methanol, and 1M EG at cooling rate of 1°C/min to -10°C and -15°C. GVBD test was also applied for following treatments: freezing with 2M and 4M Methanol at 0.5°C/min to -10°C, -15°C, -20°C and -25°C; and freezing with 2M Methanol at 1°C/min to -10°C and -15°C.

2.6 Alternative approaches for cryopreservation of zebrafish oocytes

Approaches, such as application of additional cryoprotective supplements; varying the liquid nitrogen-plunging temperatures; varying ice-seeding temperatures, and varying the pH of freezing medium have been studied. Stage III oocytes were used in these studies.

2.6.1 Application of additional cryoprotective supplements

Besides cryoprotectants, a number of other substances have been shown to have beneficial effect on cryosurvival of fish oocytes when present in the freezing medium. Major cryoprotectants (DMSO, PG, EG, methanol, glycerol etc.), which are used in high concentrations (0.5M - 4M) act colligatively by lowering the freezing point of the medium, decreasing the amount of unbound water and suppressing intracellular ice formation. Meanwhile minor supplements are used in lower concentrations and act by protecting vulnerable areas of the cell. A number of such minor additives: polyethylene glycol-400 (PEG-400), PEG-200, poloxamer-188, sucrose, glucose, dexamethasone, butylated hydroxyanisole (BHA), N-Acetyl-Cysteine, tocopherol, taurine, egg yolk suspension and acetyl salicylic acid were used in the present study. Variation of pH and composition of a freezing medium, variation of ice-seeding temperatures and liquid nitrogen plunge temperatures have also been applied in order to improve cryosurvival of oocytes.

2.6.1.1. Studies on controlled slow freezing of oocytes using KCl buffer as a medium

The oocytes were separated in KCl buffer. Oocytes were then incubated in the medium containing 4M Methanol in KCl buffer for 30 min at 22°C; they were loaded into 0.5 ml plastic straws (about 30 oocytes in each straw), and frozen using the following protocol: cooling from 20°C to -12.5°C at 2°C/min; manual seeding at -12.5°C and hold for 5 min; slow freezing from -12.5°C at 0.3°C/min to final temperatures of -10°C, -20°C, -30°C, -40°C, -50°C and -60°C. In addition, samples were also plunged into LN (-196°C) for 5 min after slow cooling (0.3°C/min) to -50°C. After reaching the final temperatures and holding at this temperature for 5 min, samples were thawed in a 27°C water bath for 20 seconds (~200°C/min). Oocytes were then washed twice in KCl buffer. Oocytes viability was assessed using TB staining and for certain conditions (-10°C and -20°C final temperature) with GVBD test. At least four replicas were used for each treatment.

2.6.1.2 Studies on oocytes controlled slow freezing using a range of supplements in the cryoprotective medium

The oocytes were kept in either Hank's solution or in KCl buffer. Control media used in these experiments were 4M methanol in Hank's or in KCl buffer. Polyethylene glycol-400 (PEG-400) (5%), PEG-200 (5%), poloxamer-188 (10mg/ml); sucrose (0.1M), glucose (0.2M), sucrose + glucose (0.1M and 0.1M respectively), dexamethasone (0.5mM), BHA (100mM), N-Ac-Cyst (0.01M), tocopherol (20mM), taurine (50mM), egg yolk suspension (3%), acetyl salicylic acid (0.5mM) were used as additives to the control media. Stage III zebrafish oocytes were incubated in the above media for 30 min at 22°C, they were then loaded into 0.5 ml plastic straws and frozen using the following protocol: cooling from 20°C to -12.5°C at 2°C/min; manual seeding at -12.5°C and hold for 5 min; slow freezing from -12.5°C to -50°C at 0.3°C/min; samples were then plunged into LN. After 10 min in LN, samples were thawed (~200°C/min) in a 27°C water bath for 20 seconds. Oocytes were then washed twice in either Hank's or KCl-buffer. Oocytes viability was then assessed using TB staining. At least four replicas were used for each treatment.

2.6.2 Use of different ice-seeding temperatures

The oocytes were incubated in a freezing medium containing 4M methanol + 0.2M glucose in KCl buffer for 30 min at 22°C, they were then loaded into 0.5 ml plastic straws (about 30 oocytes in each straw) and frozen using the following protocol: cooling from 20°C at 2°C/min cooling rate to the seeding temperature; manual seeding and hold for 5 min. Such seeding temperatures as -12.5°C; -10°C; -8.5°C were tested; as well as no ice seeding. Straws were then frozen to -50°C at 0.3°C/min, and plunged into liquid nitrogen. After 10 min in LN, samples were thawed (~200°C/min) in a 27°C water bath for 20 seconds. Oocytes were then washed twice in KCl-buffer. Oocytes viability was assessed using Trypan Blue staining (0.2% TB for 5 min). At least four replicas were used for each treatment.

2.6.3 Use of different liquid nitrogen plunge temperatures

The oocytes were incubated in the freezing medium containing 4M methanol + 0.2M glucose in KCl buffer for 30 min at 22°C, they were then loaded into 0.5 ml plastic straws (about 30 eggs in each straw) and frozen using the following protocol: cooling from 20°C to -12.5°C at 2°C/min; manual seeding and hold for 5 min. Samples were then frozen at 0.3°C/min to -30°C, -40°C, -45°C, -50°C, -60°C, and -70°C before plunging into liquid nitrogen (LN). After 10 min in LN, samples were thawed (~200°C/min) in a 27°C water bath for 20 seconds. Oocytes were then washed in KCl-buffer twice. Oocytes viability was assessed using TB staining.

Another set of experiments was also conducted with samples fast frozen from -40°C to -80°C and plunged into LN. The oocytes were incubated in the freezing medium containing 4M methanol + 0.2M glucose in KCl buffer (pH 7.4) for 30 min at 22°C, they were then loaded into 0.5 ml plastic straws (about 30 eggs in each straw) and frozen using the following protocol: cooling from 20°C to -12.5°C at 2°C/min; manual seeding at -12.5°C and hold for 5 min; slow cooling from -12.5°C to -40°C at 0.3°C/min; fast cooling from -40°C to -80°C at -10°C/min; samples were then plunged into LN. After 10 min in LN, all samples were thawed (~200°C/min) in a 27°C water bath for 20 seconds. Oocytes were then washed in KCl-buffer twice. Oocytes viability was assessed using TB staining. At least four replicas were used for each condition.

2.6.4 Use of freezing solutions with different pH values

The normal pH for KCl medium is 7.4. In these experiments, pH of KCl buffer was adjusted to: 4.4, 5.4, 6.4, 7.4, 8.4, 9.4 or 10.4 with either 1M KOH or 1M HCl. 4M Methanol + 0.2M glucose solutions in KCl buffers with different pH were prepared. Oocytes were incubated in the above media for 30 min at 22°C and frozen to LN temperature using the following protocol:

Oocytes were loaded into 0.5 ml plastic straws and cooled from 20°C to -12.5°C at 2°C/min; manual seeding at -12.5°C and hold for 5 min; slow freezing from -12.5°C to -40°C at 0.3°C/min; samples were then plunged into

LN. After 10 min in LN, samples were thawed (~200°C/min) in a 27°C water bath for 20 seconds. Oocytes were then washed either in Hank's or KCl-buffer twice. Oocytes viability was assessed using TB. At least four replicas were done for each condition.

2.7. Cryomicroscope observations on zebrafish oocytes

For cryomicroscope observations of zebrafish oocytes, Olympus BX 51 U-CMAD3 cryomicroscope (Japan) with compatible JVC color digital videocamera was used. 200 μl quartz crucible was used as container for oocytes. Stage III zebrafish oocytes were used for all cryomicroscope observations.

2.7.1 Studies on extracellular ice formation

In order to investigate processes of ice nucleation and growth in the samples, the following freezing profile was followed: 30 min incubation of stage III zebrafish oocytes in a freezing medium at room temperature; controlled slow freezing by the following profile: cooling from 20°C to -12.5°C at 2°C/min; seeding and hold at this temperature for 5 min; slow freezing from -12.5°C to -40°C at 0.3°C/min; samples are then cooled at 130°C/min (which is the maximum cooling rate for cryomicroscope) to -196°C (imitating plunge into the LN). After 5 min hold at -196°C samples are thawed (130°C/min) to room temperature.

For a number of special cases, cooling rates of 1°C/min, 2°C/min and 5°C/min were used. Pure KCl buffer, solution of 4M Methanol in KCl buffer, solution of 4M Methanol + 0.2M Glucose in KCl buffer, and solution of 4M Methanol + 0.2M Glucose in Hank's medium were used as a freezing media. In number of cases, most of the extracellular solution was removed leaving a thin layer, in order to be able to carry out microscopic observations after formation of ice crystals in the solution.

2.7.2. Studies on intracellular ice formation

All procedures for investigating the patterns of intracellular ice formation were substantially the same as in p. 2.7.1.1; but in those

experiments, where it was necessary to exclude surface-catalyzed nucleation (SCN) of oocytes by extracellular ice, extracellular medium was thoroughly removed from the sample by pipette and filter paper and replaced by transparent non-freezing silicone oil.

2.7.3. Depression of IIF temperature by penetrating cryoprotectants

In order to determine, to what extent the addition of cryoprotective agents decreases the freezing point of oocyte cytoplasm and of surrounding solution, oocytes were incubated in solutions of 4M methanol and of 4M methanol + 0.2M glucose in KCl buffer for 30 min before start of cryomicroscope observations. Cooling at rate 1°C/min from 20°C to -30°C was carried out. The microscopic pictures were made with digital camera with interval 0.3 seconds during the course of freezing; and basing on analysis of these images the temperature was determined, at which freezing of the sample occurred.

2.7.4. Damage caused to the cytoplasm by ice growth in the sample

In order to investigate the damage to the morphological structure of the oocyte, associated with freezing, oocytes were incubated in a solution of 4M methanol + 0.2M glucose in KCl buffer for 30 min before start of cryomicroscope observations, and then cooled at a rate of 1°C/min from 20°C to -30°C. In number of cases, samples were frozen to -80°C. Cooling rate of 5°C/min was used below -30°C. A thawing rate of 2°C/min from the final freezing temperature to room temperature (20°C) was used. After thawing and stabilisation of the sample temperature, thorough morphological observation of oocyte structure under microscope was carried out. In a number of cases, repeated freeze-thawing was conducted.

2.8 Assessment of ATP level of cryopreserved zebrafish oocytes

The existing tests for assessment of viability of fish oocytes are not ideal and have many disadvantages and limitations. Some of them (such as cFDA assay) do not allow to obtain a clear enough difference between viable and non-viable cells; other assess the state of plasma membrane only (such

as Trypan Blue staining), or evaluate the activity of single enzyme (MTT assay), which may lead to misinterpretations. Some methods (such as *in vitro* maturation assay) can be applied for only one stage of oocyte development. Therefore, there is a need for developing an alternative method for evaluation of viability of fish oocytes. Determining the ATP content in the cytoplasm of the oocytes could be such a method.

ATP is a vitally important substance, the main transporting agent of energy inside the living cells. The presence of ATP inside the oocytes is essential for their survival, and for normal developmental and reproduction potential. ATP content in the cell is a reliable parameter for evaluation of viability of different types of cells (Lehninger, 1982), including fish sperm cells (Kopeika et al., 1997) and fish oocytes (Boulekbache et al., 1989). For determination of ATP content in zebrafish oocytes the commercially available ATP Bioluminescent Assay Kit (FL-AA, Sigma) was used, which is based on reaction of protein luciferin with ATP, catalysed by enzyme luciferase. Product of this reaction, adenyl-luciferin, oxidises with emission of light within the optical range (λ_{max} = 560 nm).

2.8.1 Preparation of extract from oocytes

1 ml of 0.5M perchloric acid + 4mM EDTA (4°C) was added to thirty stage III zebrafish oocytes. The mix was homogenised in a centrifugeable glass tissue microhomogeniser with a conical glass pestle. The homogenate was centrifuged at 20,000g for 10 min at 0-2°C. Supernatant was separated and neutralised with 2.5M KOH to pH 7.8, and centrifuged for 5 min at 8000g. The supernatant was separated and stored at -20°C until analysis.

2.8.2 Preparation of FL-AA reagents

FL-AAM (ATP Assay Mix). Contents of one vial of ATP Assay Mix ATP ASSAY MIX (a lyophilized powder containing luciferase, luciferin, MgSO4, DTT, EDTA, bovine serum albumin and tricine buffer salts) was dissolved in 5 ml of sterile deionised water to generate a stock solution with pH 7.8. This stock solution can be stored at 0-5°C for up to 2 weeks. Immediately before experiments, FL-AAM solution was diluted with FL-AAB (ATP Assay Mix

Dilution Buffer) 25-fold. 25-fold dilution can be used for ATP concentrations of 2×10^{-10} to 2×10^{-7} mol/l.

FL-AAB (ATP Assay Mix Dilution Buffer). Contents of one vial of FL-AAB (lyophilized powder containing MgSO4, DTT, EDTA, bovine serum albumin and tricine buffer salts) were dissolved in 50 ml of sterile deionised water. This solution can last for 2 weeks when stored at 0-5°C.

FL-AAS (ATP Standard). One vial of FL-AAS contains approx. 1 mg (2.0×10^{-6} mole) of ATP. Solutions of ATP were made in sterile deionised water. Solutions are stable for 2 weeks when stored at -20°C, or up to 24 hours at 0-5°C. A number of solutions with gradually decreasing ATP concentration were prepared in sterile deionised water: 2×10^{-5}, 2×10^{-6}, 2×10^{-7}, 2×10^{-8}, 2×10^{-9}, 2×10^{-10}, 2×10^{-11}, and 2×10^{-12} M. Calibrating curve was established based on the measurements of their fluorescence, which these dilutions emitted during reaction with FL-AAM.

2.8.3 Determination of ATP level

0.1 ml of ATP Assay Mix solution was added to a reaction vial, and was gently swirled and let to stay in a room temperature for approximately 3 min. Then 0.1 ml of sample was added. The mixture was then swirled quickly and amount of produced light was immediately measured with a luminometer.

To determine the amount of background light produced, 0.1 ml of deionised water was added to 0.1 ml of ATP Assay Mix solution, the mixture was swirled and the luminescence measured. The obtained value was substracted from that obtained from the sample. The final value was proportional to the amount of ATP in the sample.

Calibration curve was established using different dilutions of *FL-AAS (ATP Standard),* and this curve was used for determining concentrations of ATP in the samples.

The luminescence was measured by a TD-20/20 luminometer (Turner Designs, USA). 10 sec integration period was used, with 2 sec delay period before each integration period. The total amount of light produced by the sample during 10 sec interval was determined. Luminescence of each sample was measured 5 times over a period of 1 minute, in order to eliminate the quenching of luminescence with time. The mean luminescence level of each sample was then calculated. For each treatment, four replicas (i.e. four samples) were measured.

The treatments were:

1. Intact oocytes freshly obtained (coded: RT).

2. Intact oocytes after 1 hour incubation in Hank's medium at room temperature (coded: RT1H).

3. Oocytes in Hank's medium were loaded to straws and frozen to -196°C by direct plunge into liquid nitrogen. They were kept in LN for 5-10 min; then thawed by immersion into 27°C waterbath for 20 sec. The oocytes were kept for 1 hour in Hank's at 22°C (coded: PLU1H).

In the following treatments, oocytes were frozen to different final temperatures by the optimal cryopreservation protocol: 30 min incubation in 0.2M Glucose + 4M Methanol solution in KCl-buff. (pH 7.4); loaded into 0.5 ml straws; cooling from 20°C to -12.5°C at 2°C/min; manual seeding and hold for 5 min; then slow freezing at 0.3°C/min to -40°C; and samples were then plunged into liquid nitrogen. Difference between the following treatments was, that the cooling was interrupted at different temperatures. After cooling was stopped, samples were thawed (~200°C/min) in a 27°C water bath for 20 sec. Oocytes were then washed twice in KCl-buffer, held in KCl buffer at room temperature for 1 hour.

4. Oocytes were cryopreserved following the optimal cryopreservation protocol all the way through to LN temperature (coded: OPT1H).

5. Oocytes were frozen following the optimal cryopreservation protocol with cessation at -10°C (coded: -10,1H).

6. Oocytes were frozen following the optimal cryopreservation protocol with cessation at -20°C (coded: -20,1H).

7. Oocytes were frozen following the optimal cryopreservation protocol with cessation at -30°C (coded: -30,1H).

8. Toxicity of 0.2M Glucose + 4M Methanol solution in KCl-buff. (pH 7.4). Oocytes were incubated in this medium for 30 min; then washed in KCl-buffer twice and kept in KCl buffer at room temperature for 1hr (coded: TOX1).

9. Oocytes were frozen following the optimal cryopreservation protocol with cessation at -20°C. 1 hr post-thaw incubation at room temperature was absent (code: -20,0H).

10. Oocytes were frozen following the optimal cryopreservation protocol with cessation at -20°C, and after thawing incubated in re-vitalising medium (see below) for 1 hr at room temperature (coded: -20,RE).

One hour post-treatment incubation at room temperature was used in the most of treatments, because of following considerations: after the cells have experienced the damaging effect of any treatment (either toxicity of cryoprotectant, or freezing injury), the intracellular ATP level would not normally change immediately. The injuries accumulated in the cells need certain amount of time to manifest themselves. 1 hour incubation of oocytes at room temperature in a medium was used in these experiments. It was presumed, that if the energy system of the cells has been seriously damaged, the ATP hydrolyses and its level would fall.

2.8.4 Experiments with a "reviving" medium

In this set of experiments, cryopreserved oocytes after thawing and rinsing from cryoprotectants were incubated in a "reviving" medium, which was designed to promote ATP synthesis in oocytes. "Reviving" medium was made using Hank's as a base medium, and supplemented by 0.05M Glucose, 1 mM adenosine, and 10 mM ADP. The aim of adding glucose is to provide oocytes with the extra-source of nutrition for synthesis of ATP. Adenosine and ADP are added to "reviving medium" in order to provide the cells with substrates for ATP synthesis, in case leakage of substances has occurred in the course of freeze-thawing through damaged plasmalemma, and cells have partially lost their stock of adenine nucleotides. In addition, the presence of elevated levels of ADP is known to enhance ATP synthesis via the mechanisms of enzyme regulation (Lehninger, 1982). Incubation time was 1 hour. While oocytes were incubated in "reviving medium", air was pumped into incubation vessels, in order to further enhance ATP synthesis.

2.9 Data analysis

2.9.1 Calculation of oocytes viability

The viability of oocytes was calculated by the formula:

$$\text{Viability (\%)} = \frac{\text{Number of viable cells}}{\text{Total number of cells}} \times 100\% \quad (2.1)$$

2.9.2 Normalisation of oocytes survival

Normalised survival was calculated in order to eliminate individual differences in initial oocyte samples. The following formula was used:

$$\text{Normalised survival (\%)} = \frac{\text{Experimental viability}}{\text{Viability of untreated control}} \times 100\% \quad (2.2)$$

2.9.3 Statistical analysis

In the present study, up to 200 oocytes were used in each tested treatment (~50 oocytes for cryomicroscopy and toxicity studies; ~200 oocytes for slow cooling studies). Each treatment was replicated for at least three times. For statistical analysis such methods as one-way ANOVA, one-way ANOVA followed by orthogonal polynomial contrasts analysis of specific comparisons, two-factor ANOVA, Kruskal-Wallis non-parametric ANOVA, Scheffe's post-hoc test and Student's t-test were used. ANOVA (confidence level <0.05) was used in order to test for significant differences between the mean values from several treatments. If ANOVA revealed the presence of statistically significant differences between the treatments in experiment, Scheffe's post-hoc test, or more rarely, orthogonal polynomial contrasts analysis of specific comparisons, was used in order to find out which groups specifically differ. In a few cases, Student's t-test (confidence level <0.05) was used to determine differences in results between two contrasting groups.

Inscription "N = ..." in graph legends indicates the total number of fsh (replicas) used for the corresponding experiment. The results of the study are presented in "mean value ± standard errors"; or in "normalised mean value ± standard errors".

CHAPTER 3. DEVELOPMENT OF SUITABLE VIABILITY ASSESSMENT METHODS FOR ZEBRAFISH (*DANIO RERIO*) OOCYTES

3.1. Introduction

The studies on cryopreservation of fish oocytes are dependant on the development of reliable vital tests for evaluation of their viability. There were no reliable tests so far for assessment of viability of unfertilised fish oocytes, although one method, namely cFDA staining, was suggested by Pearl & Arav (2000). In order to develop viability assessment methods for fish eggs, five fluorescent dyes - Calcein blue, cFDA (5-carboxy-fluoresceindiacetate), Ethidium bromide, Propidium iodide and Rhodamine 123 were tested on stage III zebrafish oocytes. In the case of Rhodamine 123, enzymatic (trypsin) defolliculation was applied to remove the follicle layer in order to improve results. Non-fluorescent methods were also tested - Trypan blue (TB) staining, MTT (thiazolyl blue) staining and *in vitro* maturation, followed by germinal vesicle breakdown (GVBD) observations.

Calcein blue (excitation wavelength 360 nm, emission wavelength 450 nm) is a fluorescent dye, which normally can easily penetrate into the cells, where it binds to calcium ions and becomes strongly fluorescent. It is retained only in viable cells which have intact oolemma. As a result, calcein blue stains live cells green, whilst dead ones remain unstained. Calcein blue has been used as a viability indicator for many cells and tissues, including corneal tissue, leucocytes and lymphocytes (Haugland, 2002).

cFDA (5-carboxy-fluoresceindiacetate), which is not fluorescent, easily penetrates cells. In live cells, cFDA is enzymatically processed by esterases to carboxyfluorescein, which emmits fluorescence (Ex / Em: 492 nm / 514 nm) and can not leave the cell because the plasma membrane is impermeable to this substance. Hence, live cells acquire green fluorescent color, while dead cells remain unstained. cFDA has been used as a vital dye for zebrafish vitellogenic oocytes, as well as for bovine oocytes (Pearl & Arav, 2000; Zeron et al., 1999; Arav et al., 1996) .

Rhodamine 123 (Ex / Em : 520 nm / 535 nm) is a cell-permeant cationic fluorescent dye which accumulates in metabolically active (i.e. charged) mitochondria and fluorescently stains them. Therefore, it can evaluate whether the energy system of the cell is working normally. It was used for assessment of mitochondrial intactness in cryopreserved pig oocytes (Wu & Lee, 1996), freshly-obtained mouse oocytes (Thouas et al., 2005), and in intact mouse oocytes maturated *in vitro* (Tokura et al., 1993).

Ethidium bromide is a fluorescent dye which stains the nuclei of dead cells, binding to DNA. This dye does not penetrate inside live cells. Molecules of ethidium bromide penetrate into the dead cells and bind to the DNA molecules of chromatine. When bound to DNA, ethidium bromide becomes red under ultraviolet light (Ex / Em : 518 nm / 605 nm).

Propidium iodide (Ex / Em : 535 nm / 617 nm) is a substance similar to ethidium bromide and from the same group of phenanthridine dyes. It has the similar to ethidium bromide mechanism of action.

Trypan blue staining is a simple and widely used non-fluorescent technique for assessment of membrane integrity of cells in biological samples. Trypan blue stains the nuclei of dead cells, and when used in higher concentrations it stains the whole cytoplasm of the dead cells. Although this vital test has certain disadvantages, it is a fast and convenient method for approximate viability assessment. Trypan blue was successfully used for assessment of viability of intact and cryopreserved porcine oocytes (Didion et al., 1990), cultured *in vitro* bovine oocytes (Fouladi Nashta et al., 1998) and for numerous other cell types.

The methyl thiazole tetrazolium (MTT) test is based on the conversion of methyl thiazole by mitochondrial enzyme succinate dehydrogenase (SDH) into insoluble formazan crystals, which are purple in color (Mosmann, 1983; Drixler et al., 2002). This reaction is only possible in viable cells with metabolically active mitochondria, so live cells acquire purple color, while dead cells remain unstained.

In vitro maturation test is based on the ability of immature vitellogenic fish oocytes to complete the process of physiological maturation *in vitro,* during incubation in a special growth medium (Leibovitz medium, L-15), supplemented with maturation inducing steroid hormone. *In vitro* maturation of fish, amphibian and mammalian oocytes (including human) has been extensively investigated (Pennequin et al., 1975; Smith, 1989; Selman et al., 1994; Pang & Ge, 1999, 2002; Shuhong et al., 1993; Ecker & Smith, 1971; Patino & Purkiss, 1993; Wallace & Jared, 1976; Campbell & Jalabert, 1979; Yi et al., 2001). Live oocytes will undergo germinal vesicle breakdown (GVBD) and progress to stage V (mature egg). During this process the cytoplasm of live oocytes loses its opacity and becomes translucent; whilst dead oocytes remain opaque. The protocol for *in vitro* maturation of zebrafish oocytes has been reported by Selman et al. (1994).

Oocytes obtained from fish ovary are aggregated in cumulus (see Chapter 2, Fig.2.9). To obtain oocytes at different developmental stages it is necessary to disaggregate the cumulus. Enzymatic disaggregation of cumulus was tested in this study as an alternative to mechanical maceration which is time consuming. It was also presumed that enzymatic disaggregation would be a more gentle method than mechanical disaggregation.

Even after cumulus disaggregation, either by mechanical or enzymatic methods, stage III (vitellogenic) zebrafish oocytes are still surrounded by a follicle layer - a structure which has nutritional, regulatory and protective function for the growing egg (Fig. 2.10). The follicle layer may decrease the permeability of oocyte to various substances, including cryoprotectants.

Enzymatic methods for defolliculation of oocytes and disaggregation of cumulus mass are described for several fish and amphibian species. In most of the cases, the proteolytic enzyme collagenase was used (Campbell & Jalabert, 1979; Miledi & Woodward, 1989), other methods such as a combination of collagenase and hyaluronidase (Choe & Sackin, 1997), and trypsin (Schultheiss et al., 1997; Sullivan et al., 1999) were also used. In some cases, bovine serum albumin (BSA) was added to enzymatic solution to protect oocytes from the lytic effect of enzymes (Campbell & Jalabert, 1979). Enzymatic disaggregation and defolliculation methods are based on the

principle that proteolytic enzymes destroy follicle cells surrounding the oocytes, and weaken connections between the cells, thus follicle layers can be easily removed from the oocyte. However, over exposure to the enzymes may lead to undesirable changes in oocytes, and even death, as proteases affect not only components of follicle cells, but also oocyte itself (Schultheiss et al., 1997).

For disaggregation of cumulus, collagenase-hyaluronidase solution was used to treat oocytes, a method which has been previously used with amphibian and fish oocytes (Choe & Sackin, 1997; Campbell & Jalabert, 1979). To carry out enzymatic defolliculation, trypsin solution was used in the present study.

3.2. Results

The results obtained in this part of the work are mainly based on microscopic observations.

3.2.1 Enzymatic disaggregation of cumulus

When enzymatic maceration of cumulus was applied, oocytes were separated well. Microscopic observation revealed high ratio of intact oocytes. However, oocytes which underwent enzymatic disaggregation can not be stained with 0.2% Trypan Blue even after incubation in high concentrations of cryoprotectants.

Experiments with GVBD-assessment of enzymatically treated oocytes showed that 15 min incubation in collagenase-hyaluronidase solution led to noticeable viability loss of the oocytes when compared to the controls (33.3% compared to 60.6%), and no oocytes survived after 30 min incubation. Addition of 5% bovine serum albumin to enzymatic medium had substantial protecting effect on oocytes during enzymatic treatment. Addition of BSA to enzymatic solution allowed 31.2% oocyte viability after 15 min incubation, and 36.3% viability after 30 min incubation with the enzymatic solution. When BSA was used in enzymatic solution, the morphological appearance of oocytes was improved with more oocytes remaining intact. The presence of BSA in solution prevented lysis of oolemma and efflux of ooplasm from the cells.

Hence, BSA is an effective protective agent during defolliculation. 10% fetal calf serum demonstrated poor ability to stop hydrolytic activity of collagenase and hyaluronidase, because the lysis of oocytes continues even after thoroughful rinsing with 10% FCS.

3.2.2 Enzymatic defolliculation of oocytes

Trypsin treatment appeared to be very damaging to oocytes, microscopic observation of trypsin-defolliculated oocytes revealed severe lysis of cells (Fig. 3.1). 10% fetal calf serum, used to stop trypsin action, was found not to be effective, and the lysis of oocytes continued during microscopic observation. Therefore, trypsinisation was not used in subsequent experiments.

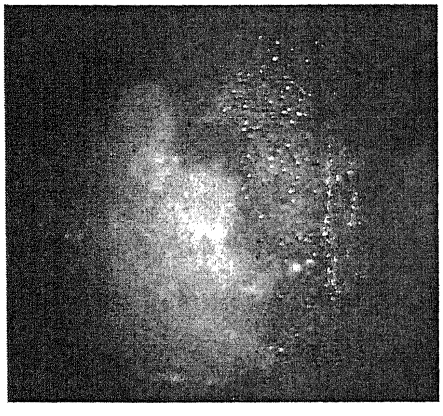


Fig.3.1. Effect of enzymatic defolliculation with trypsin on zebrafish oocytes. Stages I - III oocytes were treated with 4% trypsin for 5 min, they were then stained with 1.0 µM/L cFDA. Severe lysis of the cells was observed.

3.2.3 Ethidium bromide, Propidium iodide and Calcein blue assays

Experiments with Calcein blue, Ethidium bromide, and Propidium iodide showed that none of the oocytes, either used for positive or negative controls showed any noticeable fluorescence. Hence, these fluorescent dyes were found unsuitable as vital tests for zebrafish oocytes.

3.2.4 cFDA and Rhodamine 123 assays

The results from these experiments showed that, when oocytes were stained with cFDA or Rhodamine-123, it was not possible to distinguish

between live and dead oocytes clearly: oocytes in both positive and negative controls emitted bright green homogenous fluorescence (Fig.3.2, Fig.3.3). In the case with cFDA, there were subtle differences between the fluorescence levels of alive and dead oocytes (Fig.3.2), but the differences were not sufficient for a confident viability assessment method. In experiments with Rhodamine 123, defolliculation of oocytes with trypsin (see section 2.3.11) helped to reveal some differences between live and dead oocytes. However these differences were not sufficient to be the basis of a confident test.

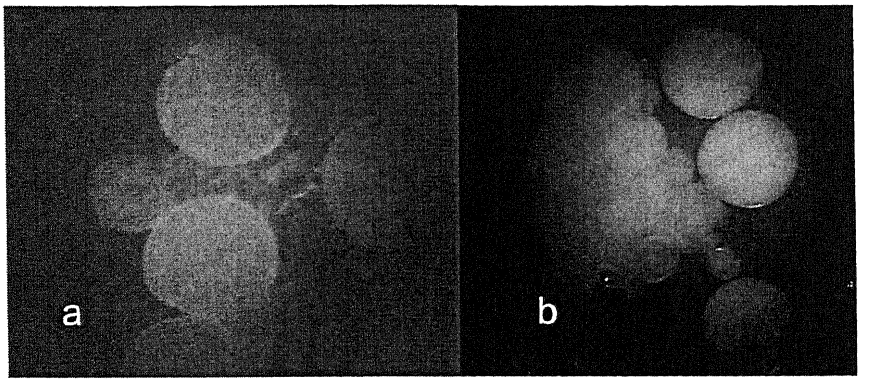


Fig. 3.2. cFDA stained stage III zebrafish oocytes. a) Intact stage III oocytes b) negative control, oocytes were treated with 100% methanol for 10 min at room temperature (22°C).

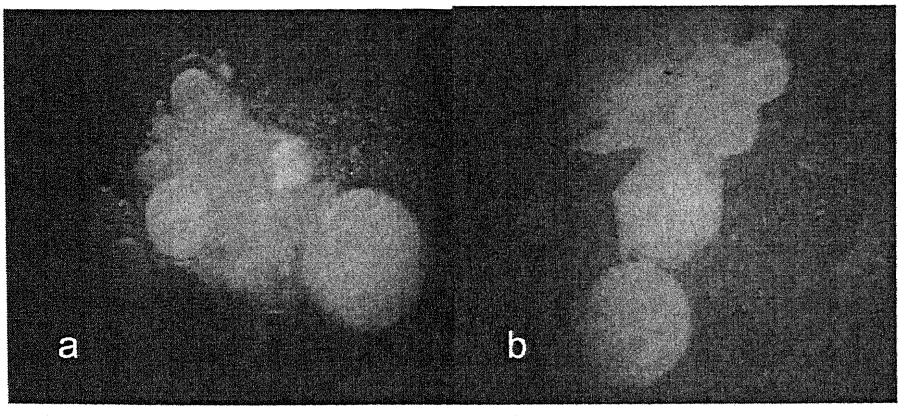
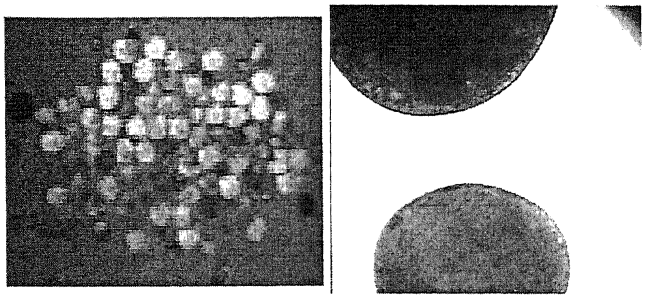


Fig. 3.3 Rhodamine-123 stained stage III zebrafish oocytes. a) Intact oocytes stained with 30 µg/ml Rhodamine 123 during 30 min at 28°C; b) Oocytes, treated with 99% methanol for 10 min at room temperature, and then stained with Rhodamine-123

3.2.5 Trypan blue (TB) staining

TB test was found to be suitable for evaluation of viability of zebrafish oocytes, as it shows significant differences between live and dead oocytes (Fig.3.4). Dead oocytes after incubation in TB acquire dark, intensive blue colour; whilst live oocytes retain natural white colour, or stain slightly blue. TB test works with all oocyte developmental stages relevant to our study (stage III, IV, V).

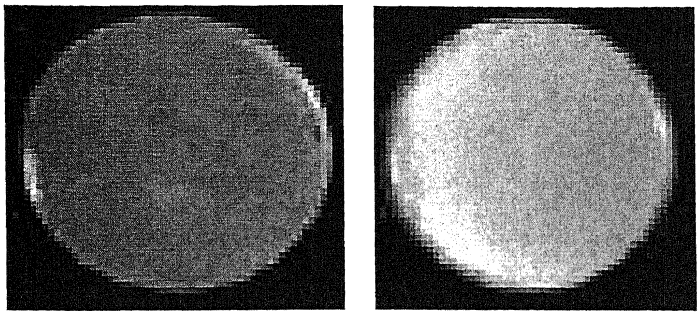


a b

Fig.3.4, a,b. TB-stained stage III oocytes: dead oocytes, stained blue, compared to unstained live oocytes.

3.2.6 *In vitro* maturation and germinal vesicle observation (GVBD) assay

Fig. 3.5 shows a stage V zebrafish oocyte, which was maturated *in vitro* from stage III oocyte. GVBD assay demonstrated clear difference between positive and negative controls: after 24 h *in vitro* maturation the cytoplasm of live oocytes becomes translucent; whilst dead oocytes remain opaque and often, lysed.



a b

Fig. 3.5. *In vitro* maturated zebrafish oocytes: viable oocyte (a), compared to non-viable oocyte (b).

3.2.7 Thiazolyl blue (MTT) assay

Experiments on Thiazolyl blue (MTT) assay have shown applicability of this method for assessment of viability of stage III zebrafish oocytes. MTT test gave easily distinguished difference between positive and negative controls in number of cases (Fig. 3.6). MTT test is not applicable to stage V oocytes: intact mature eggs remain totally unstained even after 1.5 hours of incubation with MTT. This is presumably connected with the limited membrane permeability of the plasmatic membrane of stage V eggs.

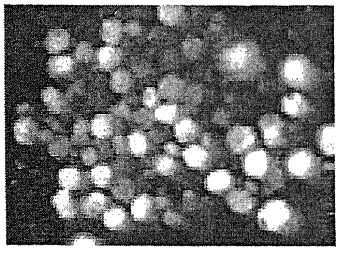


Figure 3.6. Mechanically damaged oocytes, stained with MTT. Viable cells are red, non-viable cells are white/yellow.

3.3. Discussion

3.3.1 Enzymatic disaggregation of cumulus

The enzymatic disaggregation of cumulus by enzymes collagenase and hyaluronidase resulted in deleterious changes in oocytes, which resulted in their decreased viability when assessed with GVBD, and also in changes in the plasmalemma. Enzyme-treated oocytes can not be stained by TB even after exposure to high concentrations of cryoprotectants. This suggests that incubation in collagenase and hyaluronidase significantly changed the properties of plasmatic membrane of zebrafish oocytes, which may be due to the destructions of membrane channel proteins. These destructions might be linked to the action of collagenase and hyaluronidase themselves, but they may be also the result of the action of other lytic enzymes, which probably contaminate collagenase and hyaluronidase specimens (Haynes et al., 1986). Alteration of protein components of plasma membrane probably leads to the observed loss of membrane permeability for Trypan blue. However, without additional studies the observed phenomenon can hardly be satisfyingly explained.

At the same time, the morphological characteristics of oocytes following collagenase-hyaluronidase treatment remained normal. Addition of bovine serum albumin (BSA) to the enzyme mixture resulted in increased post-treatment survival of oocytes when compared to those treated without BSA. These results are consistent with the results of other researchers, where addition of BSA to enzymatic solution allowed the protection of Salmonid fish oocytes from the lytic effect of enzymes to certain extent (Campbell & Jalabert, 1979). Addition of non-enzymatic proteins, including BSA, into cell incubation media is widely practiced in cryobiology, and has a well-reported protective effect on cells (Belous & Grischenko, 1994; Arakawa et al., 1990).

Basing on the results obtained in these experiments, mechanical disaggregation of cumulus was used in all future experiments rather than enzymatic disaggregation.

3.3.2 Enzymatic defolliculation of oocytes

Trypsin defolliculation was proven to be highly damaging for oocytes. It resulted in severe lysis of oocytes as observed with microscopic observation. Foetal calf serum, which was used as inhibitor for enzymatic reaction, failed to stop the action of trypsin. FCS is widely used in cytology and in cell culture to stop the action of lytic enzymes, because it contains a group of natural proteins – trypsin inhibitors, which inhibit trypsin and other proteinases, therefore, protecting the cells from lysis (Bundy & Mehl, 1958). In our case, the protection provided by trypsin inhibitors was not sufficient. Based on the results obtained in these experiments, defolliculation using trypsin was not applied in subsequent experiments.

3.3.3 Ethidium bromide, Propidium iodide and Calcein blue assays

When Ethidium bromide, Propidium iodide or Calcein blue dyes were used, none of the oocytes showed any noticeable fluorescence, which strongly suggests that none of the listed dyes penetrated inside zebrafish oocytes. The limited permeability of oolemma of fish oocytes (Ecker & Smith, 1971; Pennequin et al., 1975; Smith & Ecker, 1969) and the large size of these cells is the most probable explanation for this. The large size of fish oocytes results in low surface-to-volume ratio, which can slow down penetration of external compounds into the oocyte. While Ethidium bromide, Propidium iodide and Calcein blue are suitable for viability assessment of various cell types (fungi, bacteria, epithelial cells, hepatocytes, etc.) (Robinson et al., 1997; Yokoyama et al., 1997; Giraudi et al., 2005), they do not appear to be suitable for zebrafish oocytes.

3.3.4. cFDA and Rhodamine 123 assays

When cFDA or Rhodamine 123 were used, a certain degree of fluorescence was observed in oocytes; but the differences between positive and negative controls were not distinct enough, and therefore, not sufficient to be the basis of a reliable viability assessment method. As it was already mentioned in the Introduction to this chapter (Sec. 3.1), cFDA test is based on the action of a group of intracellular enzymes (esterases), which cleave two acetate groups from cFDA molecule and convert non-fluorescent cFDA to

fluorescent substance carboxyfluorescein. It is assumed that in non-viable cells the activity of these enzymes is inhibited, therefore such cells do not fluoresce, while live cells acquire green fluorescent colour. However, it has been shown, that after cell death the activity of cytoplasmic enzymes does not cease immediately, but tends to fade out gradually as time passes (Grooten et al., 1993; Leong, 2000). The same applies to mitochondrial enzymes, assessed by Rhodamine 123. This phenomenon may be the reason for unsatisfactory results obtained with cFDA and Rhodamine 123 assays when applied to zebrafish oocytes. cFDA has been previously reported as a suitable vital test for zebrafish oocytes (Pearl & Arav, 2000). However, our experiments have shown that applicability of this method for assessment of viability of zebrafish oocytes is very limited; although cFDA staining may facilitate morphological observations of oocytes under the microscope. Similar conclusions about cFDA method were made by Isayeva et al. (2004).

3.3.5 Trypan blue (TB) assay

Trypan blue staining, which is one of the most commonly used vital tests in biology (Sukach et al., 2001; Plachinta et al., 2002), was proved to be suitable for zebrafish oocytes. It was effective (i.e. able to distinguish between killed and live oocytes, although not able to confirm viability) for all oocyte developmental stages, relevant to the present study (stages III, IV and V). The advantage of using TB test is it is quick and simple. However, there are also limitations as the TB test indicates the integrity of plasmatic membrane rather than the physiological status of the whole cell, which may lead to possible misinterpretations (Giraudi et al., 2005).

3.3.6 *In vitro* maturation and germinal vesicle observation (GVBD) assay

In vitro maturation of vitellogenic oocytes, followed by germinal vesicle breakdown (GVBD) observation appears to be a sensitive viability assessment method. This protocol for zebrafish oocytes was developed by Selman et al. (1994), but was used to obtain stage V eggs, rather than as a viability test. GVBD test is a precise, functional method for viability assessment of zebrafish oocytes, as it assesses overall oocyte capability of function and development. This test showed clear difference between positive

and negative controls. None of the oocytes which were treated with 99% methanol over 10 min showed any signs of GVBD (no survival), whilst GVBD ratio of intact stage III oocytes is 60.6±9.9%. Unfortunately, GVBD test can only be applied to stage III (vitellogenic) oocytes, as stage IV and stage V oocytes had already commenced or completed the process of GVBD.

3.3.7 Thiazolyl blue (MTT) assay

MTT test indicated clear difference between positive and negative controls, and therefore was found suitable as a vital test for stage III and stage IV zebrafish oocytes. As it was already mentioned in Sec. 3.1, MTT is accumulated by metabolically active mitochondria of viable cells, where it is enzymatically converted by mitochondrial enzyme succinate dehydrogenase (SDH) to the insoluble, purple crystals of formazan. As a result, viable cells acquire purple colour while dead cells remain unstained (Mosmann, 1983). The limitation of MTT assay is that it doesn't function with stage V zebrafish oocytes: even viable intact stage V eggs remain totally unstained by MTT. The phenomenon may be explained by the decrease of oolemma permeability to many substances after maturation of oocytes, which is common for aquatic species (Ecker & Smith, 1971; Pennequin et al., 1975; Smith & Ecker, 1969).

3.4. Summary

None of the tested fluorescent dyes (cFDA, Rhodamine 123, Calceine blue, Ethidium bromide, Propidium iodide) were found to be suitable for zebrafish oocytes viability assessment.

Three methods were found to be suitable for evaluation of viability of zebrafish oocytes: trypan blue (TB) and MTT vital tests, and *in vitro* maturation assay followed by observation of germinal vesicle breakdown (GVBD). The advantages of TB test are its quickness and applicability for oocytes of all developmental stages relevant to the present study (III, IV, V). The advantage of GVBD test is its high sensitivity although it is only applicable for stage III (vitellogenic) oocytes.

Enzymatic defolliculation and disaggregation assays were found to be highly damaging for zebrafish oocytes. Defolliculation with trypsin resulted in extensive damage and lysis of oocytes. Disaggregation of cumulus with collagenase-hyaluronidase solution resulted in serious viability loss and changes in oocyte plasmatic membrane, even when bovine serum albumin was used as a protective agent. Based on these results, mechanical disaggregation method for separation of oocytes from cumulus was used in all subsequent experiments, and all work was carried out on oocytes incapsulated in follicle.

CHAPTER 4. TOXICITY OF CRYOPROTECTANTS TO ZEBRAFISH (*DANIO RERIO*) OOCYTES

4.1 Introduction

After suitable viability assessment methods were identified for zebrafish oocytes, studies on the toxicity of cryoprotectants to oocytes were undertaken. Cryoprotectants (CPAs) are substances characterised by their ability to reduce cryoinjury of biological materials during the course of freezing. Unfortunately CPAs can be toxic for cells. Hence, it is important to determine the effect of cryoprotectants on oocytes as a first step in freezing protocol design. Ideally the cryoprotectant should have low toxicity and be able to permeate the oocyte plasma membrane. Four penetrating cryoprotectants have been selected for toxicity studies, namely methanol, dimethyl sulfoxide (DMSO), propylene glycol (PG) and ethylene glycol (EG). The toxicity of sucrose and glucose was also studied, as these carbohydrates are widely used in cryobiological practice.

DMSO is the most widely used CPA and has been used in the cryopreservation of a wide range of cell types, tissues and organs. Methanol has been shown to be the most effective CPA for zebrafish embryo (Zhang et al., 1993) and sperm of several fish species (Lahnsteiner et al., 1997). EG and PG are similar compounds, which differ only by presence of the additional methyl group in PG molecule. Solutions of PG and EG promote small-granular crystallisation and amorphous solidification due to their high viscosity at low temperatures. They also lessen the quantity of unbound water within cell, therefore suppressing intracellular ice formation. Both PG and EG have been widely used for vitrification of mammalian oocytes and ovarian tissue, and also for controlled slow cooling programmes (Picton et al., 2002; Stachecki et al., 1998; Wu & Lee, 1996). Sucrose is widely used as an extracellular cryoprotectant, often in combination with permeating cryoprotectants. It induces dehydration and osmotic shrinkage of the cells, therefore lowering the risk of intracellular ice crystallisation. Glucose acts in a similar manner and was also found to be a membrane-stabilising agent, binding to lipids and proteins of the bilayer, stabilising it against compression (King et al., 1993, Suzuki et al., 1996).

Unfortunately, all known cryoprotective agents, besides with their protective properties, also have toxic effects on biological material. The generic mechanisms of CPAs toxicity were briefly discussed in Introduction, Sec. 1.2.3.5. Several following paragraphs describe specific mechanisms of CPAs toxicity, different for each of these substances.

The toxicity of methanol to organisms (Ashwood-Smith, 1987) is linked to the action of cellular enzyme alcohol dehydrogenase (ADH), which converts methanol to highly toxic compound – formaldehyde (Lehninger, 1982). Formaldehyde is an extremely strong denaturant agent due to its high chemical activity. It forms abnormal crosslinks between molecules of proteins and lipids, hence irreversibly inhibiting their normal functioning. Unconverted methanol also has certain damaging effect on cell structures. Methanol and other alcohols have been demonstrated to interact with phospholipids to destabilise the lipid bilayers of artificial membranes. Short-chain alcohols, such as methanol and ethanol, interact more directly with the polar head group of lipids in bilayers due to their low hydrophobicity. However, the short non-polar region of the alcohol can create voids between the lipid chains in the membrane interior and introduce instability within the bilayer. At high methanol concentrations the cell membrane becomes unstable resulting in lipid phase transition: lipids acyl-chains from opposing monolayers are shifted from their normal opposite-facing orientation to an interspersed arrangement that exposes the ends of the hydrophobic tails (Walsh et al., 2004).

PG generally has relatively low toxicity to most of cell types, which may be explained by the similarity of this compound to natural biochemical intermediates. Moreover, PG can be metabolised by cells and being converted into a number of non-toxic metabolites such as pyruvic acid, acetic acid, lactic acid and propionaldehyde (Ruddick, 1972).

The toxic effect of DMSO on cells has been linked to labilisation of membranes and denaturation of proteins (Henderson et al., 1975; Orvar et al., 2000). DMSO can also increase the concentration of calcium ions in cytoplasm, causing a

variety of chronic negative metabolic responses such as cytoskeleton depolymerisation and reassembly (Yamamoto, 1989). DMSO may also affect RNA splicing (Murata et al., 2003), lipid, protein and DNA synthesis (Ashwood-Smith, 1987). DMSO penetrates membranes much more slowly than water, resulting in rapid plasmolysis as water exits cells during its addition to the cells, and swelling when DMSO is removed from the extracellular medium after thawing. Therefore, osmotic stresses may aggravate the toxicity of dimethyl sulfoxide (Walsh et al., 2004).

EG has been suggested to have a destabilising effect on protein secondary structure, causing unfolding of alpha-helixes (Arakawa et al., 1990). Moreover, due to the action of enzyme alcohol dehydrogenase, EG inside the cells may be converted to a number of highly toxic derivatives, such as glycolic acid and salts of oxalic acid. Metabolites of EG suppress oxidative phosphorylation, inhibit sulfhydryl-group-containing enzymes, inhibit protein synthesis and cause severe acidification of cytoplasm.

The cell damaging effects of non-penetrating compounds, such as sucrose, are mainly osmotic. Cell shrinkage due to water loss induced by presence of non-penetrating CPAs results in undesired changes of the plasma membrane, which may include lateral separation of membrane lipids, lipid phase transitions, formation of protein clusters, spiculation and vesiculation of plasmatic membrane. Dehydration of cells may also result in formation of abnormal disulfide (-S-S-) crosslinks between protein molecules, which impairs the function of cytoplasmic and membrane proteins (Belous & Grischenko, 1994). Our studies, as well as results of other researchers, confirm that fish eggs and embryos are fairly sensitive to hyperosmotic conditions. For instance, osmotic pressure of only 480 mOsm/L (which is quite bearable for most of mammalian cells) results in 100% death of grass carp embryos (Ahammad et al., 1998).

4.2 Results

Zebrafish oocytes at three different developmental stages: stage III (vitellogenesis), stage IV (maturation) and stage V (mature) were used in this part of the study. At stage III, vitellogenesis occurs and a large amount of yolk proteins accumulate in the oocyte. During stage IV several processes occur, which lead to physiological maturation of the oocyte. Stage V oocytes are mature eggs, ready for fertilisation (Selman et al., 1993).

4.2.1 Toxicity of different CPAs to zebrafish oocytes

For present studies, the No Observed Effect Concentrations of Cryoprotectants (NOECs) were determined basing on statistical results of ANOVA and Scheffe's test. NOEC is the highest concentration of a compound used in a test, that still has no statistically significant effect on the exposed sample as compared with controls.

The No Observed Effect Concentrations (NOECs) for DMSO, methanol, EG, PG, glucose and sucrose for oocytes at three different developmental stages using three viability assessment methods: Trypan blue, MTT staining and GVBD observation, are given in Table 4.1. The summary results of stage III zebrafish oocytes viability with tested cryoprotectants using three different viability assessment methods are given in Figs 4.1, 4.2 and 4.3. The results showed that the toxicity of cryoprotectants generally increased with concentration using all three viability assessment methods. The toxicity of tested cryoprotectants increased in the order of methanol, PG, DMSO, EG, glucose and sucrose with NOECs for stage III oocytes being 2M, 1M, 1M, 0.5M, <0.25M and <0.25M respectively when assessed with GVBD observation (Table 2). Methanol is the least toxic cryoprotectant to zebrafish oocytes, the survivals assessed with GVBD and TB staining for stage III oocytes after being treated in 2M methanol for 30min at room temperature were 39.7±9.1% and 72.7±5.2% respectively when compared with 57.7±3.2% and 76.3 ±4.5% for their controls.

Table 4.1. The No Observed Effect Concentration (NOEC) of cryoprotectants to three developmental stages of oocytes obtained from different viability tests.

CPA	Stage III			Stage IV	Stage V
	TB	MTT	GVBD	TB	TB
DMSO	3M	>2M	1M	>2M	1M
Methanol	>4M	>2M	2M	>2M	-
Ethylene glycol	1M	>2M	0.5M	1M	1M
Propylene glycol	>4M	>2M	1M	>2M	-
Glucose	0.5M	0.5M	<0.25M	0.5M	-
Sucrose	0.25M	0.5M	<0.25M	0.25M	-

The highest concentration of CPAs used in the experiments was 4M except MTT test on stage III oocytes and TB test on stage IV oocytes, where the highest concentration used was 2M. N = 461.

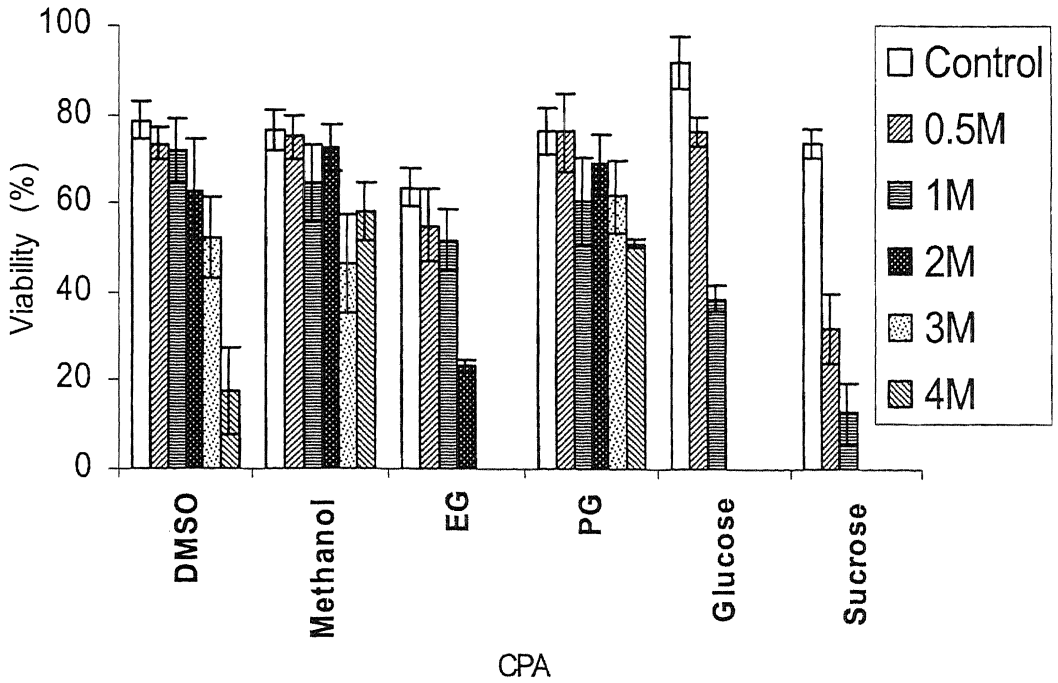


Figure 4.1. Toxic effect of cryoprotectants to stage III zebrafish oocytes assessed with TB test. Oocytes were exposed to up to 4M cryoprotectants for 30 min at room temperature. N = 250.

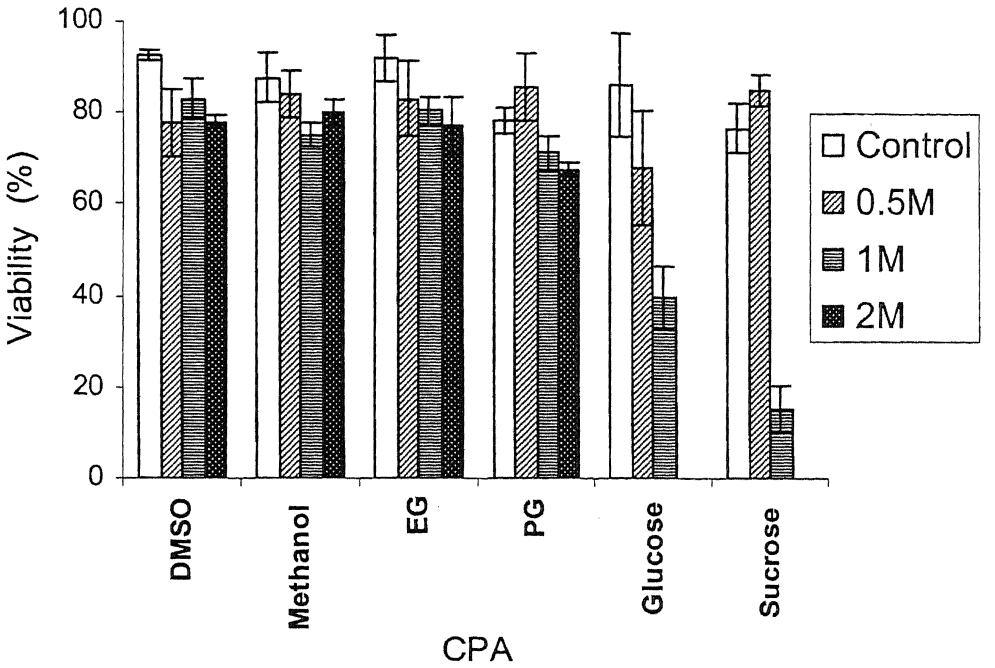


Figure 4.2. Toxic effect of cryoprotectants on stage III zebrafish oocytes assessed with MTT test. Oocytes were exposed to up to 2M cryoprotectants for 30min at room temperature. N = 80.

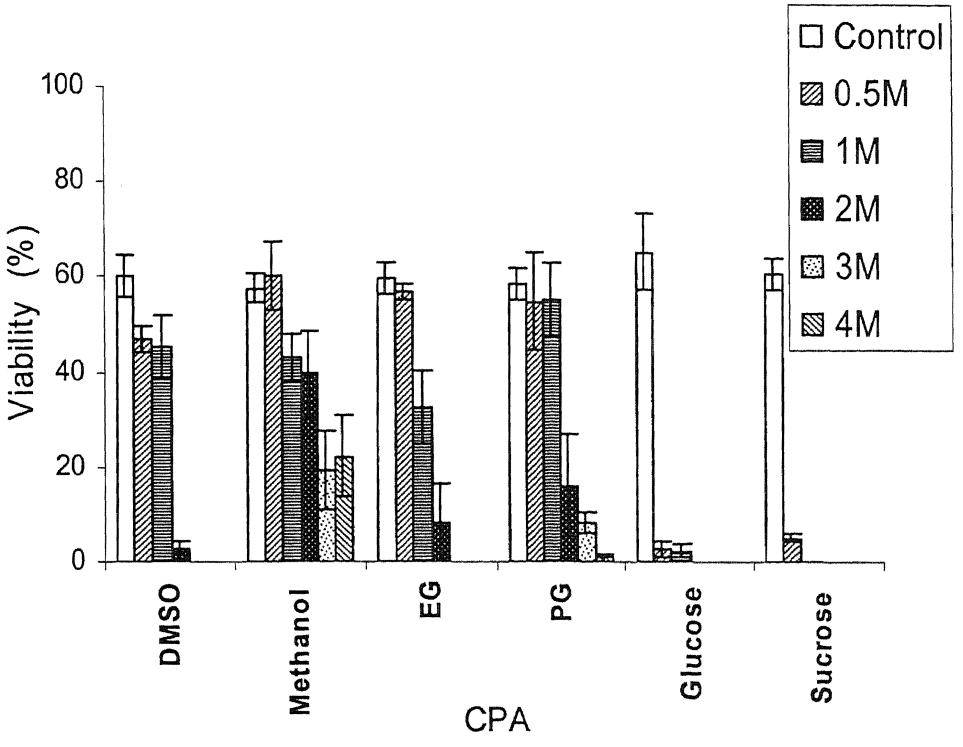


Figure 4.3. Toxic effect of cryoprotectants on stage III zebrafish oocytes assessed with GVBD observation. Oocytes were exposed to up to 4M cryoprotectants for 30min at room temperature. N = 131.

4.2.2 Effect of viability assessment method

The comparisons of the three viability assessment methods used in this study showed that the sensitivity of the viability assessment methods increase in the order of MTT, TB and GVBD (Figs. 4.4–4.9). For example, oocytes viability after 2M EG treatment for 30min at room temperature assessed with MTT, TB and GVBD were 77.3±6.2%, 23.4±1.5% and 8.3±8.3% respectively (Fig. 4.4). MTT test was relatively insensitive to concentrations of CPA that demonstrated toxic impact when tested by TB or GVBD. MTT test showed no significant differences in oocyte viability when they were exposed to DMSO, methanol, EG and PG at up to 2M for 30min at room temperature (Fig. 4.2). This method was therefore not used for stage IV oocytes.

Oocytes *in vitro* maturation followed by germinal vesicle breakdown observation (GVBD) appears to be the most sensitive viability assessment method when compared with MTT or TB.

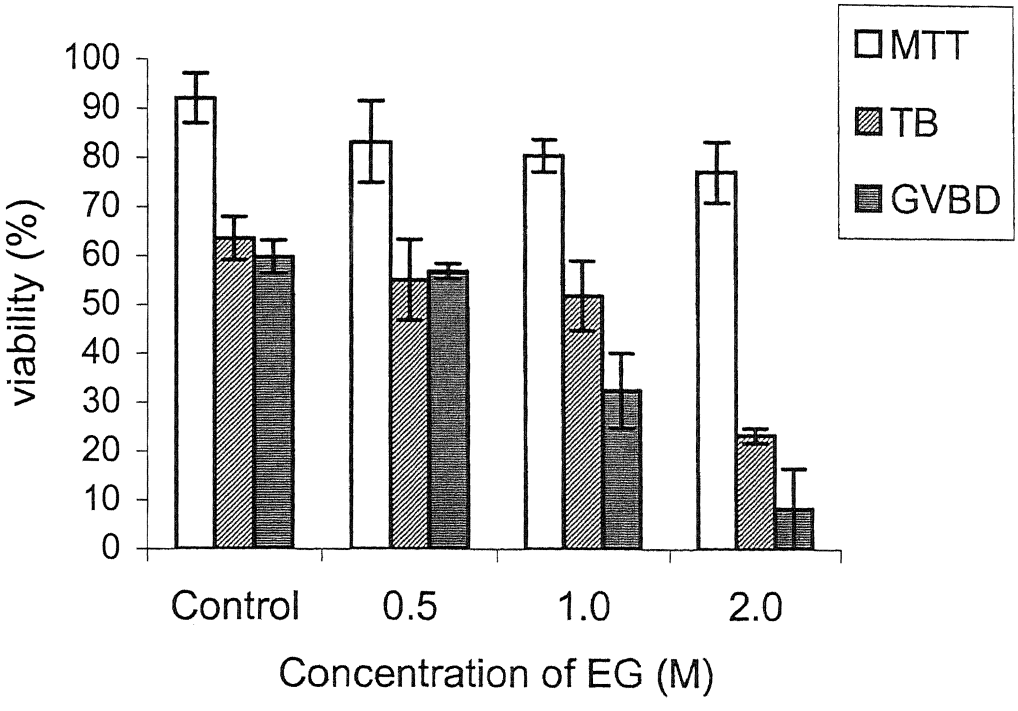


Figure 4.4. Comparisons of three viability tests: MTT staining, TB staining and GVBD observation. Stage III zebrafish oocytes were exposed to different concentrations of EG for 30 min at room temperature. N = 58.

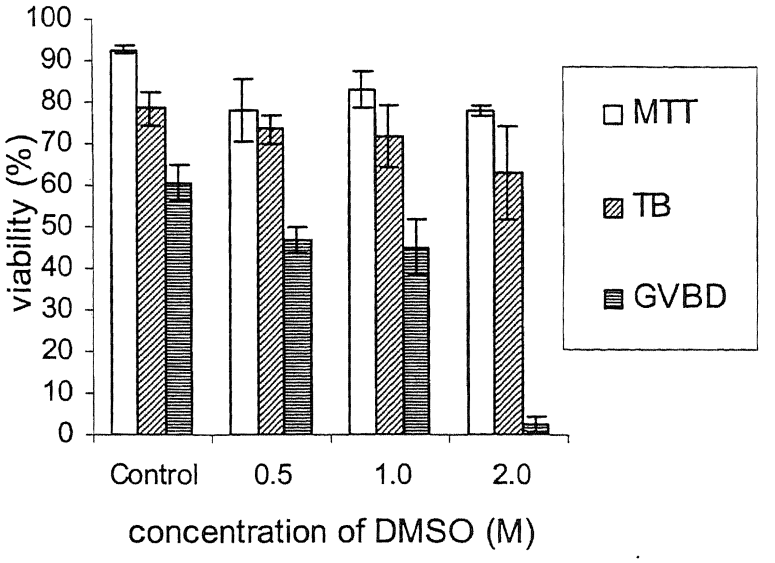


Figure 4.5. Comparisons of three viability tests: MTT staining, TB staining and GVBD observation. Stage III zebrafish oocytes were exposed to different concentrations of DMSO for 30 min at room temperature. N = 70.

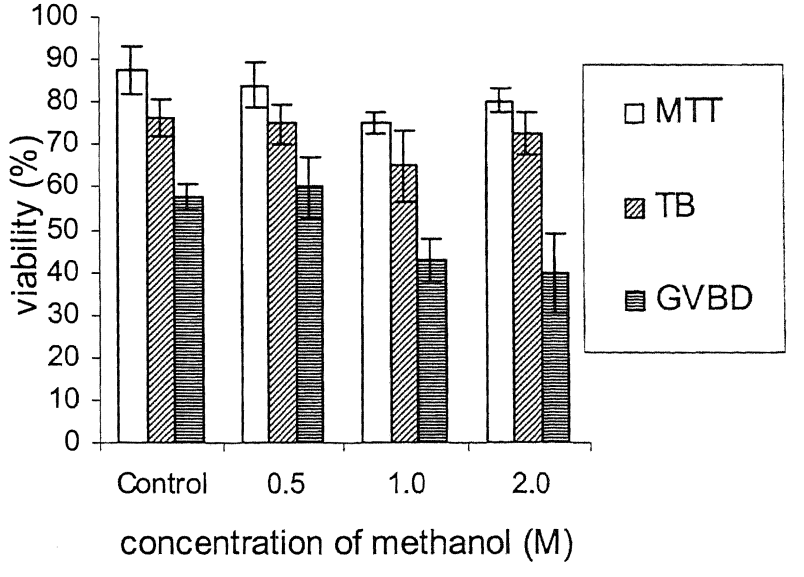


Figure 4.6. Comparisons of three viability tests: MTT staining, TB staining and GVBD observation. Stage III zebrafish oocytes were exposed to different concentrations of methanol for 30 min at room temperature. N = 69.

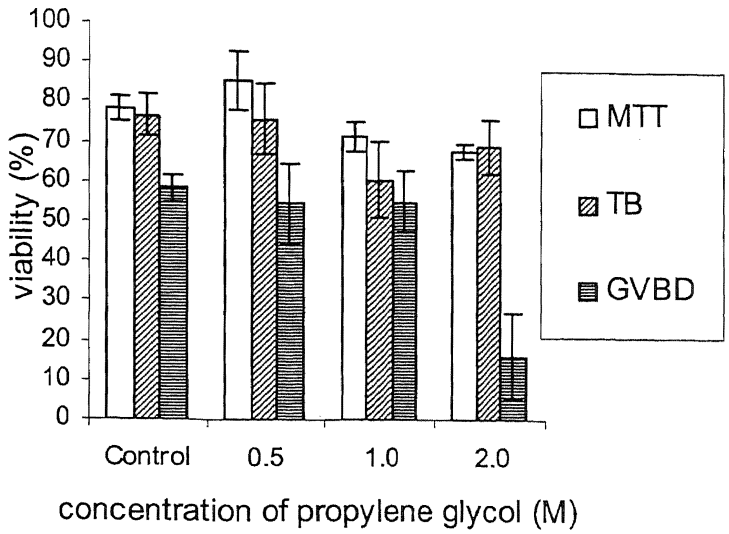


Figure 4.7. Comparisons of three viability tests: MTT staining, TB staining and GVBD observation. Stage III zebrafish oocytes were exposed to different concentrations of propylene glycol for 30 min at room temperature. N = 66.

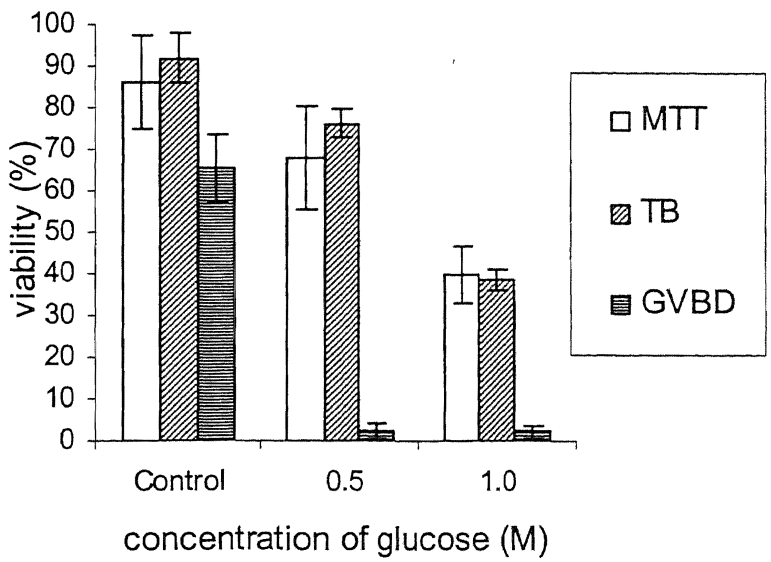


Figure 4.8. Comparisons of three viability tests: MTT staining, TB staining and GVBD observation. Stage III zebrafish oocytes were exposed to different concentrations of glucose for 30 min at room temperature. N = 43.

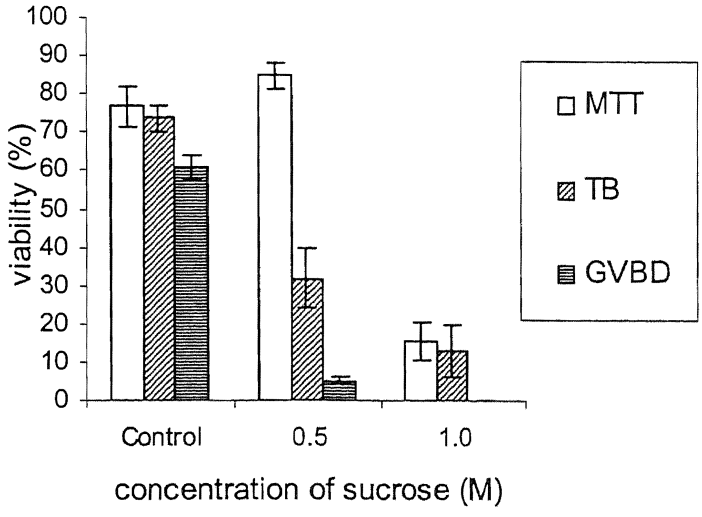


Figure 4.9. Comparisons of three viability tests: MTT staining, TB staining and GVBD observation. Stage III zebrafish oocytes were exposed to different concentrations of sucrose for 30 min at room temperature. N = 46.

4.2.3 Effect of oocytes developmental stage

Experiments showed that the sensitivity of oocytes to DMSO and EG increases with developmental stage (Figs. 4.10, 4.11). The No Observed Effect Concentrations for stages III and V oocytes after exposure to DMSO for 30 min at room temperature were 3M and 1M respectively (Table 4.1). 2 molar concentration of EG was shown to be 100% lethal for stage V zebrafish oocytes (Fig. 4.11). However the NOEC for EG remained at 1M for all stages (Table 4.1). When exposed to CPA solutions, in many cases mature oocytes (stage V) developed cortical reaction – the separation of chorion from plasma membrane and the formation of perivitelline space. The cortical reaction of mature egg in cryoprotectant solutions is not desirable as once it happens the eggs can not be fertilised. The increased sensitivity of stage V oocytes to CPA is also seen in morphological observations. When incubated in CPAs, in a number of cases stage V oocytes became swollen and distorted even though they did not stain with TB.

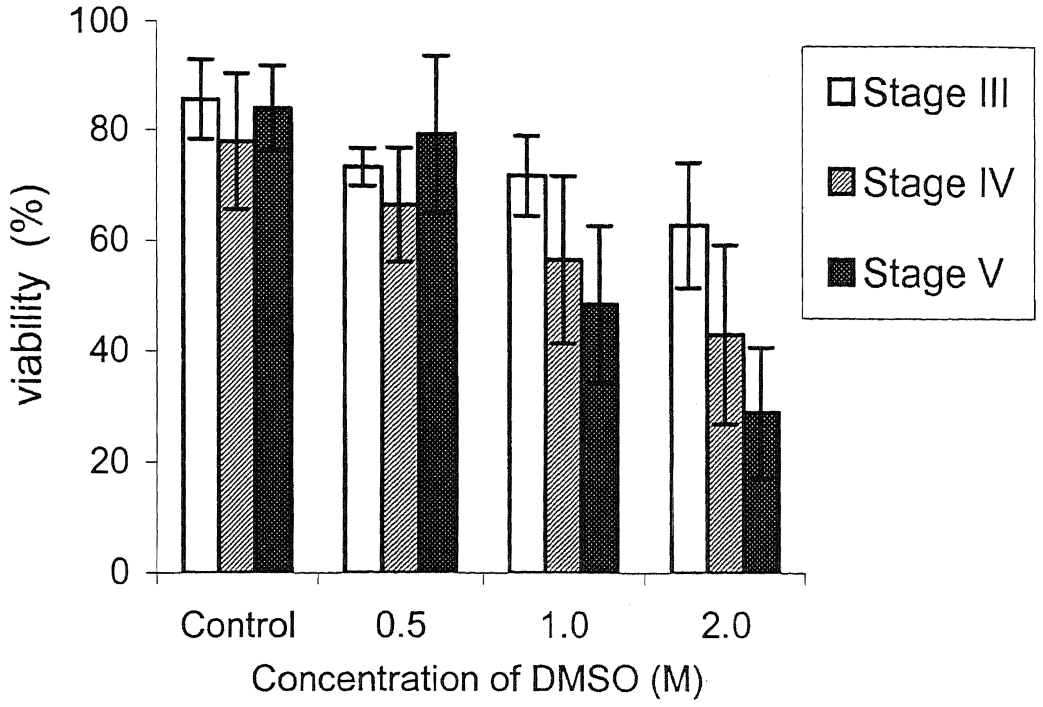


Figure 4.10. Effect of DMSO toxicity on viability of oocytes at different developmental stages, assessed with TB staining. Oocytes were exposed to DMSO for 30 min at room temperature. N = 59.

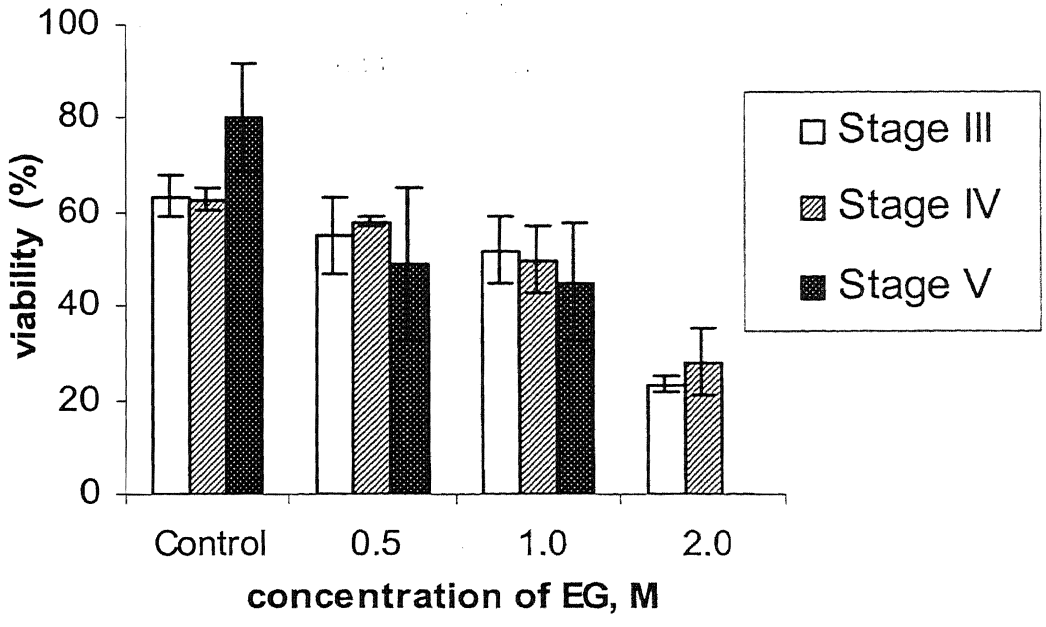


Figure 4.11. Effect of EG toxicity on viability of zebrafish oocytes at different developmental stages, assessed by TB staining. Oocytes were exposed to EG for 30 min at room temperature. N = 48.

4.3 Discussion

4.3.1 Toxicity of different CPAs to zebrafish oocytes

The results from this study have shown that the toxicity of cryoprotectants increased in order of methanol, PG, DMSO, EG, glucose and sucrose. Methanol and PG are the least toxic cryoprotectants to zebrafish oocytes when compared with other tested CPAs. These results agree well with previous findings on toxicity of CPAs to zebrafish embryos (Zhang & Rawson, 1996, Zhang et al., 1993) which also showed the relatively low toxicity of these compounds when compared with DMSO or EG. The comparisons of zebrafish oocyte and early developmental stage embryo sensitivity to methanol also indicated that zebrafish embryos were more sensitive to methanol than zebrafish oocytes. In this present study, 39.7±9.1% stage III oocytes survived 30 min incubation in 2M methanol at 22°C (according to GVBD observation), whilst previous study (Liu et al., 1998) reported no survival of 1-cell stage zebrafish embryos after similar treatment. The relatively low toxicity of methanol to fish oocyte may due to the low activity of alcohol dehydrogenase (ADH) in oocytes (Rout & Armant, 2002) which converts methanol to highly toxic compound – formaldehyde. Methanol penetrates cells very quickly (Walsh et al., 2004; Liu et al., 2003; Ashwood-Smith, 1987), thus, equilibration with it doesn't cause pronounced osmotic stress. The relatively low toxicity of PG to cells may be explained by the similarity of this compound to natural biochemical intermediates. Moreover, PG can be metabolised by cells and converted in it into a number of non-toxic metabolites such as pyruvic acid, acetic acid, lactic acid and propionaldehyde (Ruddick, 1972). DMSO and EG are more toxic than PG or methanol to zebrafish oocytes, the toxic effect of DMSO has been linked to labilisation of membranes and denaturation of proteins (Henderson et al., 1975; Orvar et al., 2000). DMSO can also increase the concentration of calcium ions in cytoplasm, causing a variety of metabolic responses such as depolymerisation and cytoskeleton reassembly (Yamamoto, 1989). DMSO may also affect RNA splicing (Murata et al., 2003). EG has been suggested to have destabilising effect on protein structure (Arakawa et al., 1990).

Degree of toxicity of different CPAs may vary considerably between different species, and identifying interspecies differences is very important in designing

cryopreservation protocol. The present study has shown that zebrafish oocytes have increased sensitivity to CPAs when compared with reproductive cells of other species. For mouse embryos, a freezing medium containing 5.5M EG + 1M Sucrose was shown to be an optimal vitrifying mixture, which has no noticeable toxicity to these embryos after exposure for 30 min at 25°C (Ali & Shelton, 1993). For zebrafish oocytes, such a medium would be lethal. At the same time, zebrafish oocytes are much more tolerant to some substances than other cell types. As shown in the present study, both with TB, MTT and GVBD tests, methanol is not very toxic for zebrafish oocytes; whilst for carp embryos concentrations of methanol >2M were lethal (Ahammad et al., 1998). A possible reason for that probably might be the increased activity of alcohol dehydrogenase in carp embryos (see Sec. 1.2.3.6). EG was the least toxic CPA of all tested CPAs (butylene glycol, propylene glycol, ethylene glycol, DMSO, glycerol) for mouse embryos, whilst PG was the most toxic (Ali & Shelton, 1993). The results obtained in the present study showed exactly the opposite for zebrafish oocytes.

Zebrafish oocytes are very sensitive to both sucrose and glucose. Stage III oocytes viability was reduced significantly after exposure to 0.25M sucrose or glucose for 30 min at room temperature. It is quite a paradoxal situation, that sucrose, which is non-permeating (extracellular) cryoprotectant, appeared to be the most toxic substance of all CPAs tested. The results of high toxicity of sucrose obtained from the present study are supported by the studies on zebrafish embryos (Zhang et al., 1993). As non-penetrating compound, the toxic effect of sucrose is mainly osmotic. Dehydration of cells, caused by sucrose solution, may result in the range of deleterious "solution effects", which were described in detail in Introduction, Sec. 1.2.2.3. Based on the results from the present study, it is clear that zebrafish oocytes have increased sensitivity to hyperosmotic conditions. For most of cell types lethal osmolarity of the medium begins at approximately 1300 mOsm (Gordienko & Pushkar, 1994), but for zebrafish oocytes, according to our GVBD test results, osmolality of about 928 mOsm (which corresponds to 0.5M sucrose in a Hank's medium, Zhang et al., 2005) is lethal in most of the cases. Fish embryos are even more sensitive to osmotic stress, probably because they are "designed" to exist in such a diluted environment as fresh water is: the increase of osmotic pressure to 480

mOsm/L results in 100% death of grass carp embryos. Maximum tolerated concentration of sucrose for carp embryos was 0.5M (Ahammad et al., 1998). It is important to point out that, as exposure to hyperosmotic conditions and dehydratation inevitably accompany biological sample in the course of slow freezing, high sensitivity of fish oocytes to dehydratation may add additional difficulties to the task of their cryopreservation.

Meanwhile sucrose doesn't penetrate inside cells and acts mainly osmotically, glucose may penetrate within the oocytes, as evidence suggests. Studies on zebrafish oocytes permeability to glucose have not been conducted, but results obtained from zebrafish embryos suggest that glucose penetrates inside these cells (Zhang, 1994). Volumetric studies of heart-beat stage zebrafish embryos show that when exposed to glucose solution (1M), embryos demonstrate typical pattern of volume changes, which is characteristic for penetrating CPAs (Fig. 4.12). (Zhang, 1994; Gordienko & Pushkar, 1994).

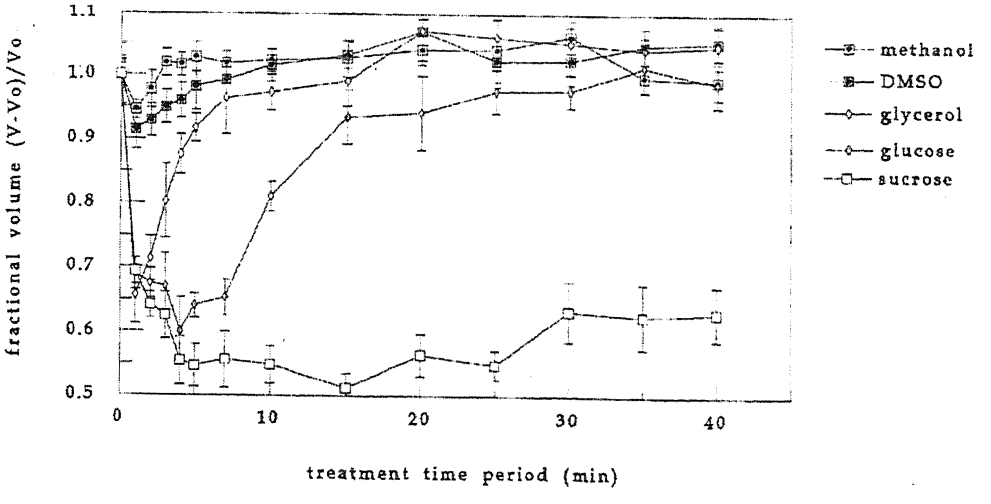


Fig. 4.12. Fractional volumes of intact heart beat stage zebrafish embryos during cryoprotectant exposure at 22°C. The concentration of methanol, DMSO and glycerol was 2M. The concentration of glucose and sucrose was 1M. (From: T. Zhang , 1994).

4.3.2 Effect of viability assessment method

The only study in the literature on zebrafish oocytes viability assessment reported the use of carboxyfluorescein diacetate (cFDA) (Pearl & Arav, 2000). However, in the present study the experiments with the use of cFDA for zebrafish oocytes viability assessment give ambiguous results, and cFDA was therefore not considered suitable for zebrafish oocytes. Initial studies on development of viability assessment methods for zebrafish oocytes were carried out using a range of vital stains and other methods (see Chapter 3). Only the three most promising methods were used in the present study: TB, MTT and GVBD. MTT test was shown to be the least sensitive testing method to zebrafish oocytes; and, in addition, it does not stain stage V oocytes at all. This may be explained by the decrease of oolemma permeability to many substances after maturation of oocytes which is common for aquatic species (Ecker & Smith, 1971; Pennequin et al., 1975; Smith & Ecker, 1969). The low sensitivity of MTT staining may due to the fact that mitochondrial enzymes including succinate dehydrogenase may remain relatively intact after CPAs treatment whilst other functional systems of the cell were damaged. It was reported, that stains of formazan group (to which MTT belongs), such as nitroblue tetrazolium, can show ambiguous results on cryopreserved human tissues (Taylor, 1986). The studies of an Israeli group of researchers confirmed, that in number of cases MTT staining may give inadequate results (Lubzens et al., 2005). Similar sequence of methods in order of increase of sensitivity (MTT → TB → GVBD) is reported by Isayeva et al. (2004) in the study about chilling sensitivity of zebrafish oocytes. *In vitro* maturation followed by observation of germinal vesicle breakdown (GVBD) is the most reliable method for assessing oocytes viability as it assesses overall oocyte capability of development after cryoprotectant treatment. It is also the most sensitive method when compared with TB or MTT. GVBD test, however, can only be applied to stage III oocytes as later developmental stages have already gone through maturation and germinal vesicle breakdown process *in vivo*. Whilst it was recognised that TB test may not be ideal as it only assesses the membrane damage as opposed to whole cell physiological status, it is the fastest test in the present study and can be used for all oocytes developmental stages which allow the comparisons to be made, therefore, it is the only suitable method for zebrafish viability assessment at the present. However, TB test should be accompanied by GVBD observation wherever possible.

4.3.3 Effect of developmental stage of oocytes

The results from the present study indicated that stage V oocytes (mature eggs) are more sensitive to DMSO and EG toxicity than earlier stages. Although there is evidence, that mature fish oocytes are less permeable to solutes than immature oocytes (Ecker & Smith, 1971; Pennequin et al., 1975; Smith & Ecker, 1969) due to loss of membrane channel proteins, these changes are unlikely to affect the permeability of stage V zebrafish eggs to EG and DMSO. Amphiphylic molecule of DMSO is very small and motile, it does not need any protein channels to penetrate through plasmatic membrane, and diffuses straightly across phospholipid bilayer (Sum & de Pablo, 2003). Diffusion of EG to cells also doesn't involve protein mechanism. Therefore, mentioned above permeability decrease of mature oocyte due to loss of membrane channels should not affect permeability of stage V oocytes to DMSO and EG. High sensitivity of mature oocyte to cryoprotectants such as DMSO and EG may be explained by the specific state of genetic material in mature oocyte. In stage V egg, the nuclear envelope is absent and chromatin is being arrested in metaphase II of meiosis (Wallace & Selman, 1981). This makes the genetic material highly vulnerable to effects such as CPA exposure and cryopreservation (Agca, 2000, Isachenko et al., 2000). Cryopreservation of immature oocytes has disadvantages when compared to mature eggs: adult female fish need to be sacrificed to obtain stage III oocytes and after post-thawing they need to be matured *iv vitro* before artificial fertilisation can take place. However, there are also advantages in using immature oocyte for cryopreservation rather than mature eggs, a number of studies have indicated that immature oocytes of aquatic species such as amphibian and teleost fishes are much more permeable to water and solutes than mature eggs (Ecker & Smith, 1971; Pennequin et al., 1975; Smith & Ecker, 1969; Seki et al., 2005). Immature oocytes are much less hydrated than mature (about 30% less hydrated in case of zebrafish oocytes) (Wallace & Selman, 1981) which poses additional benefits for their cryopreservation. Mature eggs are sensitive to the conditions of their surrounding environment and may spontaneously develop cortical reaction in absence of sperm cell, due to influence of factors such as physical contact, hypotonicity of medium and the presence of certain compounds. The cortical reaction is undesirable, as oocytes with lifted chorion can not be fertilised.

4.4 Summary

In this study, the effects of cryoprotectant toxicity to zebrafish oocytes have been reported for the first time. Results showed that the toxic effect of cryoprotectant on oocytes increased with increasing concentration. MTT test was shown to be the least sensitive testing method and gave poor correlation to subsequent GVBD results. Sensitivity of vital tests increases in the order of MTT, TB and GVBD. GVBD test showed that cryoprotectant toxicity to stage III zebrafish oocytes increased in the order of methanol, PG, DMSO, EG, glucose and sucrose. No Observed Effect Concentrations (NOECs) of different cryoprotectants were established. NOECs for stage III oocytes were 2M, 1M, 1M, 0.5M, <0.25M and <0.25M for methanol, PG, DMSO, EG, glucose and sucrose respectively. TB test also showed that the toxicity of tested cryoprotectants increased in the same order. The sensitivity of oocytes to cryoprotectants appeared to increase with development stage with stage V oocytes being the most sensitive. The results obtained in this study should provide useful information in designing protocols for fish oocytes cryopreservation.

CHAPTER 5. Controlled slow cooling of stage III zebrafish oocytes

5.1 Introduction

The present study was conducted on stage III oocytes. The reason for choosing the vitellogenesis stage for slow cooling studies is the relatively low sensitivity of this stage to the toxic effect of CPAs, as determined in previous part of this study (Chapter 4), as well as the high permeability of vitellogenic oocytes to water and CPAs (Ecker & Smith, 1971; Pennequin et al., 1975; Smith & Ecker, 1969; Seki et al., 2005; Zhang et al., 2005). At this stage the process of vitellogenesis occurs, i.e. the accumulation of large amounts of yolk proteins in the oocyte. As vitellogenic eggs are not yet mature, the dissection of female fish has to be done in order to obtain oocytes from ovary. In this part of the study, several slow cooling rates (1 – 0.1°C/min) with four intracellular cryoprotectants (ethylene glycol, propylene glycol, dimethyl sulfoxide, methanol) were tested, and two viability assessments of froze-thawed oocytes, namely trypan blue staining and *in vitro* maturation followed by GVBD observation.

5.2 Results

Four widely used penetrating CPAs, namely Dimethyl Sulfoxide (DMSO), propylene glycol (PG), ethylene glycol (EG) and methanol were chosen for this part of the study. Each of these CPAs was used at the highest No Observed Effect Concentration (NOEC) according to TB test (for stage III oocytes), which was determined in toxicity studies (Chapter 4).

5.2.1 Effect of different CPAs

The TB test showed that all of CPAs tested at -25°C gave "survival" values which were significantly higher than corresponding values of oocytes frozen in Hanks' medium (Fig. 5.1, Fig. 5.2). It appears that all of the CPAs tested have a significant protective effect on zebrafish oocytes (two-factor ANOVA with replication). Methanol was found to be the most effective cryoprotectant for all cooling rates used (ANOVA, Scheffe's test).

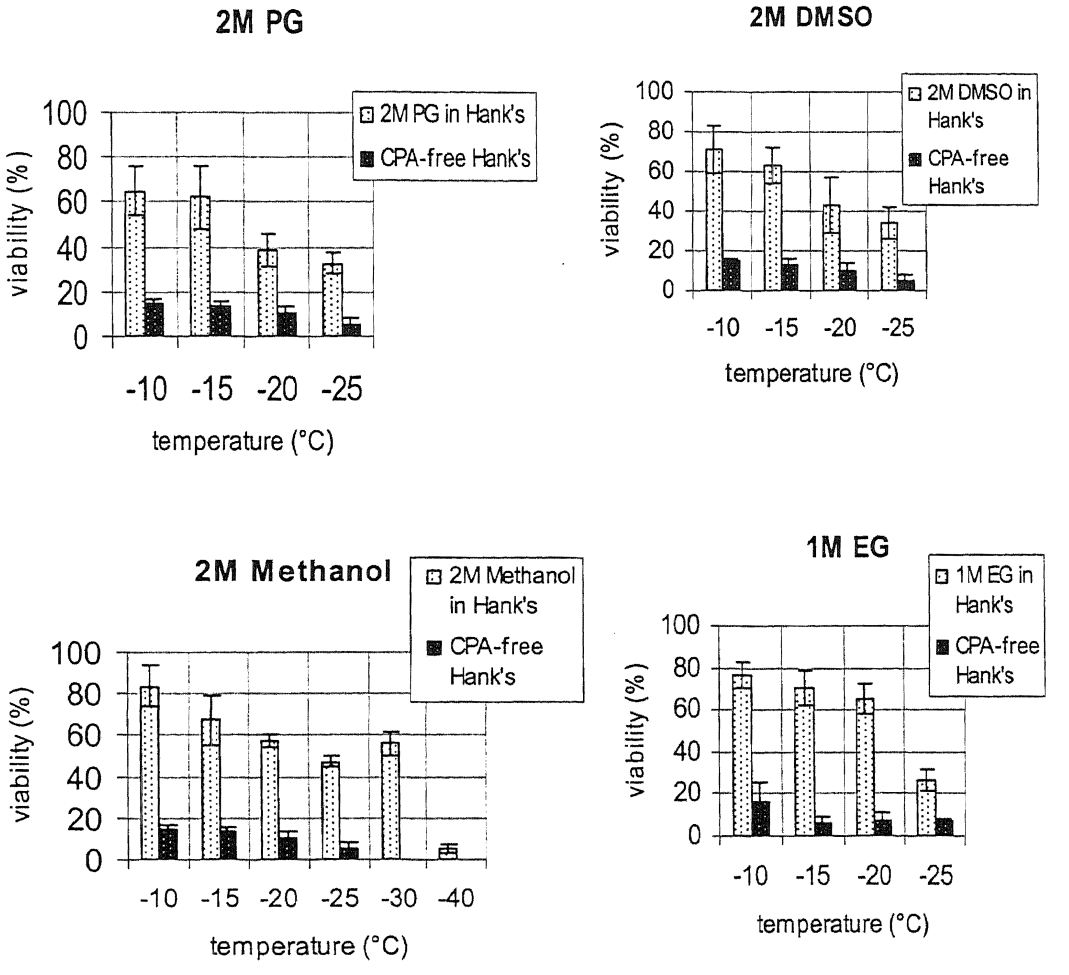


Fig. 5.1. Normalised TB-assessed viabilities of oocytes frozen in CPAs solutions (light bars) compared to viabilities of control samples, frozen in Hank's medium (dark bars). Cooling rate after seeding was 1°C/min. Oocytes survivals were normalised with respect to their room temperature survivals which were 76.4±14.6% (TB test). Error bars represent standard errors. N = 108.

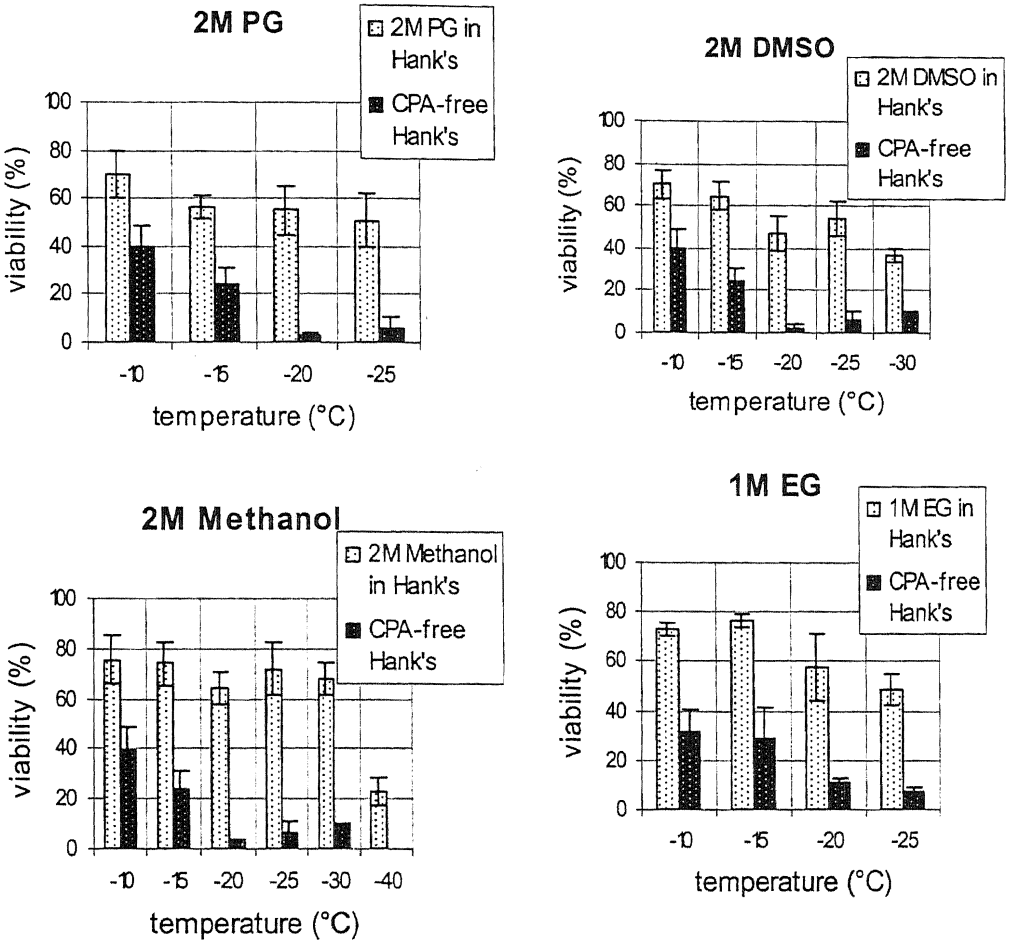


Fig. 5.2. Normalised TB-assessed viabilities of oocytes frozen in solutions of CPAs (light bars) compared to viabilities of control samples, frozen in CPA-free Hanks' medium (dark bars). Cooling rate after seeding was 0.5°C/min. Oocytes survivals were normalised with respect to their room temperature survivals which were 77.8±8.5% (TB test). Error bars represent standard errors. N = 157.

5.2.2 Effect of cooling rate

A number of cooling rates, ranging 1 - 0.1°C/min were tested. Moderate cooling rates appear to be more advantageous for cryopreservation of zebrafish oocytes compared to faster and slower ones. For all the tested CPAs, 1°C/min and 0.5°C/min cooling rates were tested, and for 2M methanol, rates of 0.3°C/min and 0.1°C/min were also tested (Fig. 5.3, Fig. 5.4). At -25°C, oocytes frozen in 2M methanol cooled at the rate 1°C/min had normalised survival of 47.4±2.4% (TB test), whilst oocytes survivals of 71.9±9.5% and 47.4±9.0% were obtained for cooling rates of 0.5°C/min and 0.3°C/min respectively. 0.1°C/min cooling rate has resulted in poor cryosurvival: 6.2±2.4% at -25°C according to TB test. At this temperature (-25°C) survivals obtained with 1°C/min and 0.5°C/min cooling rates were not significantly different from each other, but survivals obtained with 0.3°C/min and 0.1°C/min cooling rates were significantly lower than those obtained with 0.5°C/min cooling rate. The advantage of 0.3°C/min cooling rate starts to manifest at lower temperatures: at freezing temperatures as low as -40°C, 0.3°C/min cooling rate gives significantly higher survival values (51.1±1.4%) than both 0.5°C/min (22.9±5.1%) and 1°C/min (5.0±2.4%) cooling rates. It was shown that 0.3°C/min cooling rate allows significantly higher cryosurvival of oocytes at -40°C (the lowest final temperature used for 2M methanol), than both 0.5 and 0.1°C/min cooling rates (Fig. 5.4).

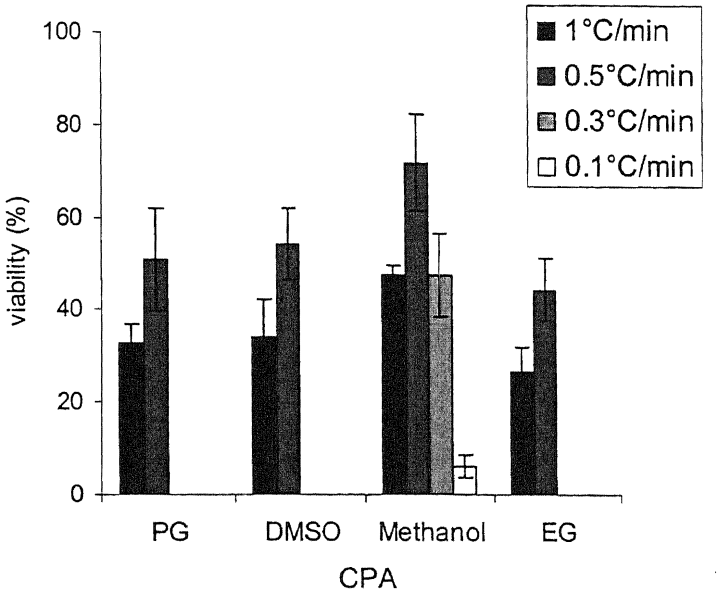


Fig. 5.3. Effect of different cooling rates (1°C/min and 0.5°C/min for 2M PG, 2M DMSO and 2M EG; and 1°C/min, 0.5°C/min, 0.3°C/min and 0.1°C/min for 2M methanol) on normalised TB-assessed viability of zebrafish oocytes. Final temperature -25°C. Oocytes survivals were normalised with respect to their room temperature survivals which were 78.9±10.9% (TB test). Error bars represent standard errors. N = 105.

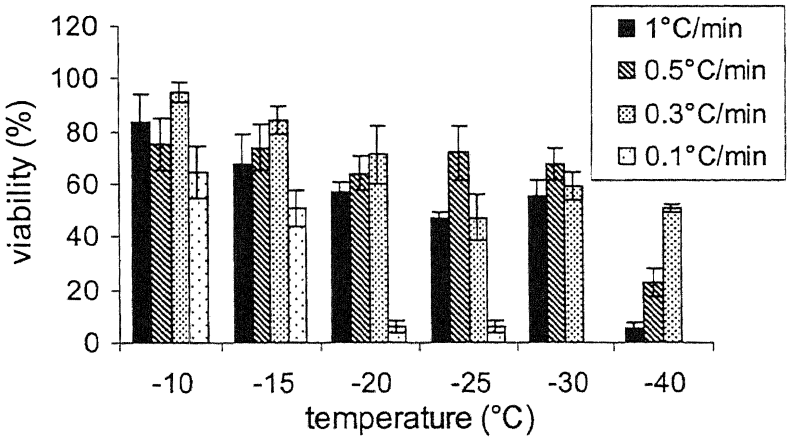


Fig. 5.4. Effect of four cooling rates (1°C/min, 0.5°C/min, 0.3°C/min and 0.1°C/min) on normalised TB-assessed viability of zebrafish oocytes, with 2M methanol, frozen to a range of different final temperatures. Oocytes survivals were normalised with respect to their room temperature survivals which were 76.4±14.6% (TB test). Error bars represent standard errors. N = 93.

5.2.3. Effect of cryoprotectant concentration

In this series of experiments, three different methanol concentrations, 2M, 3M, and 4M, were tested with 0.5°C/min cooling rate. This was done because methanol had been found to be the least toxic substance to zebrafish oocytes, and NOEC for methanol was found to be as high as >4M according to Trypan blue test (see section 4.2.1 and Tab. 4.1).

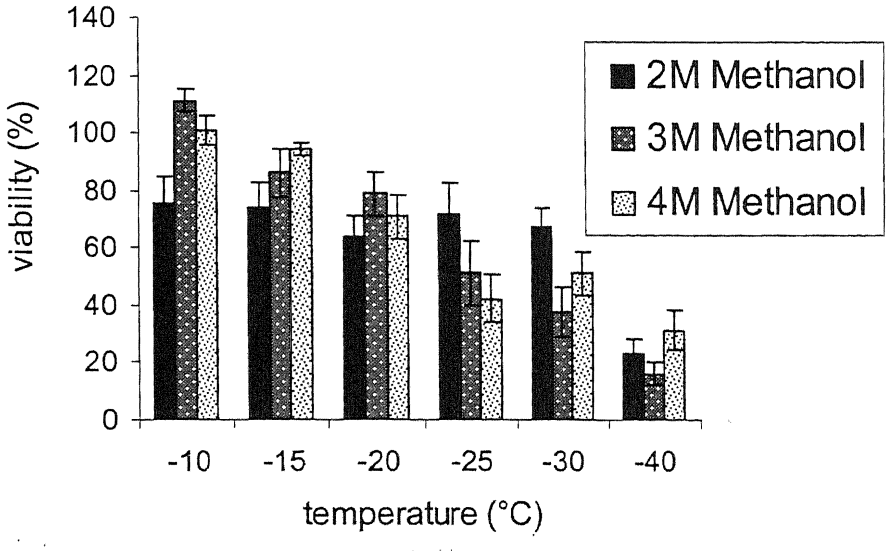


Fig. 5.5. The effect of three different concentrations of methanol (2M, 3M, 4M) on TB-assessed cryosurvival of zebrafish oocytes. Cooling rate 0.5°C/min. Error bars represent standard errors. N = 72.

At -40°C, the lowest temperature tested, the highest cryosurvivals average was obtained with 4M methanol (31.5±7.1%) when compared to 22.9±5.1% with 2M methanol and 16.3±4.0% with 3M methanol using TB test (Fig. 5.5). However, the differences between the effect of three methanol concentrations were not statistically significant (ANOVA). The advantages of using higher concentrations of methanol were supported by the results of GVBD test: with 4M methanol some cryosurvival (4.5±3.0%) was observed even at -20°C, whilst with 2M methanol, the lowest temperature at which survival was observed, was -10°C (Fig.5.8).

5.2.4 Effect of viability assessment method

In these experiments, both TB and GVBD test were used to assess oocytes survival after controlled slow cooling at 1°C/min cooling rate. The results showed that survival values with GVBD test were much lower (Fig. 5.6, 5.7, 5.8), indicating that the sensitivity of GVBD test is higher. However, when 2M methanol was used with the GVBD test, methanol appeared to be the best CPA at -10°C, but was uneffective at -15°C (Fig. 5.6). In order to investigate cryoprotective properties of methanol in more detail, further tests with this CPA were undertaken. Two cooling rates (0.5°C/min and 1°C/min), and two concentrations (2M and 4M), were tested with GVBD test (Fig. 5.7). In the present study, the lowest freezing temperature at which a level of viability of stage III oocytes is observed as assessed by the GVBD test is -20°C (conditions: 4M methanol as cryoprotectant; -0.5°C/min cooling rate) (Fig. 5.8).

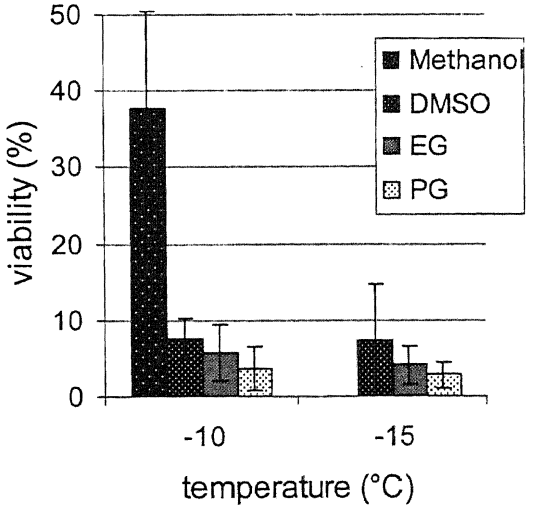


Fig. 5.6. Comparison of GVBD-assessed normalised viabilities with different CPAs (methanol, DMSO, EG, PG). Cooling rate after seeding 1°C/min. CPAs concentrations were as follows: Methanol - 2M, DMSO - 2M, EG - 1M, PG - 2M. Oocytes survivals were normalised with respect to their room temperature survivals which were 60.6±9.9% (GVBD test). Error bars represent standard errors. N = 30.

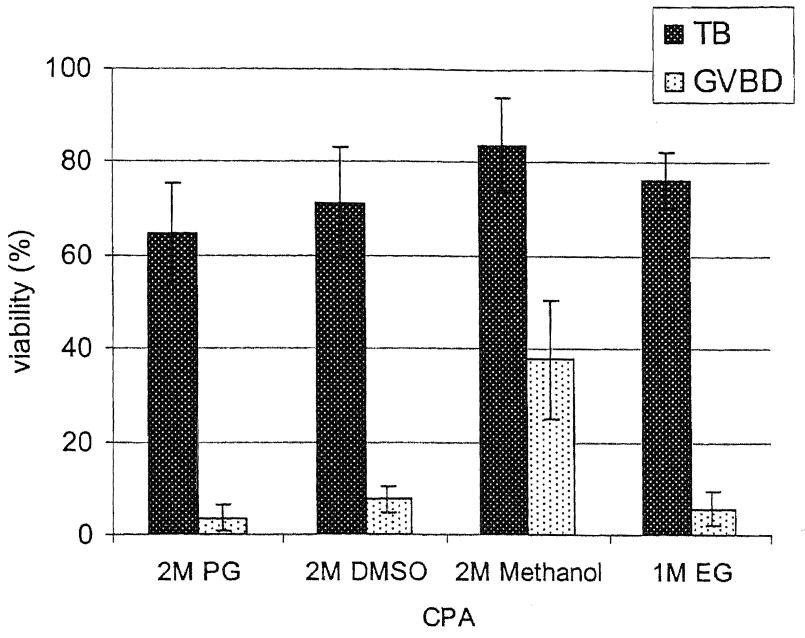


Fig. 5.7. Comparison of two viability assessment methods (TB test and GVBD test). Oocytes were cooled to -10°C at 1°C/min. Oocytes survivals were normalised with respect to their TB– and GVBD-assessed room temperature survivals which were 76.4±14.6% and 60.6±9.9% respectively. Error bars represent standard errors. N = 34.

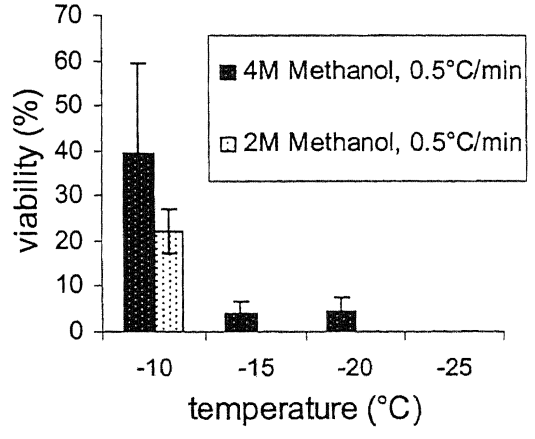


Fig. 5.8. Comparison of two freezing protocols (4M methanol versus 2M methanol; with 0.5°C/min cooling rate) assessed with GVBD test; oocytes survivals were normalised with respect to their room temperature survivals which were 60.6±9.9% (GVBD test). Error bars represent standard errors. N = 12.

5.3 Discussion

5.3.1. Effect of different CPAs

Four intracellular CPAs were used in the present study: methanol, DMSO, PG and EG. Methanol is the cryoprotectant that gave the best results in experiments on freezing of zebrafish embryos; therefore it was chosen for future work in the study. DMSO is the most widely used CPA, and is used to cryopreserve various cell types, tissues and organs. Solutions of EG and PG promote amorphous solidification due to their high viscosity at low temperatures (Belous & Grischenko, 1994). More in details, cryoprotective properties of methanol, DMSO, PG and EG were described in Introduction, Section 1.2.3.4.

There is a large volume of data relating to use of different CPAs for cryopreservation of female reproductive cells of various species. Such cryoprotectant as EG has been commonly used for cryopreservation of mammalian oocytes and embryos, especially in vitrification protocols. Mammalian oocytes and embryos have also been cryopreserved with PG (Stachecki 1998, 2000 - mouse oocytes, two-step slow cooling programme). Successful cryopreservation of human oocytes is often conducted using such cryoprotectants as PG, EG, DMSO at concentrations 1M - 1.5M, in slow cooling protocols (Picton et al., 2002, review.). In many cases freezing media were supplemented with sucrose as extracellular cryoprotectant, at concentrations 0.1-0.5M.

Information on cryopreservation of macroscopic pieces of ovarian tissue is especially useful for us, as fish oocytes are cells of large size. Macroscopic samples of ovarian tissue, taken from human, mouse and sheep are frozen mainly using DMSO, due to high penetration rate of this cryoprotectant. Yi et al. (2001) report small pieces of human ovarian tissue (1*1*0.5mm) being frozen in medium containing 1.5M DMSO and 10% serum via slow controlled cooling; and then after 6 months storage and thawing were grafted into immune-suppressed mice. As result, human oocytes successfully maturated from primordial follicles in the grafted animals, demonstrating the

survival of follicles in cryopreserved ovarian tissue. Other researchers tried several different cryoprotectants for preservation of human ovarian samples by controlled slow cooling. Small pieces of human ovarian tissue were frozen to -196°C in L15 solution, supplemented with 10% foetal calf serum and 1.5 molar concentration of DMSO / EG / PG. Cryopreservation success was evaluated by assessing the follicle survival ratio after grafting into immunodeficient mice. With DMSO, follicle survival was as high as 74%; with EG - 84%; and with PG - 44%. (Newton et al., 1996).

The results of the present study identified methanol as the best cryoprotectant for zebrafish oocytes of all the CPAs tested and at all cooling rates used. These results agree with the results on zebrafish embryos, where 2M methanol was found to be the optimal CPA, enabling 8% embryos survival at -25°C (Zhang et al., 1993a). Numerous studies on zebrafish embryos, which have been carried out in our laboratory and in other laboratories previously, have recognised the advantages of methanol as a CPA for female fish reproductive cells (Zhang et al., 1993a; Zhang et al., 1993b; Liu et al., 2001a; Liu et al., 2001b; Liu et al., 1998; Hagedorn et al., 1996; Hagedorn et al., 1997), mammalian somatic cells and embryos (Ashwood-Smith, 1987; Rall et al., 1984) and for fish sperm (Lahnsteiner et al., 1997; Horvath et al., 2003). However, it is necessary to add that interspecies differences are non-negligible. For instance, methanol was found to be the poorest CPA of all tested (EG, DMSO, PG, methanol) for slow-cooling cryopreservation of oocytes of another aquatic species, Pacific oyster, resulting in 0% post-thaw fertilisation rate (Tervit et al., 2005). The reasons for this difference are unknown, but apparently, methanol is a much more efficient CPA for fish reproductive cells than for mollusc analogues. A combination of 2M methanol with 0.5M sucrose was found to be the best medium for hypothermic (+4°C) storage of grass carp embryos (Ahammad et al., 1998). Two protocols which resulted in successful freezing of fish embryos down to liquid nitrogen temperature (-196°C) included methanol as cryoprotectant: 13% methanol (in combination with 20% PG) for vitrification of Japanese flounder embryos (Chen & Tian, 2005), and 2M methanol (8.2% v/v) (in combination with 5M DMSO, 1M EG and 10% sucrose) for vitrification of winter flounder embryos

(Robles et al., 20005). Good properties of methanol as cryoprotectant can be explained by several factors. First of all, methanol has extremely high rates of permeation into the cells of various types (Walsh et al., 2004; Liu et al., 2003; Ashwood-Smith, 1987; Rall et al., 1984; Hagedorn et al., 1996; Czlonkowska et al., 1991), including stage III zebrafish oocytes (Zhang et al., 2005). It penetrates the cells at a rate comparable with the rate of water transport, and therefore, incubation of cells with methanol doesn't lead to pronounced osmotic stresses (Zhang et al., 2005). Methanol is relatively non-toxic for fish at the organism and cellular level, as supported by numerous animal exposure experiments (http://www.epa.gov/chemfact/s_methan.txt ; Chemical Summary for methanol prepared by Office of Pollution Prevention and Toxics U.S. Environmental Protection Agency, August 1994). As this study has shown, the toxicity of methanol to oocytes at all developmental stages is lower than toxicity of the other CPAs tested (Sec. 4.2.1; Tab. 4.1.). Thus, the low toxicity and high penetration rate of methanol make this substance highly efficient as a cryoprotectant for zebrafish oocytes, allowing methanol to effectively carry out its antifreeze action inside the oocyte cytoplasm.

5.3.2. Effect of cooling rate

As already mentioned in Chapter1, slow cooling (equilibrium freezing) cryopreservation protocols are protocols that involve cooling rates less than 10°C/min. In controlled slow cooling, the crucial parameters that determine the success of cryopreservation are: cooling rate, ice-seeding temperature, liquid nitrogen plunging temperature and thawing rate. As vitellogenic zebrafish oocytes are large (diameter 0.6 - 0.9 mm), slow cooling protocols are preferable for their cryopreservation, as slow cooling rate enables sufficient cell dehydratation and, therefore, decreases the possibility of formation of intracellular ice. However, slow cooling is accompanied by various harmful effects, that have a negative impact on cell survival. These effects were discussed in detail in Chapter 1, Section 1.2.2.3. During slow cooling cells are exposed to hyperconcentrated salt solutions due to conversion of unbound water to ice crystals. Dehydratation of cells may cause denaturation and coagulation of cytoplasmic proteins, and also changes to cellular membranes (lateral separation of membrane lipids and proteins;

formation of protein clusters; increased rigidity of phospholipid bilayer and the occurrence of tangential tensions) that affect membranes permeability and may lead to their rupture. Also the leakage of lysosomal enzymes to cytoplasm; K^+ efflux from the cell (so-called 'Gardos effect'); depolymerisation of cytoskeleton may occur. These processes, occurring at sub-zero temperatures, are often referred as solution effects and osmotic shock. For most cell types the lethal osmolarity value is around 1800-2000 mOsm, and the critical shrinkage volume is 50-60% of normal cell volume. However, our previous experiments have shown that zebrafish eggs are much more sensitive to hypertonic conditions.

The optimal post-seeding cooling rate for zebrafish oocyte was shown to be 0.3°C/min. The advantage of 0.3°C/min cooling rate is increasingly evident at low freezing temperatures, such as -40°C. The fastest and the slowest cooling rates tested in this study - 1°C/min and 0.1°C/min were found suboptimal, with 0.1°C/min cooling rate giving the worst results. The advantage of moderate post-seeding cooling rates for zebrafish oocytes is to be expected with large, hydrated objects with limited membrane permeability (such as zebrafish oocyte), where slow cooling rates are preferable as they provide better dehydration in the course of freezing and lessen the risk of IIF. However, cooling rates which are too slow over expose the cells to solution effects and toxicity of CPAs (Mazur et al., 1984; Karlsson et al., 1996). Therefore, as was found in this study, 1°C/min cooling speed was too fast and didn't provide time for sufficient degree of oocyte dehydratation, which resulted in IIF and hence in high percentage of cell death; and 0.1°C/min cooling speed was too slow and over exposed the oocytes to the toxic action of the cryoprotectants and the effect of over-concentrated salt solutions during sub-zero freezing.

Fast thawing has been used for the present study, as it can prevent intracellular recrystallization and minimizes the time during which cells are exposed to CPAs. However, after cells have been thawed and rinsed clear of CPA, the possibility of post-hypertonic lysis is still present, when cells with cryodamaged membrane and sodium-enrichened cytoplasm absorb

excessive amounts of water from the medium, leading to their swelling and lysis.

5.3.3. Effect of CPA concentration

As for any cryoprotectant, a guideline for choosing the optimum concentration of methanol in a medium tends to be a balance between the toxicity of the CPA and the level required to provide its protective effect. CPAs should be used at a concentration and exposure time where their damaging effect is not pronounced, but when cryoprotective action is strong enough to protect biological material efficiently during freezing. This optimal concentration is different for each CPA and for biological material cryopreserved. Moreover, there is evidence that the optimal CPA concentration varies for different cooling rates: for slower cooling rates, lower concentration of CPAs is more suitable, and for faster cooling rates – higher concentration of CPAs. According to TB tests, the No Observed Effect Concentration for methanol with zebrafish stage III oocytes was higher than 4M (>4M). For that reason, methanol was used at three concentrations: 2M (8.2% v/v), 3M (14.3%) and 4M (16.4%). The advantage of the higher concentrations of methanol has been supported by the GVBD test. Cryosurvival values of 4.5±3.0% were observed with 4M methanol even at -20°C, which is the lowest temperature for zebrafish oocytes survival achieved to date; whilst 2M methanol failed to preserve any viability of oocytes at temperatures below -10°C (Fig.5.7).

5.3.4. Post-thaw changes in the yolk of vitellogenic oocytes

During the slow cooling studies, an interesting observation with possible important implications was made. In most of the cases after freeze-thawing, the stage III oocytes, which are normally opaque, become translucent. The same phenomenon is already reported on zebrafish oocytes by other researcher from our laboratory (Isayeva et al., 2004). There are several reports in the literature about similar post-thaw changes in oocytes of other species: coalescence of yolk granules into one transparent mass in cryopreserved pig oocytes (Nagashima et al., 1994), and yolk becoming translucent in freeze-thawed seabream (*Sparus aurata*) oocytes (Lubzens et

al., 2005). This phenomenon indicates certain important changes in the structure of oocyte yolk in the course of freeze-thawing, and these changes are unlikely to be beneficial.

The yolk of oocytes of different species is reported to be sensitive to osmotic shock and freezing. In mammalian oocytes, it has been proved, that the presence of yolk mass in the cell creates difficulties at its freezing; and the removal of part of the yolk may have benefits for cryopreservation. The authors connected the problems associated with yolk with the large amount of lipids that the yolk contains (Nagashima et al., 1994). It is reported, that tissues which are rich in non-polar lipids, such as, for example, human adipose (fat) tissue, are extremely difficult to cryopreserve (Gempt et al., 2005). Therefore, presence of large amount of lipids in the yolk of fish oocytes (McFarlane & Norton, 1999) may complicate their cryopreservation.

5.3.5. Viability assessment methods. Feasibility of cryopreservation of zebrafish oocytes

As the results of present study suggest, zebrafish stage III oocytes are somewhat more sensitive to the impact of low temperatures, than fish embryos, and far much more sensitive to freezing, than cells of most of somatic types. A sensitivity of zebrafish oocytes to chilling at hypothermic supra-zero temperatures (+4°C), which is not associated with ice formation, was previously reported in our laboratory (Isayeva et al., 2004). With zebrafish oocytes, the lowest temperature at which cryosurvival was still observed in the present study, was -20°C (according to the GVBD test), meanwhile in several studies on embryos of different fish species, including zebrafish, some embryos did survive cooling to -25°C (Rana and McAndrew, 1995; Zhang et al., 1993a; Zhang et al., 1993b; Liu et al., 2001a; Liu et al., 2001b; Liu et al., 1998; Hagedorn et al., 1996; Hagedorn et al., 1997). The trypan blue test shows high survival values of oocytes down to freezing temperatures as low as -40°C to -50°C (Fig. 5.1, Fig. 5.2); however, as pointed out already (Sec. 3.3.3), trypan blue results notify the condition of plasma membrane of the cell, rather than the viability of the cell as a whole. Whilst the integrity of plasmatic membrane is a parameter strongly linked with cell viability, a cell with relatively intact plasmalemma still may have seriously damaged cytoplasm

due to factors of cryoprotectant exposure, solution effects and intracellular ice formation. Such oocytes would remain unstained by Trypan blue, but will fail to complete GVBD. This is probably the main reason for discrepancies between TB and GVBD results. GVBD provides complete, functional assessment of the physiological status of the cell and of its ability to complete the process of normal maturation.

The critical thermal interval -20°C - -25°C, which was found to be the lower limit of stage III zebrafish oocytes cryosurvival, is probably determined by the temperature of intracellular ice formation (IIF) in the oocytes. As convincingly demonstrated in the work of Rall, Mazur and McGrath (Rall et al., 1983), low-temperature cell death is directly related to IIF, and the drastic fall of sample viability happens at exactly the same temperature interval when IIF occurs. The failure of oocytes to survive below -20°C indicates, that existing cryoprotective techniques fail to prevent IIF in zebrafish eggs at this temperature. The reason for this may be an insufficient degree of cell dehydratation achieved during freezing, which leaves more than 10% of isotonic water content in the oocyte at the moment when nucleation of ice may occur (Mazur, 1984). It is assumed, that the initiation of IIF in zebrafish oocytes takes place via surface-catalysed nucleation (SCN) (i.e. by extracellular ice), as it was shown (Karlsson et al., 1996), that volume-catalysed nucleation can occur only at temperatures below -34°C. However, further investigations, including cryomicroscopic observation, need to be carried out in order to confirm this hypothesis. It's important also to note, that in zebrafish oocytes, the temperature at which IIF can occur is much higher than in other cell types. For example, 2M glycerol and 2M DMSO were effective at preventing IIF in rapidly cooled (20°C/min) mouse embryos down to temperatures as low as -38 - -44°C (Rall et al., 1983), while for stage III zebrafish oocytes, even at slow (0.5°C/min) cooling rate, 4M methanol lowered supposed IIF temperature only down to -20°C to -25°C.

One of the ways of solving the IIF problem is to use conditions that allow a high degree of oocyte dehydratation. Use of higher concentrations of penetrating and non-penetrating CPAs would achieve this purpose. However, high sensitivity of zebrafish oocytes to dehydratation and toxic effect of CPAs,

as toxicity studies have shown (Fig. 4.3; Sec. 4.3.1) this would pose an obstacle for application of concentrated cryoprotective solutions. The ways of circumventing this difficulty would be either application of vitrification techniques, or using non-colligative ice suppressors, such as antifreeze glycoproteins (AFGPs), or another minor supplements in the cryoprotective medium.

5.4 Conclusion

The present study identifies methanol as the best CPA of those studied. The reasons for this include the low toxicity of methanol to oocytes, proved in the previous studies, and the specific properties of methanol as freeze-protectant. At all cooling rates used, methanol gave the highest (according to the TB test) viability values: 47.4±2.4% at -25°C with -1°C/min; 71.9±10.5% at -25°C with -0.5°C/min; 47.4±9.0% at -25°C with -0.3°C/min; and 6.2±2.4% at -25°C with -0.1°C/min (for 2M methanol). *In vitro* maturation test supports this conclusion: methanol gives the highest GVBD ratio (37.8±12.7%) at -10°C with -1°C/min cooling rate, which drastically differs from results obtained with other CPAs (3.6% to 7.6%). The use of higher concentrations of methanol (3M and 4M) gives better results than use of lower concentrations (2M). 4 moles per litre (4M) was the best found concentration of methanol, as it allows cryosurvival of stage III zebrafish oocytes at lowest possible temperature to date: 4.5±3.0% normalised viability at -20°C with 0.5°C/min cooling rate according to GVBD test. The cryoprotectant DMSO gives the best results after methanol: 54.1±8.0% viability with 2M DMSO at 0.5°C/min cooling rate at a temperature -25°C. Moderately slow cooling rates are better than faster and extremely slow cooling rates. -0.3°C/min cooling rate was found the optimal for st. III zebrafish oocytes. With 0.3°C/min cooling rate, survival of oocytes made up 51.1±1.4% at -40°C, which was significantly higher (Student's T-test) than cryosurvivals obtained with any other cooling rates at this temperature.

CHAPTER 6. Further studies on cryopreservation of zebrafish oocytes using controlled slow cooling

6.1 Introduction

Results of studies on controlled slow cooling of zebrafish oocytes demonstrated (Chapter 5), that freezing protocols developed so far for fish oocytes are far from satisfactory, and did not allow preservation of female fish reproductive cells at cryogenic temperatures (-80 - -196°C). In this part of the investigation, a number of alternative approaches were tested with a view to improving the results of zebrafish oocytes cryopreservation. These include: use of sodium-free cryopreservation medium; addition of minor supplements to the cryoprotective media; varying the pH of the freezing medium; varying such parameters of freezing protocol as ice-seeding temperature and liquid nitrogen-plunge temperature.

6.1.1 Solutions with low sodium content as cryopreservation media

Sodium salts (mainly sodium chloride, NaCl) are a vital component of any physiological medium. Sodium ions are crucial for maintenance of cellular homeostasis, for the functioning of the Na^+/K^+-membrane pump, and for the normal course of the majority of the biochemical reactions inside the cell. The average cell medium normally contains about 0.15M concentration of sodium chloride and other sodium salts. At the same time, the work of Stachecki et al. (1998) showed, that the presence of sodium ion in the cryopreservation medium substantially contributes to damaging "solution effects" during the course of freezing. It was found, that substitution of a normal medium to a medium with lowered NaCl content significantly improves cryosurvival of mammalian oocytes (Stachecki et al., 1998). It was also reported, that amphibian oocytes can be incubated in potassium-chloride buffer for a long periods of time without any pronounced deteriorating effects (Choe & Sackin, 1997). Incubation of *Xenopus laevis* oocytes in potassium-chloride buffer was used to shift their intracellular pH (Humphreys et al., 1995). It is known, that intacellular pH significantly changes during freezing (Belous & Grischenko, 1994), that in turn, may cause additional damage to the cells. Therefore, application of a potassium-chloride buffer as a freezing medium for fish oocyte

may also play a role in stabilising intracellular pH during cryopreservation. At the same time, it is important to add, that presence of potassium ions in the incubation medium may cause reduction of membrane potential of oocytes. For example, in studies on amphibian eggs, an increase in the concentration of K^+ ions in the medium over 0.1M caused total (0 mV) depolarisation of the oocyte membrane (the normal potential of oolemma is ~70 mV) (Maeno, 1959).

In the current work the use of the permeant potassium-chloride - potassium-acetate (KCl - KAc) buffer, which had been previously applied for toad oocytes (Choe et al., 1997b), has been investigated, Composition of the buffer is given in Chapter 2.

6.1.2 Cryoprotective supplements

In cryobiological and cryomedical practice there is considerable experience of using various cryoprotectant additives, which, whilst not being classical colligative cryoprotectants, nevertheless substantially improve the results of cryopreservation. Whilst cryoprotectants (DMSO, PG, EG, methanol, glycerol etc.) normally used in high concentrations (0.5M - 2M and higher) conduct their protective action colligatively (i.e. by lowering the freezing point of intracellular solution, decreasing the amount of unbound water and suppressing intracellular ice formation), minor supplements are used in much lower concentrations and act by protecting vulnerable areas of the cell in specific ways. A number of such substances (namely: Polyethylene glycol-400 (PEG-400), PEG-200, poloxamer-188, sucrose, glucose, dexamethasone, Butylated Hydroxyanisole (BHA), N-Acetyl-Cysteine, tocopherol, taurine, egg yolk suspension, acetyl salicylic acid) were used in order to improve cryosurvival of oocytes.

Glucose is a substance that is found naturally in the cells, where it serves as a source of energy. Naturally glucose is present in milli-molar concentration. A number of studies have shown that if applied in high concentrations (up to 0.5 mol/l), glucose may serve as an effective natural cryoprotectant. This effect was first seen in a group of freeze-tolerant frogs,

that used metabolically increased levels of glucose as a natural cryoprotective measure to overcome winter cold anabiosis (King et al., 1993; Churchill and Storey, 1996). The protective action of glucose is linked to its membrane-active properties: glucose binds to polar "heads" of phospholipids of the membrane bilayer, thereby stabilising it against compression and tangential tensions associated with freezing and dehydration (Anchordoguy et al., 1987, Suzuki et al., 1996). Presence of large amounts of glucose inside the cell also reduces the amount of unbound water, suppressing intracellular ice formation, and helps to minimize the intracellular volume reduction, thus preventing cell shrinkage during the cold season.

Sucrose is a non-permeant substance, that is used as an extracellular cryoprotectant. Normally sucrose is used in combination with an intracellular CPA, for instance, DMSO. Sucrose concentrations in cryoprotective media don't normally exceed 0.5M. The main action of sucrose as a CPA for slow cooling protocols is to create osmotic pressure, drawing unbound water out of the cell and pumping penetrating CPA into the cell (Gordienko & Pushkar, 1994). Therefore, the freezing point of the intracellular solution decreases and risk of IIF lessens. Sucrose is also a membrane-protective agent, along with other disaccharides, such as trehalose (Arakawa et al., 1990). In experiments with freeze-thawed liposomes, it was shown that disaccharides significantly stabilise the structure of phospholipid bilayer, which results in higher post-thaw integrity of liposome membranes (Anchordoguy et al., 1987). Hence, sucrose, added to a freezing medium, although not penetrating inside the cells, may well have a substantial effect of stabilising plasmatic membranes. However, uptake of certain amount of labelled sucrose by the cells after freeze-thawing cycle has been reported. This effect can be explained by influx of sucrose through ice-porated plasma membranes into the frozen cells (Shier, 1988). Sucrose is often used in vitrifying mixtures (Robles et al., 2005; Choi et al., 2002; Porcu et al., 2000; Mukaida et al., 1998; Kuleshova et al., 1999; Hong et al., 1999; Isachenko & Nayudu, 1999), because high viscosity of sucrose solutions at low temperatures promotes formation of glassified state.

Numerous membrane-stabilizing substances are widely applied in cryobiology, as cellular membranes are particularly vulnerable to low temperature injury. Membranes are considered to be one of the critical points of cryodamage. In the course of cryopreservation cellular membranes are exposed to such risks as osmotic shock, mechanical puncture by growing ice crystals, lateral phase separation of lipids, etc. As the zebrafish oocyte is a single cell of macroscopic size, any rupture of its plasma membrane (oolemma) may lead to the death of the oocyte. Moreover, the oolemma of fish oocytes is particularly sensitive due to its large surface area and low cholesterol content (MacFarlane & Norton, 1999). Membrane-protective agents, added to cryoprotective medium in small concentrations, protect membrane structures at sub-zero temperatures, and therefore significantly improve results of cryopreservation. Poloxamer-188 (Lutrol F68 ®, BASF Inc.) is an compound similar to cryoprotectants polyethyleneoxide (PEO) and polypropyleneoxide (PPO). One molecule of poloxamer-188 contains 75 ethylene oxide units on either side of domain of 30 propylene oxide units. Poloxamer-188 has strong membrane-protective properties even at low concentrations (1-10mg/ml), that is supported by experiments in which 1mg/ml solution of poloxamer-188 effectively prevented leakage of cytoplasmic contents from the cells exposed to thermal injury (Padanilam et al., 1994). Moreover, poloxamer-188, as a compound similar to polyethyleneoxide, in a freezing solution will serve as its analogue, demonstrating properties of macromolecular cryoprotectant.

Polyethyleneoxide-200 (PEO-200) and polyethyleneoxide-400 (PEO-400) are membrane-fortifying polymers. They are also known as polyethylene glycols (PEGs). These substances act in a way similar to other non-permeating macromolecular cryoprotectants (Poly-Vinylpirrolidone, HydroxyEthylStarch, dextranes, serum proteins, etc.): a polymer substance forms a sheath around the plasmalemma, stabilising it and promoting small-granular ice formation. In some cases incrustation of polymer into the plasmalemma, or a "fortification effect" takes place. Also, PEGs bind ions in solution, and therefore alleviate changes of pH and “solution effects” during freezing (Belous & Grischenko, 1994). The presence of PEGs in the freezing

medium leads to the formation of amorphous structures and hinders ice crystals. PEGs form complexes with the water of the biological structures, stabilise the hydration mesh surrounding the proteins and decrease the action of the harmful factors during the low temperature preservation (Neronov et al., 2005). The level of penetration of PEG-200 and PEG-400 inside the cells is strongly dependent on the cell type. In most cases, PEG-200 and PEG-400 act mainly as extracellular CPAs.

Macromolecular CPAs differ by the degree of polymerisation and molecular mass. These substances are widely used as cryoprotectants, usually in combination with small molecular CPAs. There is an evidence, that polyethyleneoxide-400 may penetrate inside the cells and act as an intracellular cryoprotectant and membrane fortifier (Belous & Grischenko, 1994). Therefore, polyethyleneoxide-200, which has lower molecular mass, is also likely to have similar properties. However, zebrafish oocyte and embryo are known to have limited permeability to many substances (Zhang et al., 2005; Hagedorn et al., 1996); hence, the permeation level of PEG-200 and PEG-400 to zebrafish oocytes has still to be established.

Dexamethasone is a synthetic analogue of glucocorticoid hormones with reported membrane-stabilising properties, especially relating to lysosomal membranes (Hinz & Hirschelmann, 2000; Lugovoi & Kravchenko, 1980). Lysosomal membrane cryoinjury and leakage of proteolytic enzymes into cytoplasm is one of the most damaging factors in post-thaw death of cryopreserved cells (Belous & Grischenko, 1994). Dexamethasone was tested in this study among with other additives.

Acetylsalicylic acid (aspirine), which is widely used in medicine, exhibits membrane-protecting properties, and has been applied in cryopreservation protocols (Kravchenko et al., 1974), and it was also chosen for the present study.

Taurine is a natural substance which is present in many animal tissues in milli-molar concentrations. High levels of taurine are found in oocytes of

marine fishes and in early mammalian embryos. Taurine makes up 30% of all free amino acids in oocytes. This free amino acid is an end product of cysteine metabolism, has high penetration rate through membranes, and plays the role of natural osmolyte, cryoprotectant and antioxidant (Finn et al., 2002; Hardikar et al., 2001; Dumoulin et al., 1997; Sanchez-Partida et al., 1997).

Egg yolk suspension is known for its cryoprotective properties as an extracellular CPA (Parks, 1997). Presence of 3.3% hen egg yolk suspension in a freezing medium alongside with other CPAs, strongly decreased CPA toxicity and freezing injury during mouse oocytes vitrification, and supported chromatin integrity (Isachenko & Nayudu, 1999). The yolk of hen egg is very rich in cholesterol and phospholipids, and possible mechanisms of protective action of hen yolk may include saturation of oocyte membrane with these substances, which pass to the oolemma from yolk micelles (Levitan et al., 2000). Cholesterol is an natural membrane stabiliser; and a natural moderator of homoviscous temperature adaptation in mammalian membranes (Bowler and Manning, 1994). It eliminates phase transition of membrane lipids across a wide temperature range, and, therefore, stabilises membrane structures and prevents "freeze-squeezing" of proteins in the course of cooling. Cholesterol also increases hydration of phospholipid bilayer and hence plastifies it. Membranes with low cholesterol level (for instance, mitochondrial membranes) are very vulnerable to impact of low temperatures, pH shifts and overconcentrated solutions, whilst membranes which contain optimal percentage of cholesterol (~25% of total membrane lipids weight), are well plastified and more likely to withstand the damaging factors associated with freezing (Belous & Grischenko, 1994). Artificial saturation of cell membranes with cholesterol has been successfully applied to enhance cryosurvival of bull spermatozoa (Graham et al., 2005). Cholesterol is synthesized in fish and amphibian oocytes, and is present in oolemmae of these cells in cholesterol-saturated membrane areas - so-called caveoli (Sadler, 2001; MacFarlane & Norton, 1999), but there is evidence that its content is sub-optimal for freezing purposes. Therefore, saturation of the membranes of zebrafish oocytes with cholesterol via incubation with hen yolk suspension may well have a beneficial

effect on the cryostability of these cells. Protein and phospholipid components of hen egg yolk, especially lecitine, also have a positive effect during freezing, as they help to prevent cold shock of the cells (Belous & Grischenko, 1994). It has been shown, that the stability of enzymes and of other cell proteins during freeze-thawing increases when total protein concentration in the solution is increased. Addition of a different protein also increases the stability of enzymes during freeze-thawing (Arakawa et al., 1990). Proteins contained in hen egg yolk may stabilise membrane proteins of cryopreserved cells.

Another class of biologically active substances often added to freezing media are antioxidants. It is known, that oxidative stress and lipid peroxidation are important factors contributing to cell cryoinjury (Bell et al., 1993); therefore, supplementing the freezing mixture with antioxidative agents can help to protect cell structures via reducing oxidative injury (Sputtek & Korber, 1991). N-Acetylcysteine is an antioxidant substance which acts by restoring normal glutatione content in the cell (Dunne et al., 1994; Otala et al., 2002). α-Tocopherol (vitamin E) is antioxidative compound, which also exhibits membrane-stabilising properties. Tocopherol, as well as BHA and N-acetyl-Cysteine have been successfully used in cryopreservation of cells and tissues (Janjic et al., 1996; Dunne et al., 1994; Limaye & Kale, 2001; Alvarez & Storey, 1993; Askari et al., 1994).

While some of the supplements used in this study (such as egg yolk suspension, aspirine, N-Acetyl-Cysteine or poloxamer-188) are relatively safe and non-toxic, other additives may have a toxic effect on zebrafish oocytes. For example, PEG-400 may lead to disruption of membrane structure and cause chromosome abnormalities, as well as to pathological fusion of cells (Belous & Grischenko, 1994; Biondi et al., 2002; Ashwood-Smith, 1987). Incubation of oocytes in the medium with high potassium content (i.e. in KCl buffer) may lead to reduction of their membrane electrical potential and cause other undesirable changes (Maeno, 1959). Toxicities of polyethylene glycol-400 (PEG) together with toxicity of KCl medium to zebrafish oocytes were therefore studied.

6.1.3 Varying the pH of freezing medium

The pH of the cryopreserved sample changes significantly during the course of freezing. Formation of the ice phase in samples at sub-zero temperatures leads to concentration of salts in unfrozen solution, and, as the concentration rises, some salts will precipitate, leading to the acidification of solution surrounding the cells. Thus, the extracellular pH in the "tortuous channels" of growing ice domains tends to decrease. In unbuffered saline the pH in extracellular solution falls by 1.5 -2.0 units. The presence of phosphate buffer alleviates this effect and in buffered saline pH of the liquid captured between growing ice domains falls only by 0.5-1 units. Addition of CPAs, for example DMSO or glycerol, to solution can also lessen the fluctuations of pH in the medium during freezing (Belous & Grischenko, 1994). However, the intracellular pH of the frozen cells tends to increase due to hyperosmotic shrinkage of cells and, therefore, hypertonic alkalinisation. For example, hypertonic shrinkage of *Xenopus laevis* oocyte to 83% of its initial volume due to incubation in hyperosmotic medium leads to alkalinisation of its cytoplasm from 7.3 to 7.5 (Humphreys et al., 1995). However, there are also data, indicating certain acidification of cytoplasm of the cells under hypothermic conditions, not associated with formation of ice. For example, 25 min incubation of carp (*Cyprinus carpio*) sperm at +5°C has led to acidification of pH of cytoplasm (measured by NMR method) from initial 7.52 units (room temperature) to 7.38 units (E. Kopeika, unpublished data).

During the course of freezing, when the concentration of sodium ions in a medium surrounding the cells reaches 0.5 mol/L due to ice formation and intracellular pH shifts to alkaline side, cytoskeletal proteins start to dissociate. pH increase also leads to an increase in microviscosity of soluble proteins. Changes of pH also alter the viscous properties of cellular membranes, for instance, shift of pH from 7.4 to 2.5 increases the temperature of phase transition of membrane phospholipids from 0°C to 26°C, in effect making the plasma membrane more rigid. This is due to membrane phospholipids with strongly protonated carboxyl groups tending to separate into phases and solidify (Belous & Grischenko, 1994). pH shifts may also trigger certain

physiological processes in oocytes, for example, addition of 50 mM KCl or 10 mM ammonia chloride to the medium, which results in basification of pH, causes activation of maturation in eggs of surf clam (*Spisula solidissima*) mollusc. This may be connected to the fact that maturation of oocytes of the majority of species (as well as fertilisation) is accompanied by the increase of their intracellular pH (Dube & Eckberg, 1996). The pH value plays an important role in formation and supporting the structure of the oocyte yolk. Yolk vesicles maintain acidic internal pH which might be important for stabilisation of the crystal lattice of yolk proteins stored in yolk vesicles: yolk proteins, like phosvitin and lipovitelline, bear high negative charge, and the presence of protons may be required to neutralise the forces of repulsion inside the protein crystal. Therefore, the excessive change of intracellular pH may negatively affect the structure of oocyte yolk (Fagotto & Maxfield, 1994).

By controlling extra- and intracellular pH of the sample, it may be possible to avoid various undesired complications which may emerge during cryopreservation of fish oocytes. Incubation of *Xenopus laevis* oocytes in permeant KCl buffer was primarily used to shift intracellular pH of the cells (Humphreys et al., 1995). Thus, application of KCl buffer as a freezing medium for fish oocyte, besides avoiding harmful effects of sodium ions in course of freezing, may also allow control and stabilisation of intracellular pH of oocytes during cryopreservation. The pH of KCl buffer normally used was 6.4 units. In this study different pH values, both basic and acidic were tested, in order to determine how the freezing in media of different pH values affected the post-thaw viability of stage III zebrafish oocytes.

6.1.4 Varying the ice-seeding temperature and plunge temperature

Ice seeding is a procedure of deliberate initiation of ice nucleation, commonly practiced in cryobiology, and aiming to avoid excessive supercooling of samples and therefore lessen the risk of IIF. Ice seeding is commonly conducted by touching the sample straws with forceps pre-cooled by immersion into liquid nitrogen. This causes the local cold spot on the wall

of the straw, leading to ice nucleation in the adjacent solution, and the ice front spreads throughout the sample.

According to the principles of physical chemistry (Raoult's law), addition of 1 mol/l of non-dissociating solute to water solution lowers the freezing point of the solution by 1.86°C (for details, see Section 7.1.2.1, formula 7.2). This value is constant for any water solutions and is called cryoscopic constant. Therefore, cryoscopic constant of water is 1.86 K*kg/mol. Taking this into consideration, we can calculate, for example, the freezing point of 4M Methanol + 0.2M Glucose solution in KCl buffer. The calculated osmolarity of this solution is 4.5 osmol (4 osm added by methanol; 0.2 osm added by glucose; 0.3 osm added by the components of KCl buffer). This corresponds to the freezing temperature of -8.37°C. Therefore, the seeding temperature for the given solution should be lower than -8.37°C. Normally, throughout this study the following empirical rule was used for determination of seeding temperature: addition of each mole of CPA into freezing medium needs the lowering of seeding temperature by 2.5°C. Therefore, for 1M concentration of CPAs seeding is done at -5°C; for 2M - at -7.5°C; for 3M - at -10°C; and for 4M - at -12.5°C. This rule is grounded on the necessity to allow certain degree of supercooling for seeding (Zhang, 1994).

In the literature there are several reports supporting the view that by varying ice seeding temperature it is possible to substantially improve the results of cryopreservation. For instance, when human oocytes were cooled in 1.5M PG and seeded at -8°C, -6°C, or -4.5°C, the incidence of intracellular ice formation was 78%, 33%, and 0% respectively, and 24h post-thaw survival was 32%, 56%, and 93% respectively. The authors propose that ice seeding temperature should be quite close to freezing temperature in order to obtain better cryosurvival (Trad et al., 1999). For the purpose of cryopreservation of mouse follicles with 1.5M DMSO as cryoprotectant, -5°C seeding temperature was optimal and gave significantly higher cryosurvival values than those with lower seeding temperatures (49±4% with seeding temp. -5°C; 26 ±1% with seeding temp. -7°C; 28 ±3% with seeding temp. -9°C) (Newton & Illingworth,

2001). Similar results were obtained by Miyamoto & Ishibashi (1981): on freezing mouse embryos, use of high (-4°C) seeding temperature gave dramatically higher results than the use of low (-13°C) seeding temperature: 74% against 45% survival. It was also reported, that changing the seeding temperature significantly affects the cryosurvival of mouse oocytes cryopreserved in 1.5M PG + 0.1M sucrose: post-thaw survival of oocytes which were seeded at -20°C was only 5.8% (measured by development to blastocyst stage) comparing to 43% survival of oocytes which were seeded at conventional temperature of -7°C (Stachecki & Willadsen, 2000).

According to data of differential scanning calorimetry (DSC), spontaneous intracellular ice formation in zebrafish embryos (on 6-somite stage) occurs at -13.0°C (cooling rate 10°C/min), and changing of cooling rate to 2°C/min did not have any significant effect on this nucleation temperature. Incubation of 6-somite embryos for 2 hrs in 2M methanol decreases the temperature of intracellular crystallisation by 4°C (Liu et al., 2001b). In the work of Rall & Mazur on mouse embryos (1983) it was shown, that when embryos are rapidly cooled in saline which doesn't contain any cryoprotectants, the formation of intracellular ice occurs at temperatures ranging from -10°C to -15°C; meanwhile addition of glycerol or DMSO in 1-2 molar concentration lowers the temperature of intracellular ice initiation to -38°C - -44°C. In a study of Muldrew & McGann (1990) it was shown, that for 0.5M concentration of glycerol optimal seeding temperature (which fully prevents IIF) is -6°C; for 1M glycerol - -8°C; and for 2M concentration - -10°C. Their study has also shown (which is consistent with results obtained in our laboratory (Liu, 2000; Liu et al., 2001a; 2001b) that the temperature of IIF doesn't depend on the rate, at which the cells are cooled, but is strongly dependent on concentration of CPAs in the medium. At the same time, the percentage of cells which underwent IIF is strongly dependent on cooling rate (Muldrew & McGann, 1990). In this study several ice seeding temperatures are used in combination with 4M + 0.2 Glucose in KCl buffer freezing medium (-12.5°C; -10°C; -8.5°C; and no ice seeding at all), to determine how ice seeding temperature affects the cryosurvival of stage III zebrafish oocytes.

Another important parameter in cryopreservation protocols is the choice of LN plunge temperature. Plunge temperature is a temperature at which slow cooling of the cryopreserved samples terminates, and the containers with samples are plunged into liquid nitrogen for long-term storage. Plunge temperatures used in cryobiological practice are normally lower than eutectic temperature of water-salt-cryoprotectant system (-20°C - -40°C). Choice of an optimum plunge temperature should be determined by the "golden mean" rule. If the plunge temperature is too high, cells do not have time to dehydrate sufficiently, and IIF will occur during the plunge into liquid nitrogen. On the other hand, if plunge temperature is too low, the cells will be overexposed to cryoprotectants and "solution effects", and, moreover, intracellular ice is more likely to form during initial cooling because slow cooling rates afford sufficient time for water molecules to aggregate into stable ice nuclei. Karlsson et al., (1996) give D-shaped curve of acceptable combinations of plunge temperatures (vertical axis) and dehydration cooling rates (horizontal axis). Within this "D"-area lethal IIF should not occur, and ideally, at the moment of liquid nitrogen plunge, intracellular solution should be converted into an amorphous glass-like phase, even though slow cooling technique is used (Mazur, 1984; Woods et al., 2003; Gordienko & Pushkar, 1994). For this desirable event to occur, three conditions must be combined: 1) a temperature should be below freezing point 2) intracellular water content should be <10% of its isotonic value; and 3) level of supercooling of intracellular solution should not exceed 2°C (Mazur, 1984). The first condition is easy to comply, as freezing point of most of cryoprotective media is not lower than -10°C, and the plunge temperature which is used in majority of protocols nowadays is far below that point. The matching to other two conditions is achieved by sufficient dehydratation of the cells in the course of slow cooling, which precedes the plunge into LN. If one of the listed conditions is not fulfilled, intracellular ice formation may occur after plunge into liquid nitrogen, with possible lethal consequences for the cells (Mazur, 1984; Rall et al., 1983). To avoid that, a plunge temperature should be chosen carefully, and correlated with dehydration cooling rate and other factors. In a study of Karlsson et al., 1996, a theoretically designed D-shaped graph is given for the

acceptable range of plunge temperatures (i.e. which allow IIF in less than 5% of cells) for mouse oocytes with different pre-plunge (dehydration) cooling rates. This graph gives good correlation with experimental results. However, it's worth noticing that for zebrafish oocytes any dependence probably would be significantly different. There are several studies, investigating the effect of different plunge temperatures on viability of freeze-thawed cells. In a study of Stachecki and Willadsen (2000) with mature mouse oocytes, three following plunge temperatures were examined: -10°C; -20°C and -33°C. The lowest one (-33°C) was found the best of all three, with more than 90% oocytes surviving cryopreservation. In a report of Czlonkowska et al. (1991), which studied slow cooling of sheep embryos with 3M methanol, it was shown, that the plunge temperature of the sample must not be lower than -45°C; otherwise the survival of embryos dramatically falls. In the work of Abbeel et al. (1994) it was shown, that optimal plunge temperatures for cryopreservation of mouse embryos lay within the range of -35°C - -50°C. Even narrower plunge interval (-30°C - -40°C) for freezing of mouse embryos is defined in work of Rall et al. (1984). Plunge temperatures such as -24°C (Visintin et al., 2000); -30°C (Lassalle et al., 1985); -33°C (Bass et al., 2004); -55°C (Harp et al., 1994); -70°C (Yi et al., 2001), -80°C (Cox et al., 1996; Crister et al., 1988; Carroll et al.,1993; Karlsson et al., 1996) and even -140°C (Salle et al., 1999) have been successfully applied. Obviously, the choice of optimal plunge temperature largely depends on the nature of frozen cells or tissue, its permeability and hydration properties and biochemical structure.

It is common practice in cryobiology and cryomedicine, after a phase of slow cooling, to add a stage of fast (usually -10°C/min) cooling down to temperatures ranging -80°C--150°C; and after that the samples are plunged into liquid nitrogen (Newton et al., 1996; Candy et al., 2000; Picton et al., 2002). The reason for the additional step is to bring the liquid nitrogen plunge temperature to the lowest possible point in specific freezing protocols; and at the same time to avoid dangers of prolonged slow cooling.

Based on the given data, a range of different plunge temperatures (-30°C - -70°C) has been tested in the present study in addition to standard plunge temperature (-50°C), that has been used in all previous experiments.

6.2 Results

6.2.1. Low-sodium medium

The results of KCl buffer toxicity to zebrafish oocytes using two viability assessment methods (TB test and GVBD test) are shown in Fig. 6.1. Statistical analysis (ANOVA and Scheffe's test) shows that KCl buffer did not have detrimental effect on zebrafish oocytes after 30 min incubation period using TB staining, irrespective whether separation of oocytes was conducted in Hank's medium or in KCl medium ($F_{2/22}$ = 0.282, P = 0.76). However, with the GVBD test, 30 min incubation in KCl buffer did have a deleterious effect on zebrafish oocytes ($F_{2/21}$ = 12.99, P = 0.00021), and this effect was more pronounced when separation of oocytes was conducted in Hank's medium and then transferred to KCl buffer (S = 4.58, $S_{critical}$ = 2.63); comparing to those oocytes, which were incubated in KCl buffer throughout (S = 3.01, $S_{critical}$ = 2.63) (Fig. 6.1).

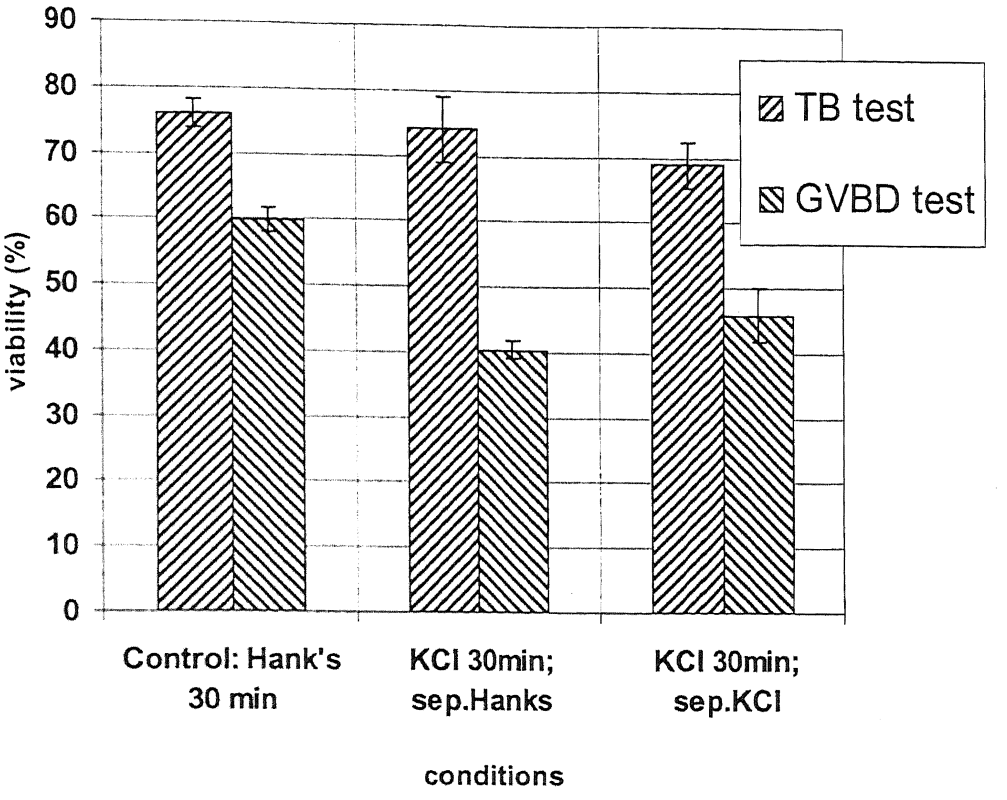


Fig. 6.1. Effect of 30 min incubation in KCl buffer and Hank's on TB- and GVBD-assessed viability of stage III zebrafish oocytes.
Labels "sep. Hank's" and "sep. KCl" mean that the oocytes were separated from the fish ovary in Hank's medium or in KCl buffer, respectively. Error bars represent standard errors. N = 49.

6.2.2 Cryoprotective supplements

6.2.2.1 Toxicity of supplements

The experiments on toxicity of methanol solutions showed, that when assessed with TB, toxicity of 2M and 4M methanol in KCl buffer did not differ significantly from toxicity of methanol solutions of the same concentrations in Hank's medium. However, GVBD test showed, that 4M methanol in KCl buffer was less toxic than methanol in Hank's medium ($F_{1/8} = 6.62$, $S = 2.37$, $S_{critical} = 2.30$) (Fig. 6.2.).

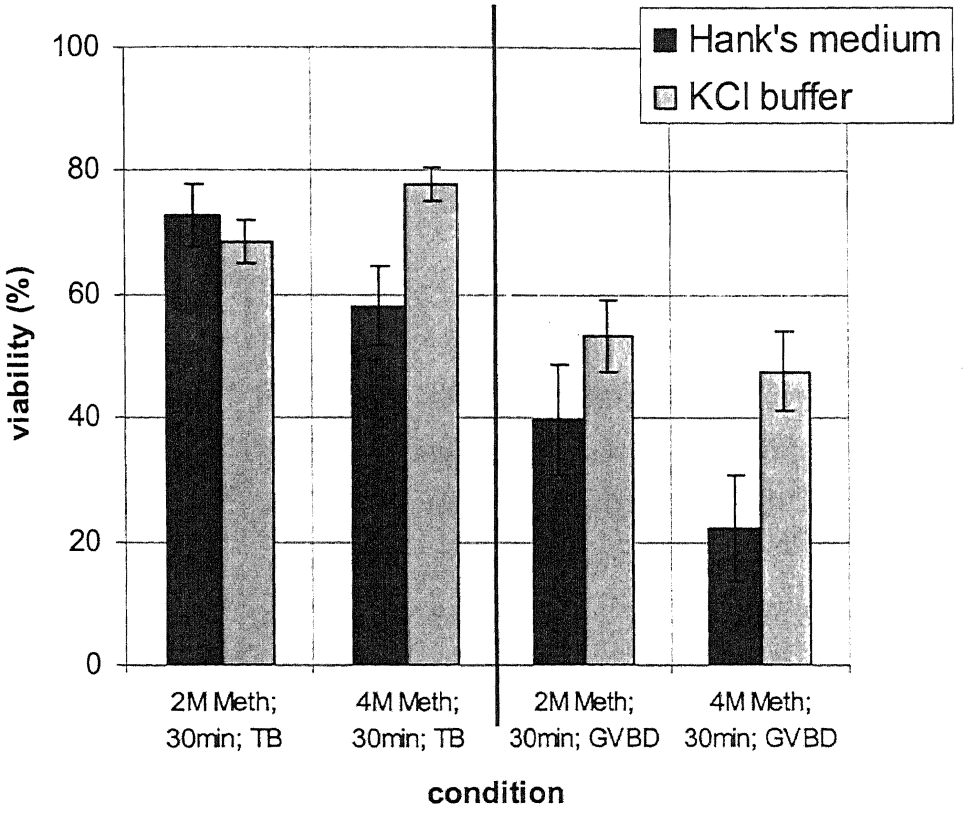


Fig. 6.2. Effect of 2M and 4M methanol solutions in KCl buffer and Hank's medium on TB- and GVBD-assessed viability of stage III zebrafish oocytes. Incubation time 30 min at 22°C. N = 29.

Experiments on toxicity of PEG-400 showed, that incubation of stage III zebrafish oocytes in 5% solution of PEG-400 in Hank's medium over 30 min

or 60 min did not have any statistically significant (ANOVA, Scheffe test ; Student's t-test) damaging effect on oocytes, according both to TB staining and GVBD observation (Fig. 6.3.).

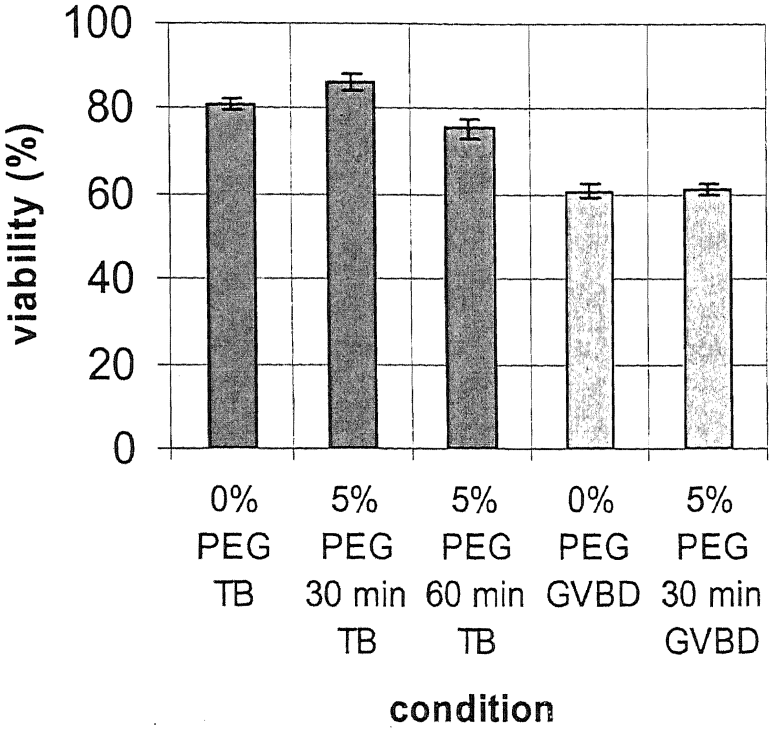


Fig. 6.3. Effect of 5% PEG-400 solutions in Hank's on TB- and GVBD-assessed viability of stage III zebrafish oocytes, compared to control. Incubation time 30 min or 60 min at 22°C. N = 54.

6.2.2.2 Freezing with supplements

Fig. 6.4. shows the TB assessed viabilities of stage III zebrafish oocytes frozen to different final temperatures in a freezing medium consisting of 4M methanol in KCl buffer; compared to the corresponding viabilities of oocytes in a conventional freezing medium (4M methanol in Hank's medium). At -50°C, oocyte viabilities were significantly higher (Student's t-test) in KCl buffer than those obtained with Hank's medium: viability of oocytes frozen with 4M Methanol in KCl buffer was 36.2±6.8% comparing to 16.2±5.2% when 4M Methanol in Hank's was used. At -196°C, viabilities of oocytes frozen with 4M Methanol in KCl buffer and Hank's were 8.7±1% and 5.5±1.5% respectively. However, these differences are not statistically significant (Student's t-test).

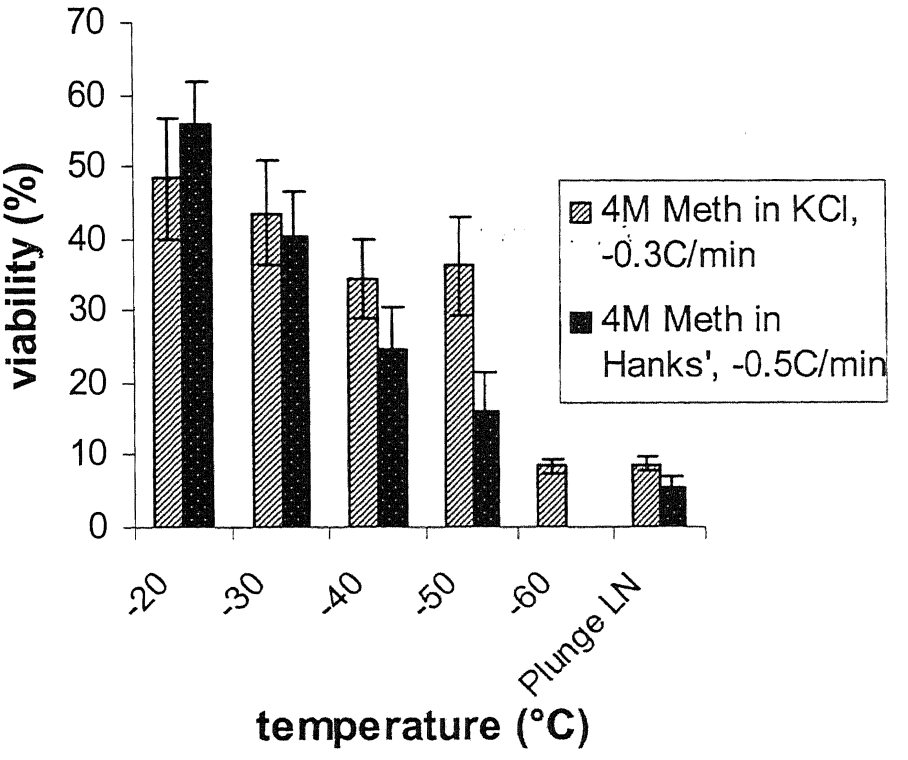


Fig. 6.4. TB-assessed viabilities of oocytes frozen in 4M Methanol KCl-buffer solution compared to viabilities of the samples, frozen in 4M Methanol Hank's solution. Error bars represent standard errors. N = 39.

With GVBD test, viabilities of oocytes frozen to -10°C with 4M Methanol in KCl buffer or Hank's medium were 16.9±2.8% and 24±12% respectively. The viabilities of oocytes frozen to -20°C with KCl buffer or Hank's medium were 0% and 2.7±1.8% respectively (Fig. 6.5.). According to Student's t-test, there were no significant differences between the effect of KCl buffer and Hank's medium either at -10°C or at -20°C (P = 0.422 and P = 0.217 respectively).

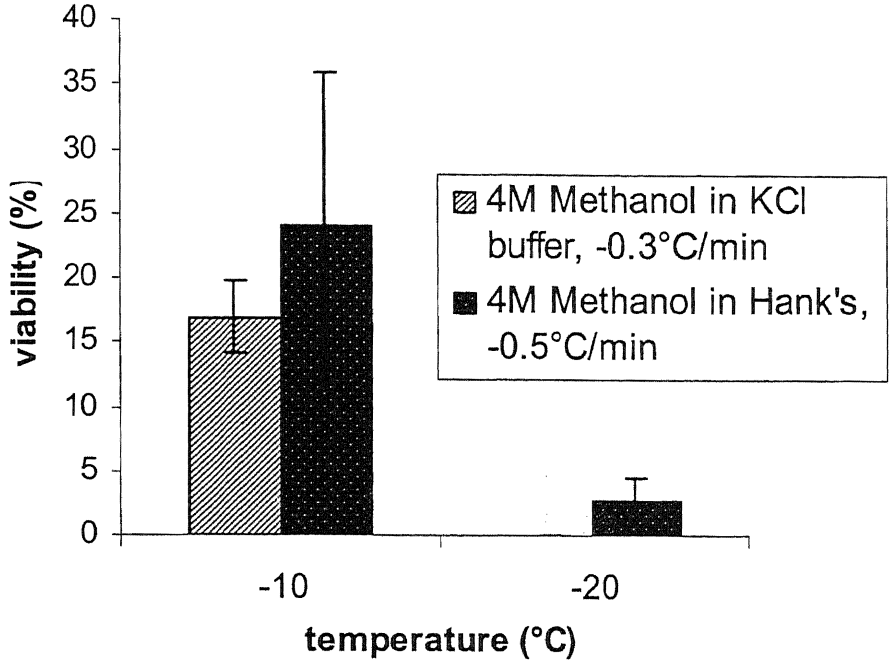


Fig. 6.5. GVBD-assessed viabilities of oocytes frozen in 4M Methanol KCl-buffer solution compared to viabilities of the samples, frozen in 4M Methanol Hank's solution. Error bars represent standard errors. N = 16.

The results of experiments where oocytes were exposed to freezing at -196°C at a cooling rate of 0.3°C/min, with 4M methanol in KCl buffer, and with addition of various supplements to a freezing medium are shown in Fig. 6.6. The best cryosurvival values were obtained with: 4M Methanol + 0.2M Glucose (16.3± 4.2%); 4M Methanol + 0.2M Glucose + 5% PEG-200 (16.3± 4.3%); 4M Methanol + 0.1M Sucrose (11.3± 3.2%), and with 4M Methanol + 5% PEG-200 (11.1± 2.3%). Seeding temperature was -12.5°C, LN plunge temperature was -50°C, pH 7.4.

One-way ANOVA followed by orthogonal polynomial contrasts analysis of specific comparisons revealed that of all supplements tested, only the additions of 0.2M Glucose, and 0.2M Glucose + 5% PEG-200, had a statistically significant ($F_{1/76}$ = 4.123, P = 0.0458) positive effect on TB-assessed cryosurvival. None of the other membrane-stabilising or antioxidizing supplements had any significant positive effect on cryosurvival, and the addition of 100mM BHA has led to total loss of membrane integrity (0% TB-assessed survival) of frozen oocytes.

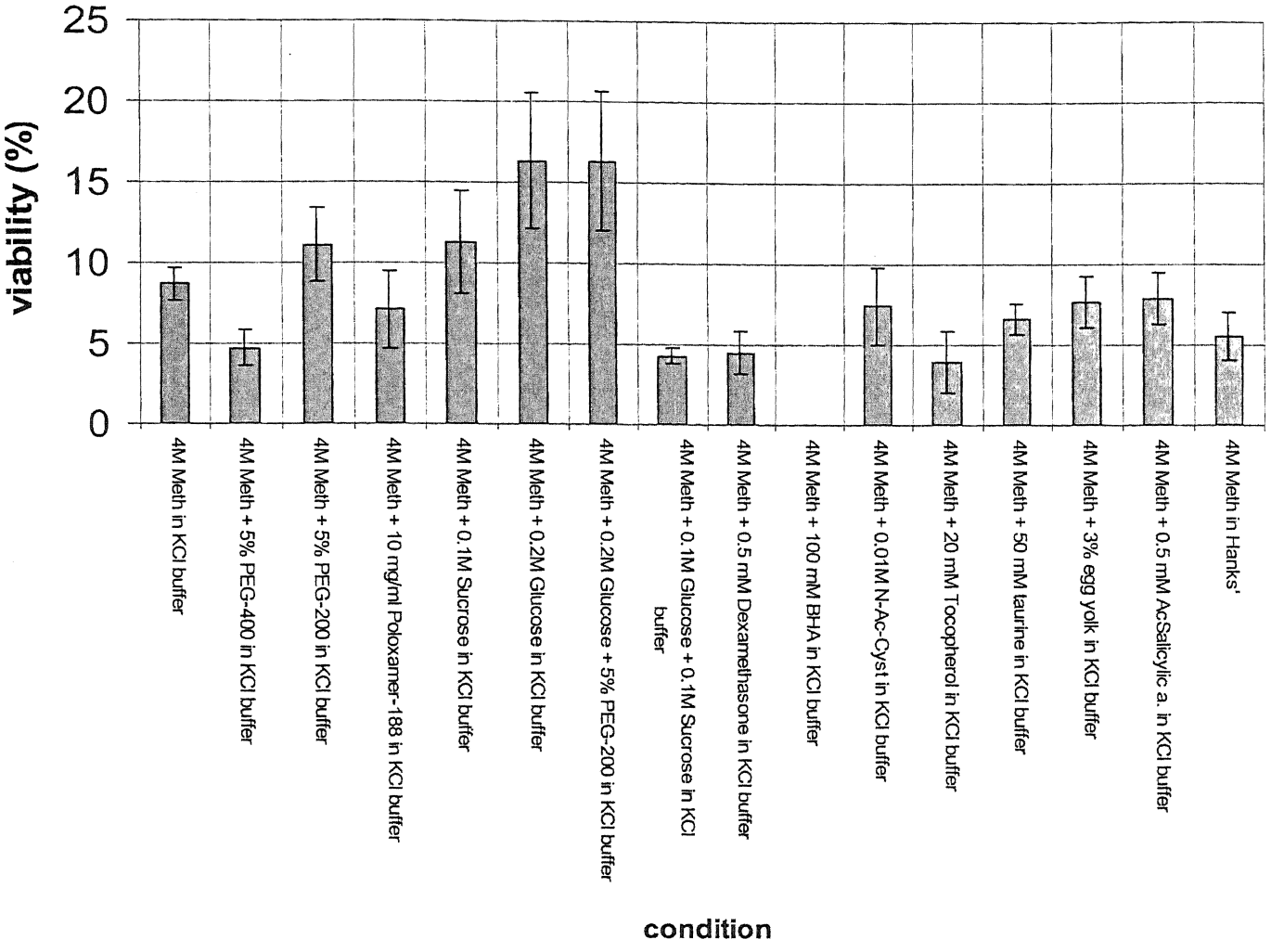


Fig. 6. 6. TB-assessed viabilities of oocytes frozen to -196°C in 4M Methanol KCl-buffer solutions and in 4M Methanol Hank's solutions with added supplements. Error bars represent standard errors. Freezing protocol: stage III zebrafish oocytes were incubated in the above media for 30 min, then loaded into 0.5ml plastic straws and frozen using the following protocol: cooling from 20°C to -12.5°C at 2°C/min; manual seeding at -12.5°C and held for 5 min; slow freezing from -12.5°C to -50°C at 0.3°C/min; samples were then plunged into LN. After 10 min in LN, samples were thawed (~200°C/min) in a 27°C water bath. Oocytes were then washed either in Hank's or KCl-buffer twice. Oocytes viability was then assessed using Trypan Blue staining (0.2% for 5min). N = 92.

6.2.3 Effect of ice-seeding temperature

The effect of different ice-seeding temperatures on TB-assessed quality of cryopreserved stage III zebrafish oocytes, frozen to -196°C, is presented in Fig. 6.7. The results showed that although mean values of oocytes survivals were higher when oocytes were seeded at -12.5°C (standard) or -10°C than those at -8.5°C or no seeding at all, the differences between any of the tested ice-seeding conditions were not statistically significant (ANOVA, Kruskal-Wallis non-parametric ANOVA).

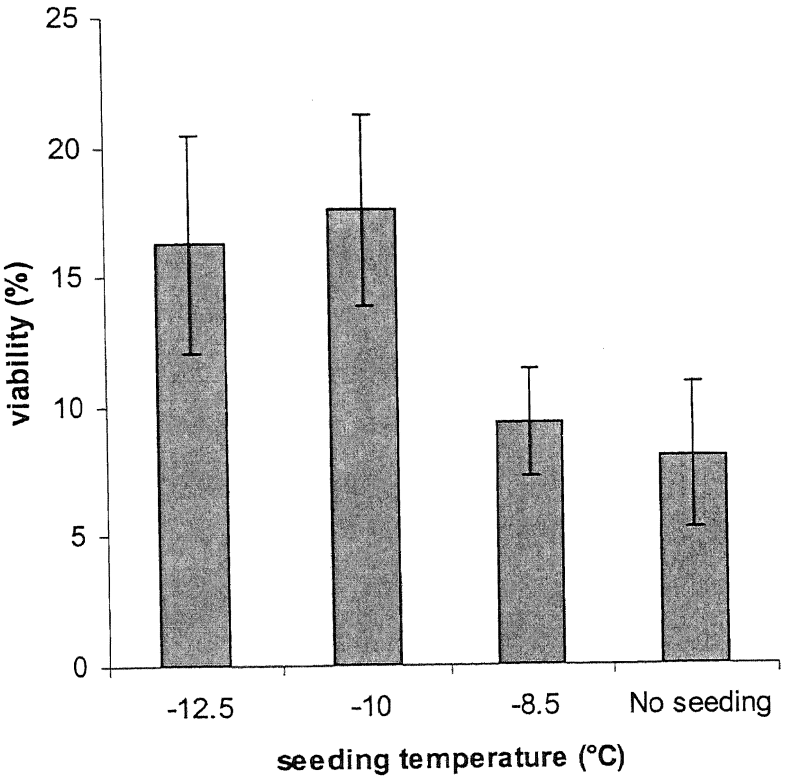


Fig. 6.7. Effect of different ice-seeding temperatures on TB-assessed quality of cryopreserved stage III zebrafish oocytes, frozen to -196°C following standard freezing protocol (0.3°C/min post-seeding cooling rate; plunge to LN at -50°C; 4M Methanol + 0.2M Glucose in KCl buffer as a freezing medium). N = 21.

6.2.4 Effect of liquid nitrogen plunge temperature

Fig. 6.8. shows the effect of different liquid nitrogen plunge temperatures on TB-assessed cryosurvival of stage III zebrafish oocytes, frozen to -196°C. Moderate plunge temperatures (-40 to -60°C) resulted in higher mean cryosurvival values than higher or lower plunge temperatures (-30°C and -70°C), with -40°C plunge temperature provided the highest mean survival (Fig. 6.8). However, the differences between any of tested plunge temperatures were not statistically significant (ANOVA, Kruskal-Wallis non-parametric ANOVA).

Adding a fast (10°C/min) cooling stage to -80°C before plunge to LN significantly (Student's t-test, $P<0.05$) decreased the viability of oocytes comparing to those samples which were plunged into LN at -40°C (Fig. 6.9). Post-thaw TB-assessed survival of stage III zebrafish oocytes frozen by the optimum protocol (4M Methanol + 0.2M Glucose in KCl buffer; slow cooling to -40°C; then direct plunge into LN) was 19.6±8%. In contrast post-thaw TB-assessed survival of stage III zebrafish oocytes frozen by the optimum protocol with added phase of fast cooling was 9.2±1%. As a result of these findings, fast cooling stage was not used in subsequent experiments.

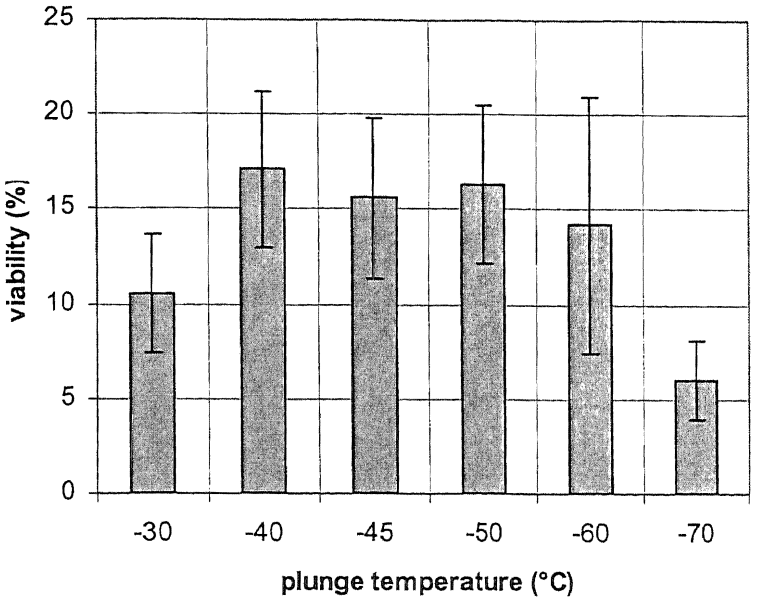


Fig. 6.8. Effect of different LN-plunge temperatures on TB-assessed quality of cryopreserved stage III zebrafish oocytes, frozen to -196°C following standard freezing protocol (seeding at -12.5°C; 0.3°C/min post-seeding cooling rate; 4M Methanol + 0.2M Glucose in KCl buffer as a freezing medium). N = 37.

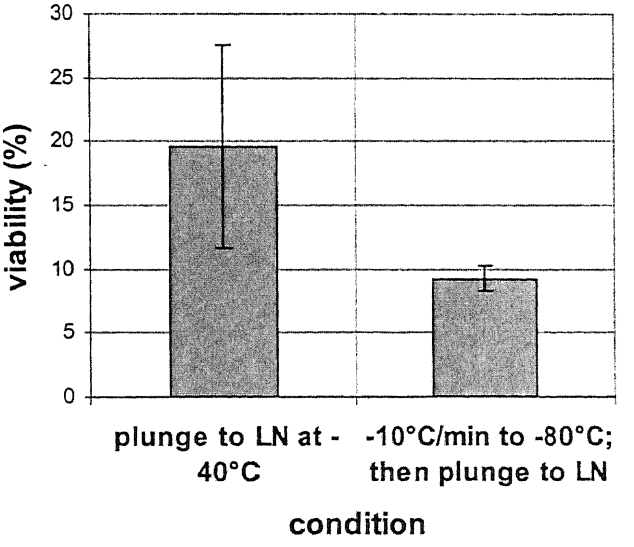


Fig. 6.9. Effect of adding a fast cooling stage on TB-assessed quality of cryopreserved stage III zebrafish oocytes, frozen to -196°C (modified procedure: seeding at -12.5°C; cooling at rate 0.3°C/min to -40°C; cooling at 10°C/min to -80°C; plunge to LN at -80°C) comparing to oocytes frozen using

standard cooling profile (seeding at -12.5°C; 0.3°C/min post-seeding cooling rate, plunge to LN at -40°C; 4M Methanol + 0.2M Glucose in KCl buffer as a freezing medium). N = 15.

6.2.5 Effect of pH of freezing medium

Fig. 6.10 and Fig. 6.11 show the effect of different pH values of freezing medium on TB-assessed quality of stage III zebrafish oocytes, frozen to the temperature of liquid nitrogen. As it can be seen, close to neutral values of pH of the freezing medium result in higher cryosurvivals of oocytes. According to one-way ANOVA followed by orthogonal polynomial contrasts analysis of specific comparisons, extreme acidic (pH 4.4, 5.4) and extreme alkaline (pH 9.4, 10.4) freezing media have statistically significant ($F_{2/33}$ = 6.42, P = 0.00443) damaging effect on oocytes when compared to media with close-to-physiological (6.4 – 8.4) pH values (Fig. 6.10, Fig. 6.11).

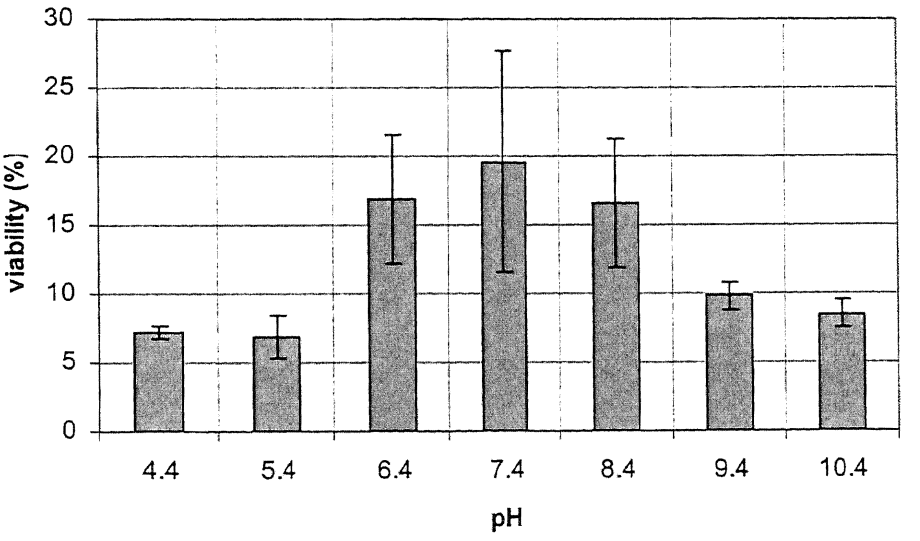


Fig. 6.10. Effect of pH of freezing medium on TB-assessed quality of cryopreserved stage III zebrafish oocytes. N = 36.

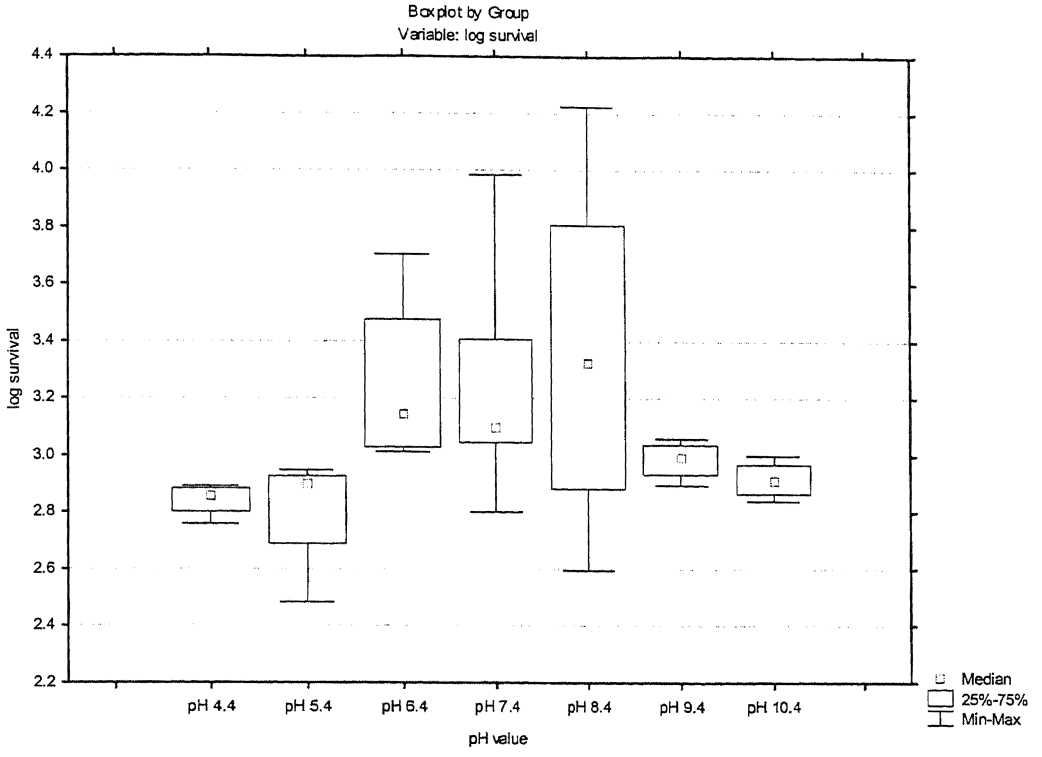


Fig. 6.11. Box-and whiskers graph of logarithmic forms of TB-assessed viabilities of zebrafish oocytes, frozen to -196°C in KCl buffer with different pH values. Whiskers represent minimum and maximum values of the sample, boxes – a range between 25th and 75th percentiles, a square marks – median of the given sample.

6.3 Discussion

6.3.1. Low-sodium medium

As the results of toxicity trial of KCl medium suggest, although KCl buffer has statistically significant damaging effects on zebrafish oocytes (Fig. 6.1), the best results after cryopreservation were obtained with oocytes in KCl based solutions. As with cryoprotectants, the toxic effect of KCl buffer may well be compensated by its beneficial effect during slow cooling. Toxicity studies of KCl buffer have also shown, that change of composition of the incubation medium by itself leads to the loss of viability of the cells (Fig. 6.1); therefore, it is better to use the same medium all the way through experiment. For instance, if KCl buffer is used as the freezing medium, separation of oocytes and disaggregation of cumulus should also be carried out in KCl buffer. These results are in agreement with the theory of osmotic stress, and

with the view that the cells should not be exposed to changing conditions of the incubation media for too many times (Belous & Grischenko, 1994).

The results with freezing of zebrafish oocytes using KCl medium have shown, that KCl medium has advantages in cryopreservation, comparing to Hank's medium. In a number of cases, it allowed to achieve significantly higher TB-assessed cryosurvival of oocytes (Fig. 6.4). However, according to GVBD results, KCl medium resulted in slightly poorer results than those obtained with Hank's (Fig. 6.5), although this difference was under statistically significant level. Possible reason for this might be that membrane-protective action of KCl buffer (indicated by TB results) is counterbalanced by its deteriorating effect on the cytoplasm of the cells (which is reflected by GVBD results). The reason for mild damaging effect of KCl buffer on oocytes may lie in reduction of membrane potential (Maeno, 1959) caused by increased concentration of potassium ions in this medium. Also, increased concentration of K^+ ions in extracellular solution may have disbalancing effect on mechanisms of cell volume regulation (Volkl & Lang, 1990). Apparently, certain amount of Na^+ ions in extracellular medium is necessary for supporting the normal cell functions. Therefore KCl buffer should be used cautiously as a medium for cryopreservation of zebrafish oocytes. The solution for the mild deteriorating effect of KCl buffer on the oocytes may be in the use of mixtures of KCl buffer and Hank's medium.

6.3.2. Effect of cryoprotective supplements

In the experiments on freezing oocytes to liquid nitrogen temperature and with 4M Methanol as the base cryoprotectant, the best cryosurvivals were obtained with 0.2M Glucose and 0.2M Glucose + 5% PEG-200 (Fig. 6.6). These results demonstrate that the addition of low concentrations of penetrating sugars (0.1-0.2M) to the freezing medium has a significant positive effect on oocytes cryosurvival. Glucose acts mainly as an intracellular membrane stabiliser, and also decreases amount of unbound water within the cell (Anchordoguy et al., 1987, Suzuki et al., 1996). Whilst PEG-400 had little effect on cryosurvival of oocytes, PEG-200 was shown to be more effective. The differences might be explained by different

permeability of oocyte plasma membrane to these substances: PEG-200 which has smaller molecular size, presumably penetrates membranes better, than heavier PEG-400 (Ghandehari et al., 1997).

The lack of any positive effect of various tested antioxidants (BHA, N-Acetyl-Cysteine, tocopherol, taurine) leads to the conclusion that the main damage to the oocytes under freezing conditions is not the oxidative stress. Oxidative stress may well contribute to the cryoinjury of fish oocytes, but it is not the crucial factor as the present study showed. This assumption is in agreement with results obtained on human sperm (Alvarez & Storey, 1993). Similarly, membrane-protectors and membrane-stabilisers, such as PEG-400, poloxamer-188, dexamethasone, egg yolk suspension and acetyl salicylic acid did not have pronounced beneficial effects on post-thaw viability of oocytes, which suggests, action of these additives is either insufficient, or the mechanisms of membrane cryodamage of zebrafish eggs are affecting different targets in the membrane than protective action of listed supplements covers.

6.3.3. Effect of pH of freezing medium

The experiments showed that the shift of pH of the freezing medium to above or below physiological values did not have beneficial effect on cryosurvival of stage III zebrafish oocytes and on stability of oocyte plasmatic membranes. At contrary, it has led to significant deterioration of oocytes quality (TB test). The presumption was that shift of pH to alkaline values would lead to plastification of plasmatic membranes (Belous & Grischenko, 1994), which in turn, would result in higher cryostability of membranes (Graham et al., 2005) and therefore, in better cryosurvival of the cells. However, as present results suggest, the normal physiological pH value of 7.4 appears to be the optimal pH value for cryopreservation of zebrafish oocytes. In previous studies on zebrafish embryos, although no special studies on pH impact were carried out, all cryoprotective media were prepared on base of "embryo medium" – a slightly modified Hank's solution with close-to-neutral pH value: 7.2 (Zhang et al., 1993a). Based on obtained results (Fig. 6.10), it

can be stated that physiological, close-to-neutral pH values of freezing medium are optimal for cryopreservation of zebrafish oocytes and should be used in all further experiments

6.3.4 Effect of ice-seeding temperature

The main reason for these experiments was the assumption that -12.5°C seeding temperature (normally used in our experiments) was too low and hence allowed IIF to occur. This was because in a number of cases, when the straws were taken out of cooling chamber for manual seeding at -12.5°C ice initiation had already occurred in some of the straws. It was therefore decided to test higher seeding temperatures at -10°C and -8.5°C, as well as no seeding at all.

The experiments showed that the highest mean TB-assessed survivals were observed at seeding temperatures -10.5°C (17.6±3.7%) and -12.5°C (16.3±4.2%). The less favorable conditions were seeding at -8.5°C (9.4±2.1%) and no seeding at all (8.1±2.8%). However, the differences between the effect of all these conditions were not statistically significant. These results are consistent with the results obtained by Tervit et al. (2005), who investigated controlled slow cooling of Pacific oyster (*Crassostrea gigas)* eggs. It was found that for oyster eggs the optimal seeding temperatures for 10% v/v (~2M) EG and 15% v/v (~2M) DMSO were -10°C and -12°C respectively. Higher seeding temperatures (-8°C, -6°C) and 'no seeding at all' were disadvantageous conditions. The possible explanation of these results is given as following: as mentioned in Section 6.1.4, the freezing point of a solution, containing 4M Methanol + 0.2M Glucose in KCl medium is -8.37°C. Evidently, seeding temperature -8.5°C doesn't allow a certain degree of a supercooling (2-3°C below freezing point) which is needed for the seeding to be effective. When samples were not seeded, the cryoprotective solution and intracellular medium would be significantly supercooled before ice nucleation, which lead to IIF and massive loss in viability of cells. Based on results obtained from these experiments, the seeding temperature of -12.5°C was continued to be used in all further experiments.

6.3.5. Effect of liquid nitrogen plunge temperature

Plunge temperature of -40°C provided highest mean oocytes cryosurvival among all plunge temperatures tested, whilst plunge temperatures below -50°C provided the lowest oocytes survival. The reason for this may be that the low plunge temperatures resulted in exposure of cells to concentrated salt and cryoprotectant solutions for extended periods of time, and therefore, over dehydratation. The results obtained from these experiments are consistent with the results obtained by Rall et al. (1984), who found that methanol loses its effectiveness as a cryoprotectant for slow cooling at temperatures below -45°C, and therefore, plunge to the LN should be done above this temperature. They also indicated that the loss of methanol's properties as a CPA below -45°C when methanol was overconcentrated was due to the crystallisation of ice. On the other hand, if plunge temperature is too high, it doesn't allow sufficient degree of cell dehydration. Our results are also consistent with the results of other researchers, who reported preferred moderate sub-zero plunge temperatures for cryopreservation of mammalian oocytes and embryos (Czlonkowska et al., 1991; Stachecki and Willadsen, 2000). Although there were no statistically significant differences between any plunge temperatures tested in the present experiment, based on the average survival, plunge temperature -40°C was considered the optimal for stage III zebrafish oocytes, and was used in all further experiments.

6.4 Summary

Although KCl buffer had moderate toxic effect on oocytes, the experiments with freezing of oocytes to LN temperature showed that KCl buffer was the best freezing medium for these cells, giving highest TB-assessed cryosurvival values. The problem of a mild deteriorating effect of KCl buffer on oocytes may be solved by using the mixtures of KCl buffer with Hank's medium.

In experiments with freezing of oocytes to the temperature of liquid nitrogen and with 4M Methanol as a basic cryoprotectant, the highest average cryosurvival values were reached with such supplements as 0.2M Glucose

and 0.2M Glucose + 5% PEG-200 (16.3±4.2% and 16.3±4.3% respectively). Obtained results indicate, that addition of small concentrations of glucose (0.1-0.2M) and/or of PEG-200 (5%) to a freezing medium had a well-established positive effect on the success of cryopreservation.

The preferable seeding temperatures for cryopreservation of zebrafish oocytes in 4M methanol + 0.2M glucose were -10.5°C (17.6±3.7% experimental viability) and -12.5°C (16.3±4.2% experimental viability). Based on this, the seeding at temperature -12.5°C was continued to be practiced in all further experiments. Plunge temperature -40°C was found to be the optimal among all plunge temperatures tested, and the physiological pH value 7.4 appears to be the optimal pH value of the freezing medium for cryopreservation of zebrafish oocytes.

From the obtained data, the optimal protocol for cryopreservation of stage III zebrafish oocytes is: 30 min incubation of stage III zebrafish oocytes in freezing medium containing 4M Methanol + 0.2M Glucose in KCl buffer (pH 7.4) at room temperature before loading into 0.5 ml plastic straws, they should then be frozen as follows: cooling from 20°C to -12.5°C at 2°C/min; manual seeding at -12.5°C and hold for 5 min; slow freezing from -12.5°C to -40°C at 0.3°C/min; samples should then be plunged into LN (-196°C). Fast thawing (~200°C/min) in a 27°C water bath is recommended, and double washing in cryoprotectant-free KCl-buffer. Oocytes should then be transferred to Hank's solution or to another incubation medium. However, even with this optimal protocol, TB-assessed survival of oocytes doesn't exceed 20%. Furthermore, GVBD experiments showed that none of the cryopreserved at LN oocytes can be maturated *in vitro*, indicating that successful cryopreservation of fish oocytes at -196°C still remains elusive.

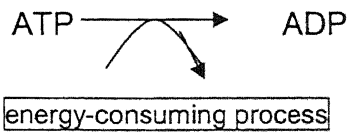
CHAPTER 7. FURTHER ASSESSMENTS OF QUALITY OF ZEBRAFISH OOCYTES AFTER CRYOPRESERVATION USING CONTROLLED SLOW COOLING

7.1. Introduction

7.1.1. ATP test

7.1.1.1. Assessment of ATP content in cells

Adenosine 5'-triphosphate (ATP) is the main energy carrier inside the cells of all animal species. It mediates the transfer of energy from nutritional substances (such as sugars and lipids) to numerous energy-consuming processes, such as the synthesis of molecules which constitute the cell, chemical reactions necessary for cell, transfer of ions and molecules across the membranes, creation of chemical gradients, etc. High ATP content is required for progression of immature oocytes through maturation stage, as meiosis and a number of associated processes (cytoskeleton rearrangement, formation of meiotic spindle, motion of chromosomes, etc.) consume large amounts of energy. As adenosine 5'-triphosphate discharges, the macroergic bond hydrolyses, and inorganic phosphate is produced:



(7.1)

Adenosine 5'-diphosphate (or ADP), can be reused for synthesis of ATP during glycolysis, cell respiration, lipid oxidation and a number of other catabolic processes. But if the vital energy systems of the cell (e.g. mitochondria, their membrane and enzyme components, enzymes of glycolysis and Krebs cycle and β-oxidases of fatty acids) have been damaged, this may not happen, and ADP degrades further to adenosine 5'-monophosphate (AMP) and other substances (Lehninger, 1982).

7.1.1.2. Cryopreservation and cellular ATP content

Mitochondria of the cells are very vulnerable to low temperature injury and to the impact of pH shifts and solution effects, as mitochondrial membranes contain low level of cholesterol (Walder, 1970; Belous & Grischenko, 1994). Low-temperature induced poration of the inner mitochondrial membrane leads to swelling of mitochondria, pathological uncoupling of oxidative phosphorylation and major failure of cell ATP synthesis. Glycolytic enzymes, enzymes of Krebs cycle and enzymes of pentose-phosphate pathway are less vulnerable to the effect of low temperatures, but can also be impaired by freezing injury. Kopeika et al., (1997) reported a significant fall of ATP level, as well as other macroergic substances, in cryopreserved sperm of common carp (*Cyprinus carpio*) comparing to the fresh controls. Researchers tend to link ATP decrease in fish sperm to cell membrane damage resulted from lipid phase transitions during freezing.

Depletion of cellular ATP in cells induces disruption of microfilaments, as well as cessation of many other vitally important processes, and therefore impairs the normal course of oocyte development. ATP depletion in oocytes can prevent the normal assembly of cytoskeleton and spindle contractile proteins and compromise blastomere cleavage and differentiation in future embryogenesis. The mortality of embryos obtained from fertilisation of ATP-starved eggs was reported to be significantly higher (80-90% comparing to 10-20%) than of those obtained from ATP-rich oocytes (Boulekbache et al., 1989). Hence, the presence of ATP inside the oocytes is essential for their survival, normal developmental and reproductive potential. ATP content of the cells is a useful parameter for evaluation of viability of the sample.

7.1.1.3. Enhancement of ATP synthesis in the cryopreserved cells

A new method for stimulating ATP synthesis in freeze-thawed zebrafish oocytes was developed in the present study. In order to test ATP synthesising activity of cryopreserved cells, the freeze-thawed oocytes are incubated in a specially designed medium. The medium uses Hank's solution as a base and contains glucose, ADP (adenosine 5'- diphosphate) and adenosine (see Chapter 2, Section 2.7.2.4). The purpose of glucose was to provide oocytes

with extra-source of nutrition for synthesis of ATP. Adenosine and ADP were added in order to provide the cells with substrates for ATP synthesis. This is especially important if plasmalemma was damaged during freeze-thawing and cells partially lost their stock of adenine nucleotides. In addition, the presence of elevated levels of ADP is known to enhance ATP synthesis via the mechanisms of enzyme regulation.

Elevated levels of ATP in cryopreserved oocytes incubated this way, compared to those incubated in normal cell medium, would indicate that cryopreserved cells had certain unaffected elements in the energy system; and if there are no elevated ATP levels in the "re-vived" cells, it would indicate the irreparable damage to mitochondria and the death of the cells.

7.1.2. Cryomicroscopic observations of zebrafish oocytes

Cryomicroscopy is a powerful method allowing visual observation of biological materials during the course of freezing. In majority of the cases, cryomicroscopy is used by researchers to determine: temperatures of extracellular and intracellular ice formation, forms of ice crystals, patterns of spreading of the ice in the cells, the effect of cryopreservation on morphological structures of the cell, etc. Determinations of these features are of great importance for cryobiologist, because the temperature and character of intracellular ice formation and the type of ice forming would have direct impact on cryosurvival of the cells.

7.1.2.1. Extracellular and intracellular ice formation (EIF and IIF)

Ice nucleation may occur at temperatures below so-called freezing point, which is determined by Raoult's law, The equation for temperature of crystallisation, i.e. freezing point, is shown below :

$$T_{cryst} = -K^* N , \qquad (7.2)$$

where T_{cryst} is crystallisation temperature; K is cryoscopic constant of water (K = 1.86°C/mol) and N is the osmolarity of solution (Keune et al., 1975).

From this equation a very important conclusion can be drawn, that the freezing (crystallisation) temperatures of water solutions do not depend on the nature of dissolved substance, but is determined by the molar quantity of this substance in the solution and by dissociation constant of this substance. Hence, for every mole of non-dissociating solute dissolved in 1 litre of pure water, the freezing temperature will be depressed by 1.86°C.

In some cases nucleation and ice formation occur straight away at the freezing point; but more often phenomenon of supercooling takes place. Supercooling happens when a liquid is cooled below its freezing point without freezing. Supercooled state normally is promoted either by high viscosity of the solution (which makes difficult for ice crystals to form and expand), or by absence of centres of crystallisation in the solution. The supercooled state of a liquid is not stable (metastable) and any kind of disturbance of physical conditions (vibration, fluctuation of temperature, emergence of ice crystals or impurities) may cause nucleation of ice and crystallisation of solution. Any heterogenities in the solution may become the centres of crystallisation: ice crystals, solid particles, cells or debris of cells, air bubbles and even the molecules of certain substances (e.g., silver iodide). The presence of such impurities in the supercooled solution initiates breakage of meta-stable state and solution freezes: ice nucleates and grows. Such process is called heterogeneous ice nucleation. But even in the case that heterogeneous nucleators are absent in the solution, when supercooling reaches critical level, homogeneous ice nucleation occurs.

There is an experimentally obtained formula, presented by Rasmussen and MacKenzie (1972) for temperature of homogeneous nucleation (T_n) :

$$T_n = -36 - (2 * \text{total}\Delta T_{fr}). \qquad (7.3)$$

-36°C is the temperature of homogeneous nucleation of water droplet with diameter of 47 μm; totalΔT_{fr} is depression of freezing point provided by all cytoplasmic solutes and cryoprotective additives.

However, for objects larger than 47 μm, the temperature of homogeneous nucleation is expected to be much higher than calculated by the given formula. For pure water maximum temperature of supercooling is -36°C. Addition of extra-high concentrations of CPAs to solution and application of high cooling rates (~1500°C/min) allows prevention of crystallisation in solution even at -196°C for unlimited periods of time, resulting in preservation in a "glass-like" state. This process is known as vitrification and is being widely used in the modern cryobiological practice. Vitrification allows the detrimental effects associated with ice formation inside the biological samples to be avoided (Muldrew & McGann, 1997). However, in most of the cases, ice nucleation in the liquid does occur. The germs of ice crystals appear in solution and new water molecules join their crystal lattice, leading to expansion of the ice crystals. In majority of the cases ice initiation occurs in the extracellular medium, but later ice crystals are likely to appear inside the cells as well. This phenomenon is called intracellular ice formation (IIF). Several theories have been proposed for mechanisms of IIF (Mazur, 1984; Toner et al., 1990; Muldrew & McGann, 1990; Rall et al., 1983; Acker et al., 2001; Karlsson et al., 1996). We have reasons to believe that under different cooling conditions the initiation of IIF can occur in different ways.

The main mechanisms offered for initiation of IIF are:

a) freezing of intracellular solution occurs through heterogeneous nucleation by intracellular agents
b) freezing of intracellular solution occurs through homogeneous nucleation due to critical supercooling of cytoplasm: volume-catalysed nucleation (VCN) (Muldrew & McGann, 1990)
c) initiation of IIF occurs through heterogeneous nucleation by extracellular ice crystals, which can grow through water pores in membrane and "seed" the cytoplasm (Acker et al., 2001)
d) initiation of IIF occurs through heterogeneous nucleation by extracellular ice crystals, which can contact cytoplasm through damaged plasmatic membrane (electrically damaged by charged ice crystals, osmotically damaged by hyperconcentrated salt solutions or

mechanically punctured by extracellular crystals of hexagonal ice). Also, external ice crystals can alter the formation of cell plasmatic membrane resulting in its inner surface becoming an effective ice nucleator, inducing IIF (Toner et al., 1990). All these mechanisms are named as surface-catalyzed nucleation (SCN)

We can now reject the hypothesis **a** with confidence, as it has been proved that the cytoplasm of vast majority of cell types doesn't contain any effective nucleators (Rall et al., 1983). Hypothesis **c** seems somewhat doubtful for a number of authors as there are discrepancies in experimental results (Muldrew & McGann, 1990). Therefore, two well-proven models for initiation of IIF, **b** and **d**, remain. With low concentrations of penetrating cryoprotectants, the initiation of IIF occurs through heterogeneous nucleation by external ice - surface-catalysed nucleation (SCN). With high concentrations of penetrating cryoprotectants in the medium and inside the cells, the initiation of IIF presumably occurs by homogeneous, volume-catalysed nucleation (VCN), as cryoprotective agents have the property to prevent heterogeneous nucleation by external ice (Rall et al., 1983). Also, VCN is believed to occur at low temperatures (below -34°C), whilst SCN occurs mainly at temperatures above this point (Karlsson et al., 1996). According to Mazur's assumption, for IIF to occur, three conditions should be met: 1) temperature should be below freezing point, at ice-nucleation zone; 2) intracellular water content should be >10% of its isotonic value; and 3) intracellular solution should be at least 2°C supercooled. The probability of IIF increases exponentially with the increase of degree of supercooling (Mazur, 1984). Ideal cryopreservation protocol, which excludes IIF and results crystal-free solidification of cytoplasm during LN plunge, should be designed in a way to avoid coincidence of these three conditions. A protocol is considered successful if it allows IIF in 5% or less of all the cells in the sample (Mazur, 1984).

7.1.2.2. Forms of ice

The water ice has about 9 structural types, which differ by density, structure of crystallic grid and shape of crystals. The most widespread type of ice, which forms at cryobiologically relevant conditions is type I hexagonal ice (Ih). Every oxygen atom in this form of ice is situated at the center of a tetrahedron formed by four oxygen atoms. The distance between oxygen atoms is 2.7å. The important aspect of the structure of Ih ice is the presence of vacant "pipes", or pencil-like liquid-filled areas within the crystals. Hexagonal ice is dangerous to cells due its relatively large and sharp-shape crystals. Moreover, Ih ice is prone to recrystallisation and formation of even larger crystals at temperature range as low as -50 to -150°C. So-called small-granular ice types, which include cubical ice (Ic), cellated ice, and so-called disappearing spherulites, are less dangerous for cells. Cubical ice (Ic) forms from ice type II at -107°C. Small-granular forms of ice are strongly promoted by presence of cryoprotectants in solution and are formed from dendritic type of ice during cooling (Belous & Grischenko, 1994; Muldrew & McGann, 1997).

The surface of ice crystal is surrounded by a quasi-liquid electrically active layer of water molecules and other ions (H^+, OH^-, Na^+, Cl^-), driven out to unfrozen solution by growing ice crystal. This phenomenon is called "Workman-Reynolds effect". The magnitude of the electrical potential difference between the ice phase and liquid phase at the interface is proportional to the velocity of ice formation. The electrically-charged "film" surrounding the ice crystal is of great importance for growth, motility of ice crystals and their electrical properties. At temperature range -40°C to -80°C quasi-liquid layer disappears, but at lower temperatures it appears again and can be registered at temperatures as low as -130°C.

7.1.2.3. Damages of cells by ice formation

Ice crystals, especially when they are formed inside the cells (intracellular ice formation, IIF), cause dramatic damage to cell structures: mechanical puncture of cell membrane organoids by ice crystals and disarrangement of cytoskeleton with lethal consequences. Mechanical

squeezing and squashing of the cells between growing ice fronts may also occur. In addition electric poration and disarrangement of membranes by ice crystals may occur, as ice crystals are electrically charged and may carry strong electromagnetic fields around them. Although most of cells during the course of freezing are pushed to solution-filled "tortuous channels" by growing ice front, some of them may get trapped into isolated cavities inside the ice phase. Pressure in such ice cavities may reach up to 10^8 Pa. Such hyperbaric conditions also damage the cells. Gas bubbles are also likely to form in such cells after thawing (Gordienko & Pushkar, 1994).

Despite all these factors, many cell types can survive formation of intracellular ice if small-granular "cubical" '1c' ice forms and the sizes of ice crystals do not exceed so-called "critical radius", which is cell type dependent. The *total* amount of intracellular ice formed is no less crucial parameter for cell survival than the size of crystals. It is important to note that during thawing intracellular small-granular ice may recrystallise to large hexagonal ice crystals resulting in cell death. Therefore careful control of thawing process in order to prevent this unwished phenomenon is just as important as the control of freezing process.

The main objectives of cryomicroscopic studies on stage III zebrafish oocytes are:

- to study how "optimal" cryopreservation protocol affects the processes of ice formation
- to study the patterns of ice formation and types of forming ice
- to determine temperatures of extracellular and intracellular ice formation, both by SCN and VCN mechanism
- to determine whether IIF coincides with EIF
- to study morphological changes in cytoplasm after cycle of freeze-thawing

7.2. Results

7.2.1. ATP content in intact and cryopreserved stage III oocytes

7.2.1.1. Effect of cryopreservation on ATP content in stage III zebrafish oocytes

Figure 7.1 shows the calibration curve that was established using standard ATP solutions of known concentrations. This calibration curve provides correlations between the amount of luminescence produced and the concentration of ATP in the sample. Therefore it can be used to determine the amount of ATP in the samples knowing produced by them amount of luminescence. Once the concentration of ATP in the sample is known, the amount of ATP in a single oocyte can be calculated, as each sample contains fixed amount of oocytes (30 oocytes in 1.15 mL of solution). The average amount of ATP in a single oocyte in a sample can be calculated by the following formula:

Amount of ATP in a single oocyte [Mol] =
Concentration of ATP in the sample [M, or Mol/L] * **1.15*10^{-3}** [L] / **30** [cells]

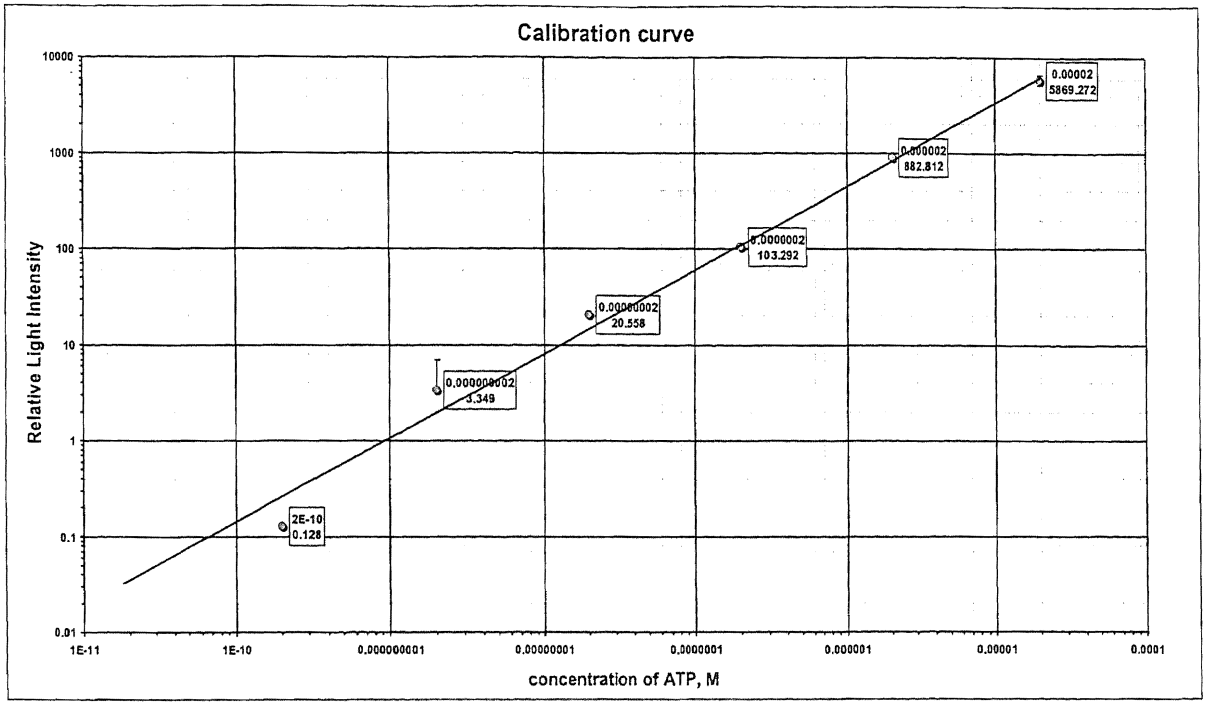


Fig.7.1. Calibration curve for 25-fold dilution of FL-AAM mix. ATP concentrations range – $1*10^{-10}$ M to $1*10^{-5}$ M.

The abbreviations which are used in the graphs for this section are explained below (this list can also be found in Chapter 2, Materials and Methods, Sec. 2.8.3):

1. Intact oocytes freshly obtained (coded: RT).

2. Intact oocytes after 1 hour incubation in Hank's medium at room temperature (coded: RT1H).

3. Oocytes in Hank's medium were loaded to straws and frozen to -196°C by direct plunge into liquid nitrogen (LN). They were kept in LN for 5-10 min; then thawed by immersion into 27°C waterbath for 20 sec. The oocytes were kept for 1 hour in Hank's at 22°C (coded: PLU1H).

4. Oocytes were cryopreserved following the optimal cryopreservation protocol: 30 min incubation in 0.2M Glucose + 4M Methanol solution in KCl-buffer (pH 7.4); loaded into 0.5 ml straws; cooling from 20°C to -

12.5°C at 2°C/min; manual seeding and hold for 5 min; then slow freezing at 0.3°C/min to -40°C; and samples were then plunged into LN. After 5-10 min in LN, samples are thawed (~200°C/min) in a 27°C water bath for 20 sec. Oocytes were then washed in KCl-buffer twice, kept in KCl buffer at room temperature for 1 hour (coded: OPT1H).

5. Oocytes were frozen following the optimal cryopreservation protocol, but only to -10°C: 30 min incubation in 0.2M Glucose + 4M Methanol solution in KCl-buffer (pH 7.4); cooling from 20°C to -10°C at 2°C/min. Samples were then thawed (~200°C/min) in a 27°C water bath for 20 sec. Oocytes were then washed in KCl-buffer twice and kept in KCl buffer at room temperature for 1 hour (coded: -10,1H).

6. Oocytes were frozen following the optimal cryopreservation protocol, but only to -20°C: 30 min incubation in 0.2M Glucose + 4M Methanol solution in KCl-buffer (pH 7.4); cooling from 20°C to -12.5°C at 2°C/min; manual seeding and held for 5 min; then slow freezing at 0.3°C/min to -20 °C. Samples were then thawed (~200°C/min) in a 27°C water bath for 20 sec. Oocytes were then washed in KCl-buffer twice and kept in KCl buffer at room temperature for 1hour (coded: -20,1H).

7. Oocytes were frozen following the optimal cryopreservation protocol, but only to -30°C: 30 min incubation in 0.2M Glucose + 4M Methanol solution in KCl-buffer (pH 7.4); cooling from 20°C to -12.5°C at 2°C/min; manual seeding and held for 5 min; then slow freezing at 0.3°C/min to -30 °C. Samples were then thawed (~200°C/min) in a 27°C water bath for 20 sec. Oocytes were then washed in KCl-buffer twice and kept in KCl buffer at room temperature for 1 hour (coded: -30,1H).

8. Oocytes were frozen following the optimal cryopreservation protocol, but only to -40°C: 30 min incubation in 0.2M Glucose + 4M Methanol solution in KCl-buffer (pH 7.4); cooling from 20°C to -12.5°C at 2°C/min; manual seeding and held for 5 min; then slow freezing at 0.3°C/min to -40 °C. Samples were then thawed (~200°C/min) in a 27°C water bath for 20 sec.

Oocytes were then washed in KCl-buffer twice and kept in KCl buffer at room temperature for 1 hour (coded: -40,1H).

9. Toxicity of 0.2M Glucose + 4M Methanol solution in KCl-buffer (pH 7.4). Oocytes were incubated in this medium for 30 min; then washed in KCl-buffer twice and kept in KCl buffer at room temperature for 1 hour (coded: TOX1).

One hour post-treatment incubation was used in most of the treatments, because of the following considerations: after the cells had experienced the damaging effect of any treatment (either toxicity of cryoprotectant, or freezing injury), the intracellular ATP level would not normally change immediately. The injuries accumulated in the cells need certain amount of time to manifest themselves. 1 hour incubation of oocytes at room temperature in KCl buffer was used in these experiments. It was presumed that if the energy system of the cells had been seriously damaged during freezing, the ATP hydrolyses and its level would fall.

Figure 7.2 shows the effect of different cryobiological procedures, including cryoprotectant exposure, controlled slow cooling to different final temperatures and direct plunge into the liquid nitrogen, on the ATP content in zebrafish vitellogenic oocytes, compared to the amount of ATP in intact oocytes.

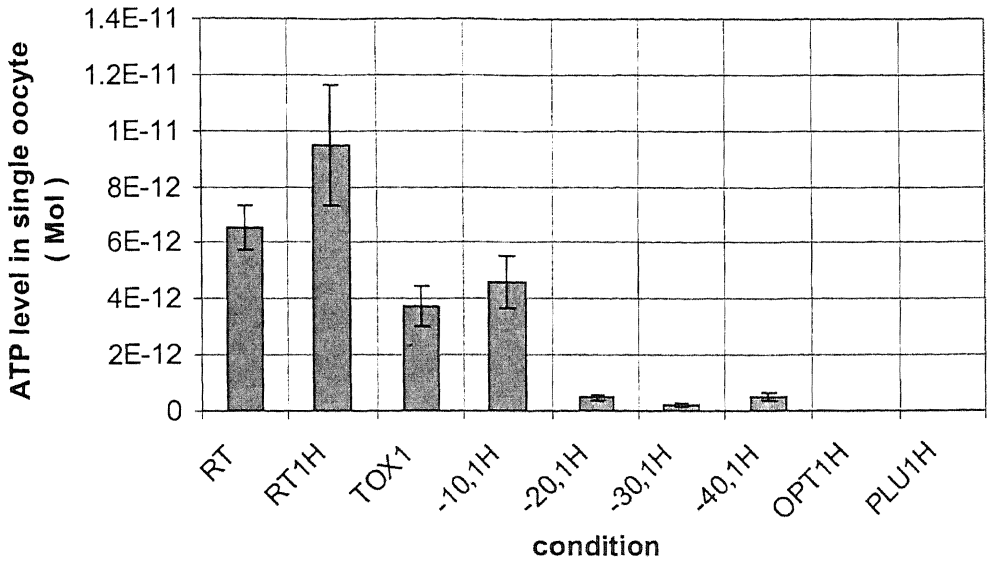


Figure 7.2. ATP levels in stage III zebrafish oocytes after cryoprotectant exposure and freezing. Error bars represent standard errors. N = 36.

As the values of treatments OPT1H and PLU1H are diminishingly low and can not be seen on the previous graph, a separate graph in a smaller scale is built in order to show the differences between the effect of these two treatments:

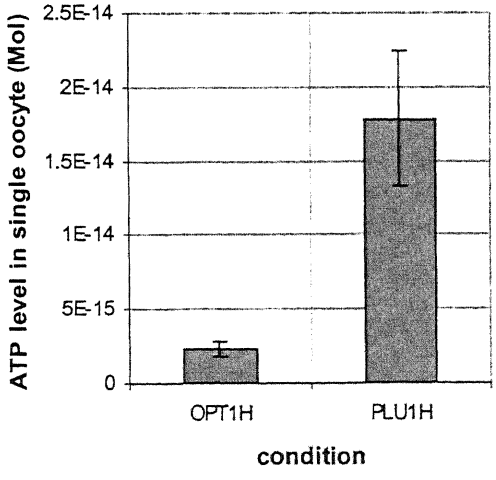


Fig. 7.3. ATP levels in stage III zebrafish oocytes after freezing to temperature of LN by two different methods: following the optimal cryopreservation protocol (OPT1H) and by direct CPA-free plunge into LN (PLU1H). Error bars represent standard errors. N = 8.

Results of statistical analysis (ANOVA and Scheffe's post-hoc test) showed that there are no significant differences of ATP level between the following treatments: intact, "fresh" controls (RT); incubation of freshly separated oocytes for 1 hour at room temperature in Hank's medium (RT1H); 30 min incubation of oocytes in 4M methanol + 0.2M glucose (TOX1); freezing oocytes following the optimal cryopreservation protocol to -10°C (-10,1H). Meanwhile, cooling to lower temperatures led to dramatic fall of ATP content in oocytes, and the ATP content in the samples which were cooled to -20°C (-20,1H), - 30°C (-30,1H), -40°C (-40,1H), -196°C (OPT1H) following the optimal cryopreservation protocol and of those, which were directly plunged into the liquid nitrogen (PLU1H), significantly differs from the ATP content in the intact fresh oocytes. Indeed, the ATP content in oocytes frozen to -40°C (-40,1H) was nearly 20 times lower than in room temperature control (RT1H); and the ATP content in oocytes frozen to -196°C (OPT1H) was nearly four thousand times lower than that in RT control (RT1H) (Fig.7.2).

Student's t-test showed that the ATP content in the oocytes frozen by the optimal protocol (OPT1H : 30 min incubation in 4M Methanol + 0.2M Glucose in KCl buffer (pH 7.4); loading into 0.5 ml plastic straws; fast cooling at 2°C/min to -12.5°C; manual seeding and hold for 5 min; slow cooling at 0.3°C/min to -40°C; plunge to liquid nitrogen (-196°C); fast thawing at approx. 200°C/min) was significantly lower ($P<0.05$) than in the samples frozen by direct plunge into LN without addition of any CPAs (PLU1H).

7.2.1.2. Effect of the "recovering" medium on ATP content in cryopreserved oocytes

The results of experiments, involving the use "reviving medium" are presented in Figure 7.4. The abbreviations which are used in this graph are explained below:

1. Oocytes were frozen following the optimal cryopreservation protocol, but only to -20°C: 30 min incubation in 0.2M Glucose + 4M Methanol solution in KCl-buffer (pH 7.4); cooling from 20°C to -12.5°C at 2°C/min; manual seeding and hold for 5 min; then slow freezing at 0.3°C/min to -20°C. Samples were then thawed (~200°C/min) in a 27°C water bath for 20 sec. Oocytes were then washed in KCl-buffer twice (code: -20,0H).

2. Oocytes were frozen following the optimal cryopreservation protocol, but only to -20°C: 30 min incubation in 0.2M Glucose + 4M Methanol solution in KCl-buffer (pH 7.4); cooling from 20°C to -12.5°C at 2°C/min; manual seeding and hold for 5 min; then slow freezing at 0.3°C/min to -20 °C. Samples were then thawed (~200°C/min) in a 27°C water bath for 20 sec. Oocytes were then washed in KCl-buffer twice and kept in KCl buffer at room temperature for 1 hour (coded: -20,1H).

3. Oocytes were frozen following the optimal cryopreservation protocol, but only to -20°C: 30 min incubation in 0.2M Glucose + 4M Methanol solution in KCl-buffer (pH 7.4); cooling from 20°C to -12.5°C at 2°C/min; manual seeding and hold for 5 min; then slow freezing at 0.3°C/min to -20 °C. Samples were then thawed (~200°C/min) in a 27°C water bath for 20 sec. Oocytes were then washed in KCl-buffer twice and incubated in "recovering medium" (see below) for 1 hour (coded: -20,RE).

The results showed that cooling to -20°C led to dramatic fall of ATP level in oocytes. According to ANOVA and Scheffe's test, all treatments (cooling to -20°C, thawing and measurement of ATP straight after this - -

20,0H ; cooling to -20°C, thawing, 1 hour incubation in KCl buffer, followed by measurement of ATP - **-20,1H**; and cooling to -20°C, thawing, 1 hour incubation in "recovering" medium, followed by measurement of ATP - **-20,RE**) differ significantly from the control (**RT**). No significant differences were present between any of the frozen samples (**-20,0H; -20,1H; -20,RE**) according to Student's t-test. This means that neither 1 hour post-thaw incubation of freeze-thawed oocytes in pure Hank's medium, nor their 1 hour incubation in "recovering" medium had lead to any significant changes in ATP level.

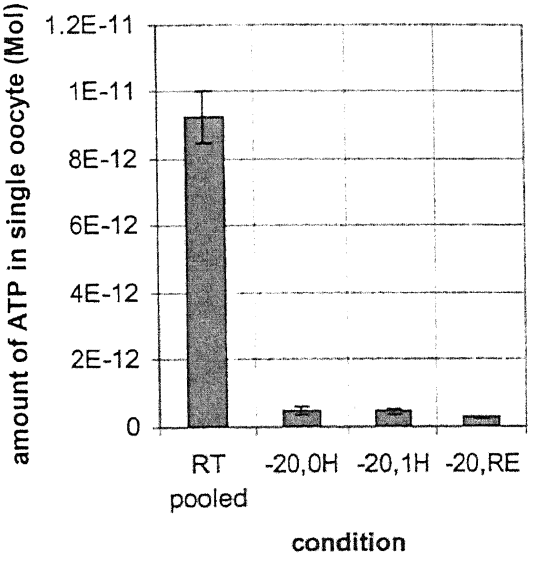


Figure 7.4. Effect of "recovering" medium on the ATP content in the stage III zebrafish oocytes, compared to the ATP content in intact oocytes. Oocytes were frozen to -20°C using controlled slow cooling. Incubation time in "recovering" medium was 1 hour at room temperature. Error bars represent standard errors. N = 28.

7.2.2. Cryomicroscopic observations of stage III zebrafish oocytes

7.2.2.1. Extracellular ice formation (EIF) and intracellular ice formation (IIF)

In order to investigate patterns of extracellular ice formation, zebrafish oocytes were frozen on a cryomicroscope using the "optimal" cryopreservation protocol as follows: 30 min incubation of stage III zebrafish oocytes in KCl buffer (pH 7.4) containing 4M Methanol + 0.2M Glucose at room temperature; controlled slow freezing from 20°C to -12.5°C at 2°C/min; seeding and hold samples at -12.5°C for 5 min; slow freezing from -12.5°C to -40°C at 0.3°C/min; plunge the samples into LN. Fast thawing (130°C/min) to RT (20°C).

In a number of cases, when mentioned specifically, different freezing media were used, for instance: 4M Methanol + 0.2M Glucose in Hank's; 4M Methanol without Glucose in KCl buffer or in Hank's; or KCl buffer. Also, in a number of experiments different cooling rates were used. In some experiments, in order to exclude the initiation of IIF by external ice, the extracellular solution was removed and replaced by silicone oil.

7.2.2.1.1. Temperature and patterns of extracellular ice formation

Cryomicroscope observations showed, that the formation of dendritic extracellular ice took place during freezing (Fig. 7.5). With 4M Methanol + 0.2M Glucose in Hank's as a freezing medium, the temperature of extracellular ice formation (EIF) was identified as -13.5 ± 1.2°C; n=6. Varying cooling rates from 0.3°C/min to 1°C/min did not alter the pattern of EIF.

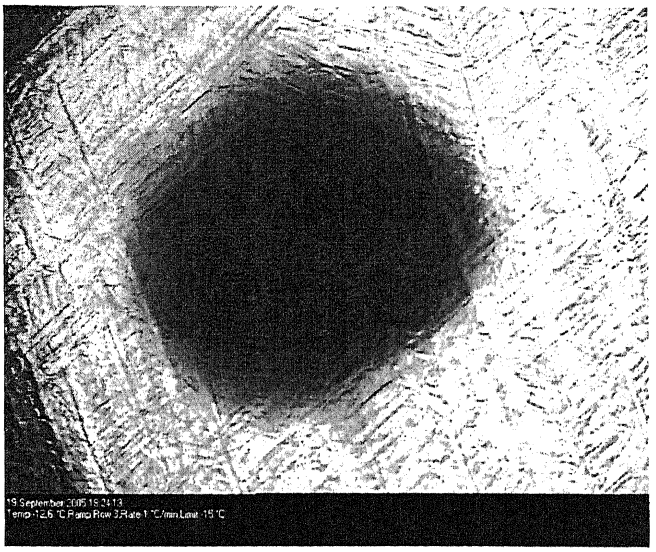


Fig. 7.5. Extracellular ice formation. Cooling rate 1°C/min. Freezing medium 4M Methanol + 0.2M Glucose in Hank's.

7.2.2.1.2. Temperature and patterns of intracellular ice formation

Initially, when a thick layer of solution was used, the events occurring inside the oocyte could not be seen clearly because the thick layer of forming ice obstructed the area of vision. To circumvent this problem, a thin layer of solution was used. The images in Fig.7.6 (taken with 0.3 sec interval) showed that EIF and IIF occur simultaneously at -12.3°C, even before ice seeding procedure at -12.5°C. IIF can be identified because the cytoplasm of the cell becomes noticeably darker (a visual effect which is often referred in cryobiology as "flashing" (Muldrew & McGann, 1997; Muldrew & McGann, 1990). As the experiments showed, with the "optimal" cooling protocol the IIF coincided with EIF in nearly 100% of cases. In cryoprotective medium containing 4M Methanol + 0.2M Glucose in KCl buffer, the average temperature of IIF + EIF is -13.5 ± 1.2°C (n=6).

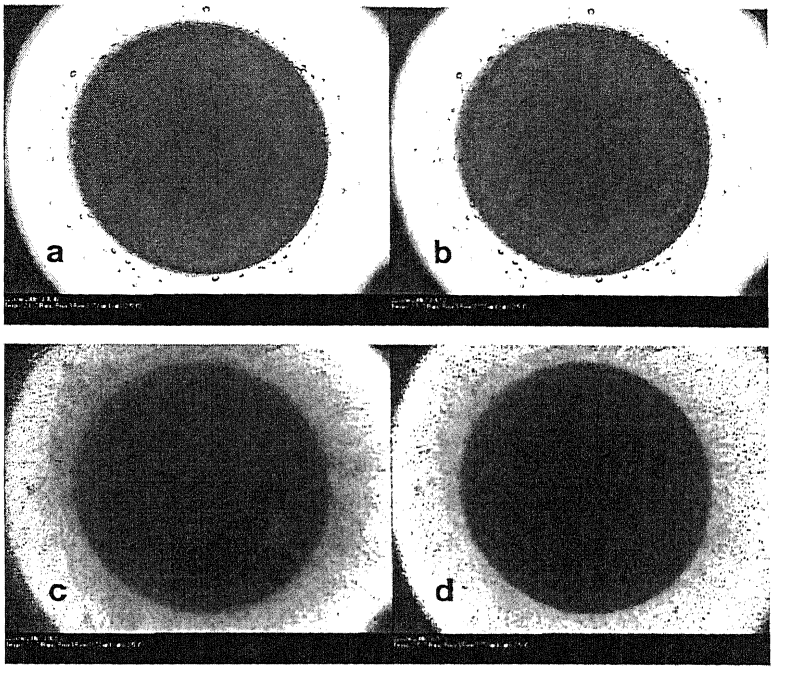


Fig. 7.6. a-d shows the moment of nucleation. Optimal cooling protocol. Cooling rate at the moment of nucleation 2°C/min. 4M Methanol + 0.2M Glucose in KCl buffer as a cryoprotective medium.

7.2.2.1.3 Coincidence of EIF and IIF at different cooling rates

In order to study the effect of the cooling rate on EIF and IIF, several additional experiments with different cooling rates were carried out.
Fig. 7.7. shows the coincidence of EIF and IIF at cooling rate of 1°C/min.

Fig. 7.8. shows the EIF and IIF at cooling rate 2°C/min. In this case, a short delay of IIF was observed (~0.3 sec) after EIF (Fig. 7.8. b and c). Recorded temperature for both EIF and IIF was -6.2°C.

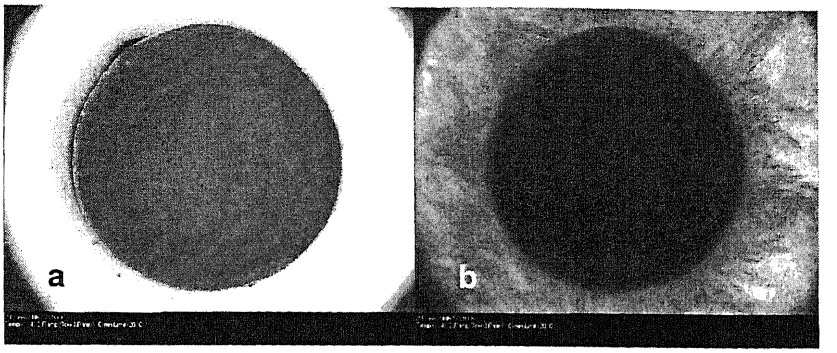


Fig.7.7 a,b. The moment of EIF and IIF. Cooling rate 1°C/min. 4M Methanol + 0.2 Glucose in Hank's was used as the cryoprotective medium. Images were taken with 0.3 sec interval. IIF & EIF occurred at –11.4°C.

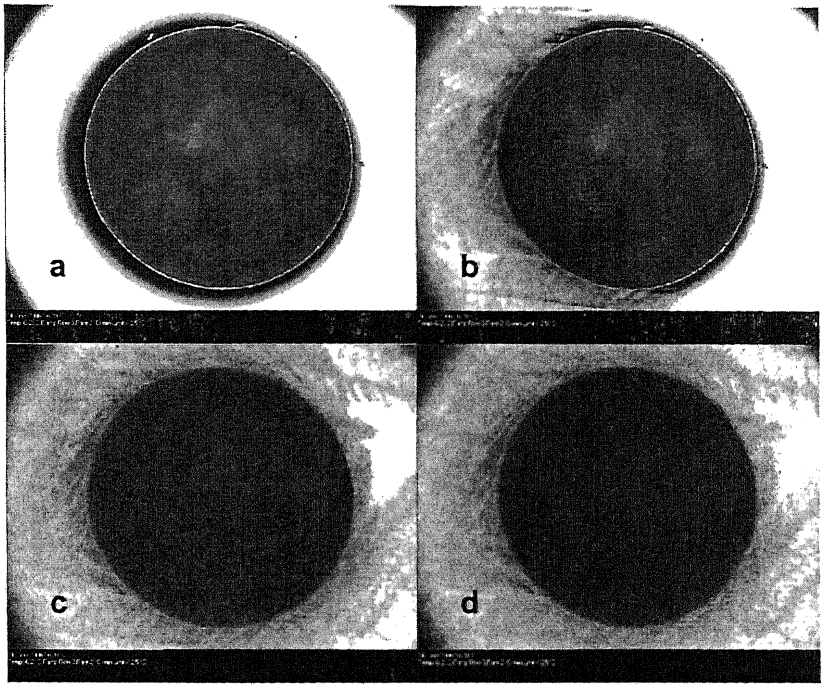
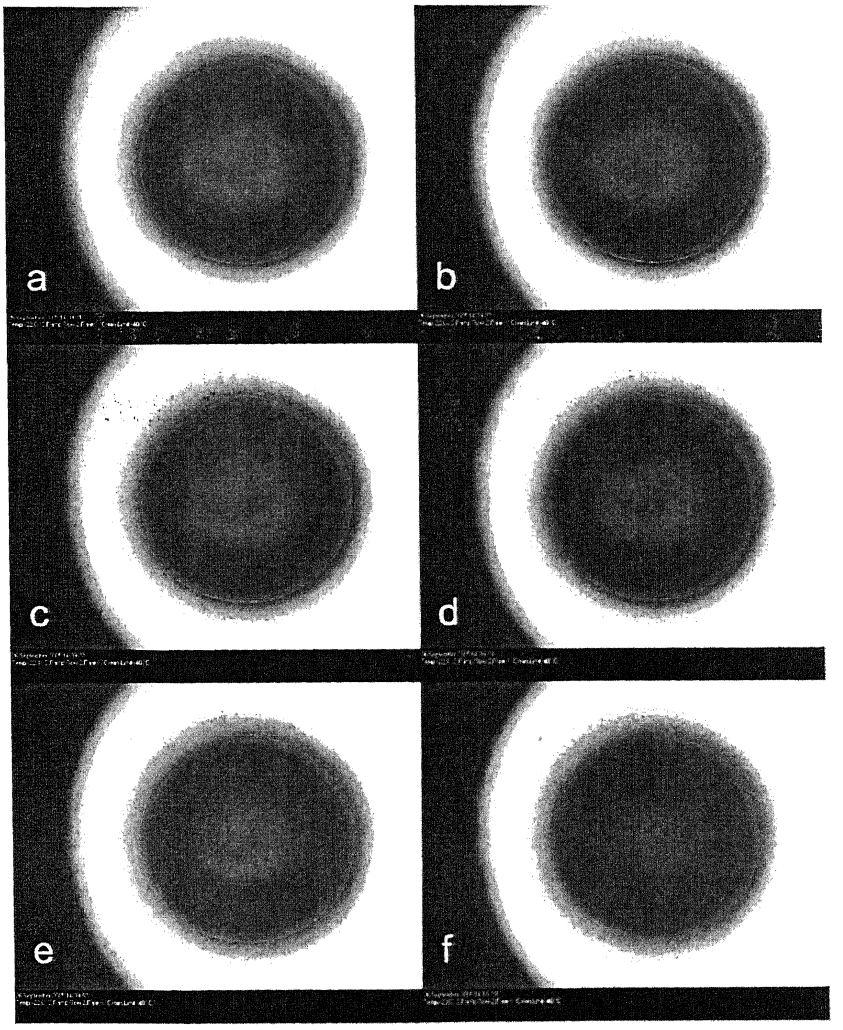


Fig. 7.8 a-d. The moment of EIF and IIF. Cooling rate 2°C/min. 4M Methanol in Hank's was used as the cryoprotective medium. Images were taken with 0.3 sec interval. IIF & EIF occurred at –6.2°C.

Although coincidences of EIF and IIF were observed in most of experiments, it is worth noting that in one case when 4M Methanol in KCl buffer was used with 1°C/min cooling rate, *gradual* IIF (not coinciding with EIF) was observed (Fig. 7.9). In this case EIF occurred at -22.1°C (Fig. 7.9.

c), but didn't initiate IIF immediately. IIF started to occur at -22.6°C (Fig. 7.9. e) and was completed at -24°C (Fig. 7.9. j). The process of IIF in this case was not "flash-like", but gradual. The mechanism of intracellular ice nucleation in this case is SCN (surface-catalysed nucleation), and because it happened some time after EIF, a dehydratation of cytoplasm took place, and the supercooling of cytoplasm was prevented. This would result in formation of relatively safe, small-granular form of intracellular ice according to cryobiological theory (Belous & Grischenko, 1994). Unfortunately, such a desirable event as gradual IIF takes place only in rare cases.



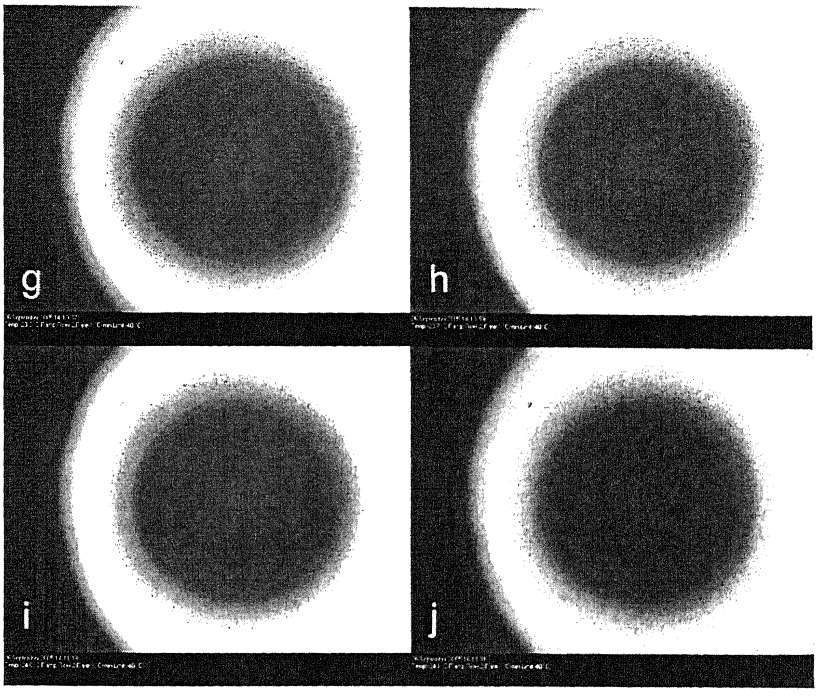


Fig. 7.9 a-j. Gradual IIF. 4M Methanol in KCl buffer and a thin layer of solution was used. Cooling rate was 1°C/min. Images were taken with 0.3 sec interval.

7.2.2.1.4. Temperature of homogeneous intracellular ice nucleation

To determine the temperature of intracellular *homogeneous* nucleation (i.e. no initiation by extracellular ice) the following set of experiments was conducted: after 30 min incubation in cryoprotective medium, the solutions surrounding oocytes were removed and substituted by silicone oil. Oocytes were then frozen with different cooling rates.

Fig. 7.10 shows the images of stage III zebrafish oocytes when frozen following treatment in 4M Methanol + 0.2M Glucose in KCl buffer, at maximum cooling rate (130°C/min), and with extracellular medium substituted by silicone oil. The moment of intracellular nucleation (so called "flashing") can be seen clearly.

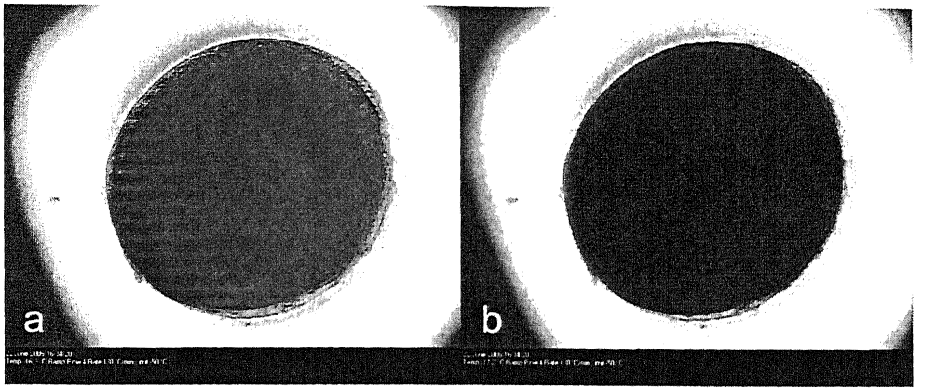


Fig. 7.10 a, b. IIF by homogeneous mechanism (volume-catalysed nucleation, VCN). 4M Methanol + 0.2M Glucose in KCl buffer as a cryoprotective medium. After 30 min incubation, all extracellular medium was substituted by silicone oil. Cooling rate was 130°C/min. Images were taken in 0.3 sec interval. Temperature of IIF was -16.7°C.

Average temperature of homogeneous intracellular nucleation in oocytes immersed in silicone oil and pre-incubated with 4M Methanol + 0.2M Glucose in KCl buffer was -17.4 ± 2.6°C (n = 6).

Fig. 7.11 and Fig. 7.12 also show the moment of IIF ("flashing") in experiments with the extracellular solution replaced by silicone oil. In addition, on these images the diffraction of transmitted light in ice crystal can be observed, as well as the polygonic geometrical structure of the crystals.

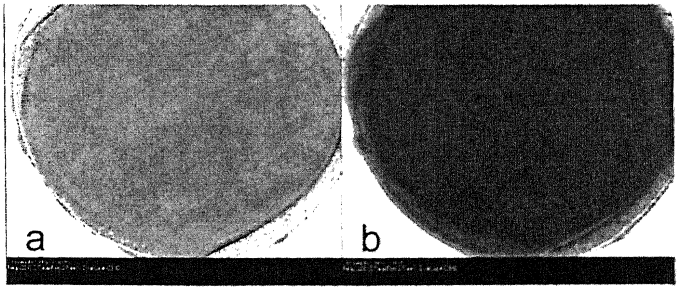


Fig. 7.11 a, b. IIF by homogeneous mechanism (VCN). 4M Meth in KCl buffer was used as cryoprotective medium. After 30 min incubation, all extracellular medium was substituted by silicone oil. Cooling rate was 1°C/min. Images were taken in 0.3 sec interval. Temperature of IIF was -22.8°C.

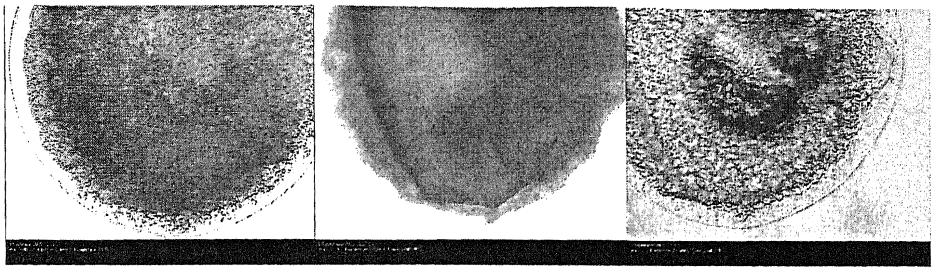


Fig. 7.12 a-c. IIF by homogeneous mechanism (VCN). CPA-free KCl buffer was used as cryoprotective medium. After 30 min incubation, all extracellular medium was substituted by silicone oil. Cooling rate was 1°C/min. Images were taken with 0.3 sec interval. Fig. 7.12c shows the same oocyte after thawing.

7.2.2.2. Other cryomicroscopic observations of stage III oocytes during freezing and thawing

7.2.2.2.1 Damage of cytoplasm after freeze-thawing

Fig. 7.13 shows the oocyte before, during and after cryopreservation cycle. Changes in yolk and cytoplasm after freeze-thawing can be seen: yolk acquires transparent, granular and heterogeneous appearance.

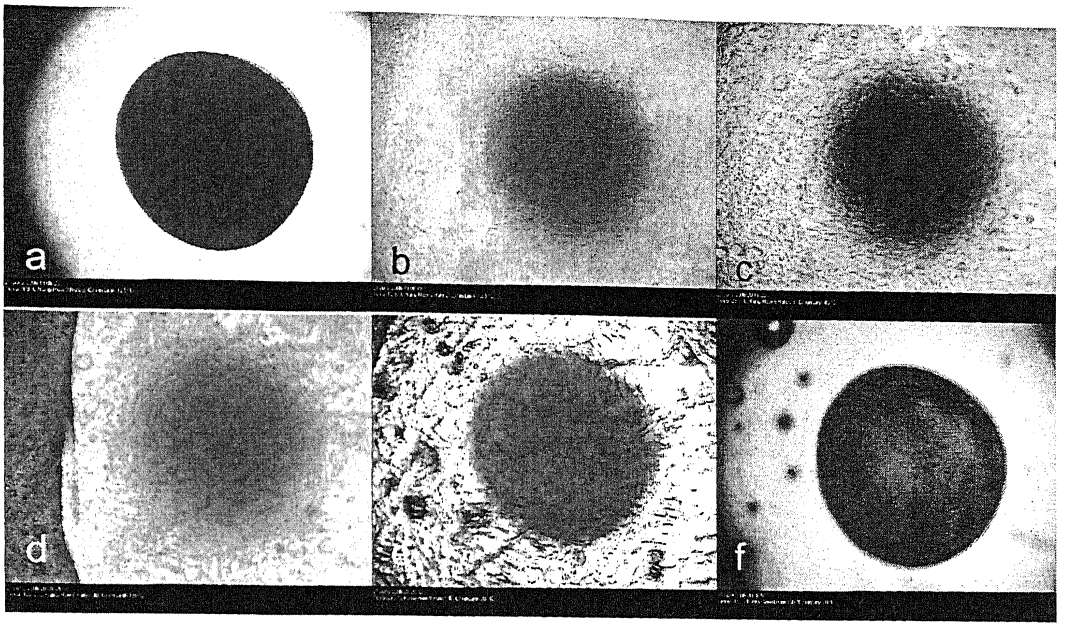


Fig. 7.13 a-f. Different stages of cryopreservation cycle. Cryopreservation medium 4M Methanol + 0.2M Glucose in KCl buffer. Oocytes were frozen using the optimal cryopreservation protocol.

a: oocyte before the nucleation of ice occurred

b: oocyte immediately after the moment of EIF (-10.7°C).
In this case, nucleation occurred before ice-seeding.

c: oocyte during the stage of "dehydratation" slow cooling (-0.3°C/min)

d: oocyte during the "plunge" stage (cooling rate 130°C/min)

e: oocyte during the stage of thawing (warming rate 130°C/min)

f: oocyte after cycle of freeze-thawing.

Fig. 7.14 shows that apart from the changes in the cytoplasm, the radial pattern of melting of intracellular ice can also be observed. The large centrically situated "cavity" in the cytoplasm of oocyte, formed by destruction of structures of the cell by intracellular ice can be observed.

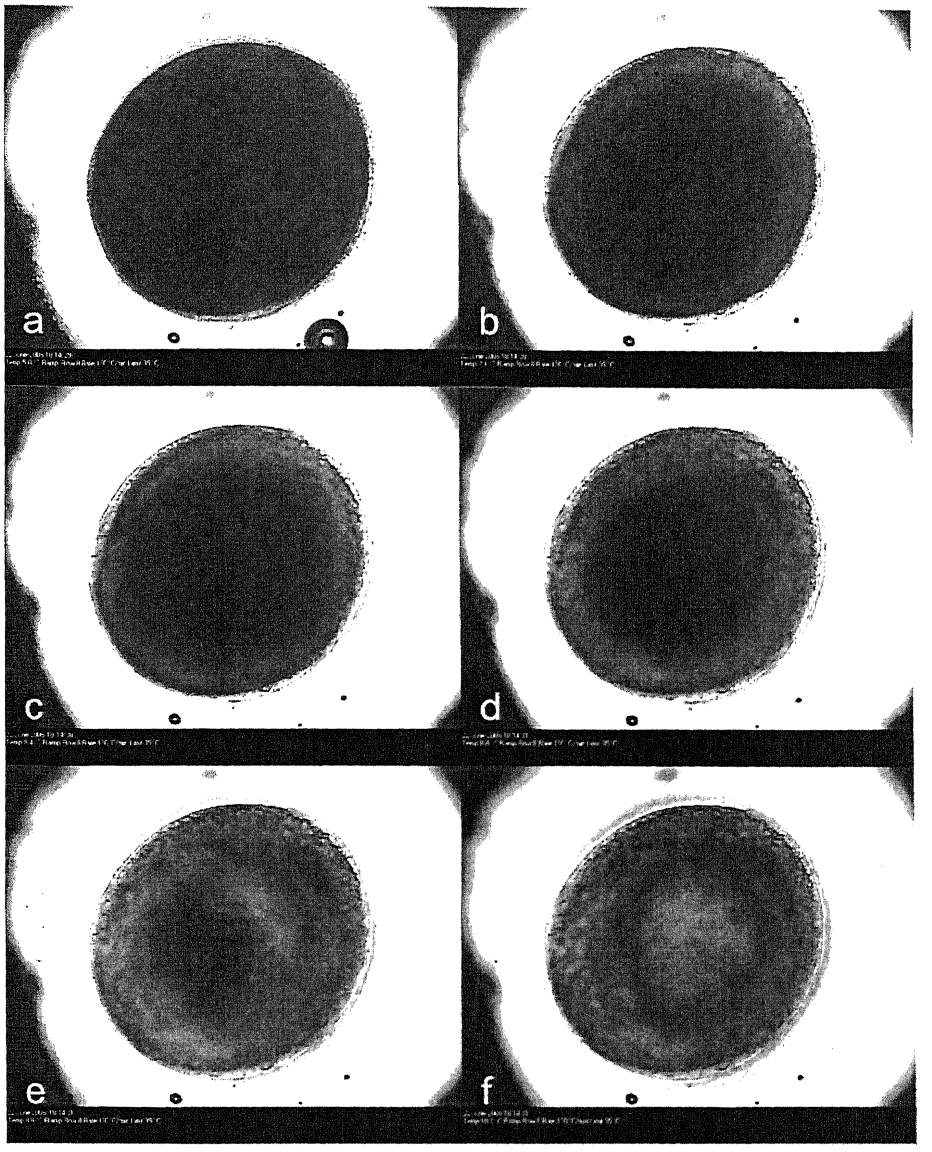


Fig. 7.14, a-f. Oocyte during the stage of thawing (130°C/min). 4M Methanol + 0.2M Glucose in KCl buffer as cryoprotective medium; after 30 min incubation extracellular medium is substituted by silicone oil.

7.2.2.2.2. Cracking of ice during freezing

It is important to point out the occurrence of cracks of ice, which form at temperatures below -100°C. These cracks indicated the presence of substantial mechanical tensions in the frozen phase at low temperatures, which can, alongside with other factors, adversely affect the integrity of the cryopreserved cells.

Images of Fig. 7.15 were taken during the course of freezing using the optimal cryopreservation protocol, with 4M Methanol + 0.2M Glucose in Hank's as a cryoprotective medium. During the fast cooling stage (130°C/min) formation of cracks in the ice mass was observed at temperature range -120°C - -170°C.

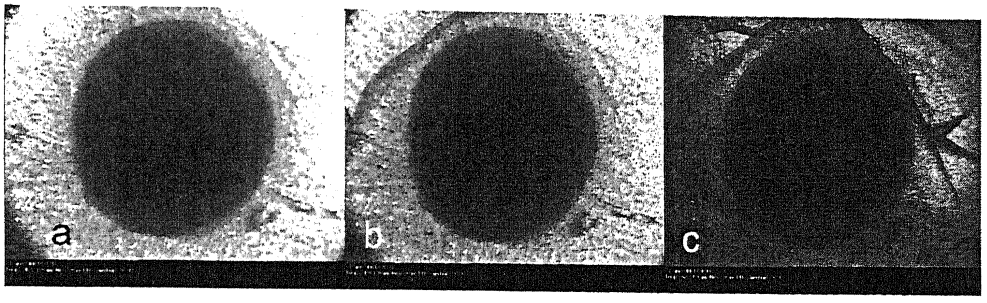


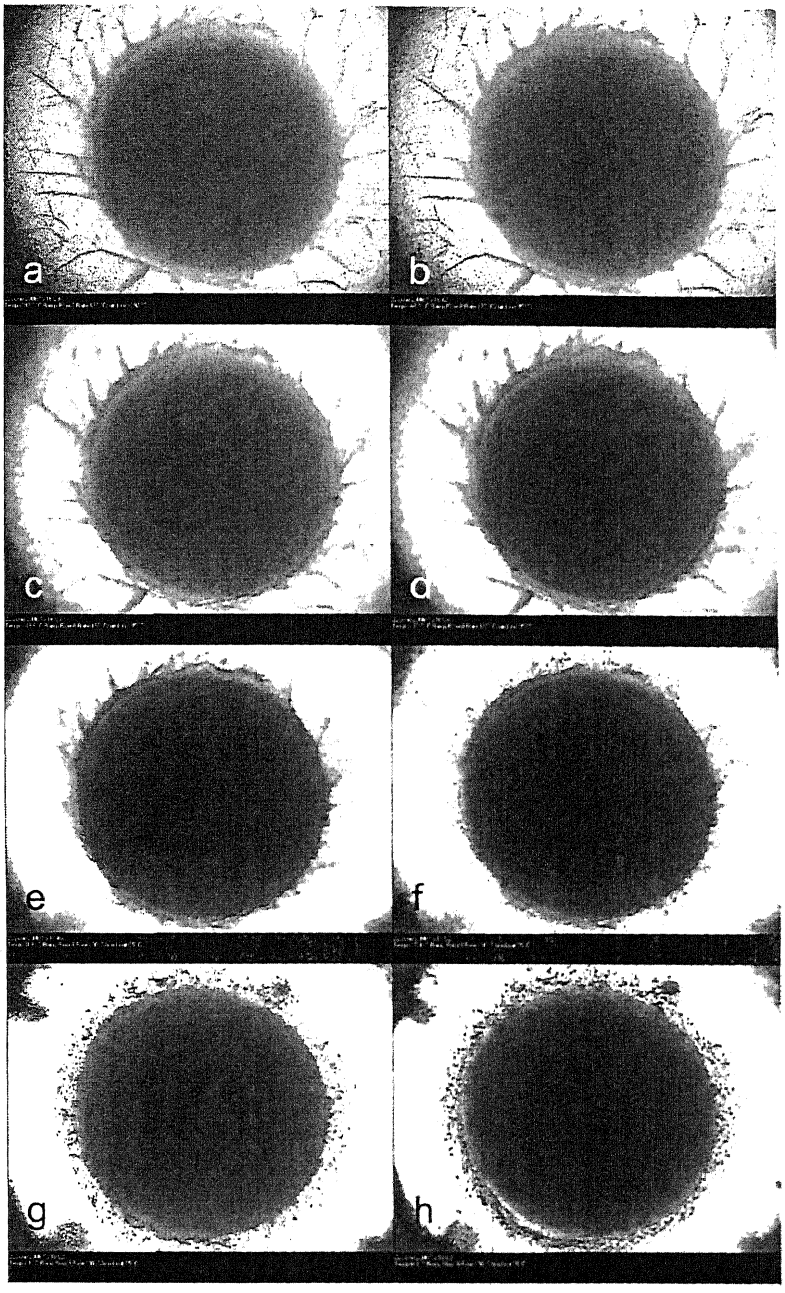
Fig. 7.15. a,b,c. Formation of cracks in the ice at temperatures below -100°C. 4M methanol + 0.2M Glucose in Hank's was used as a freezing medium. "Plunge" stage (cooling rate 130°C/min).

7.2.2.2.3 Formation of gas bubbles during thawing

During the course of freezing, some cells may be caught in the isolated cavities inside the ice phase. Pressure in such ice cavities may reach up to 10^8 Pa. Such hyperbaric conditions result in direct damage to the cells, and also in the formation of gas bubbles after thawing (Gordienko & Pushkar, 1994).

The composition of gas in the bubbles is believed to be a mixture of oxygen and nitrogen (35% : 65%) subject to solubility in water. There may also be a small amount of water and cryoprotectant (methanol) vapour.

Fig. 7.16 shows the gas bubble formation during thawing when oocytes were thawed at 130°C/min after freezing with 4M Methanol + 0.2M Glucose in KCl buffer following the optimal cryopreservation protocol.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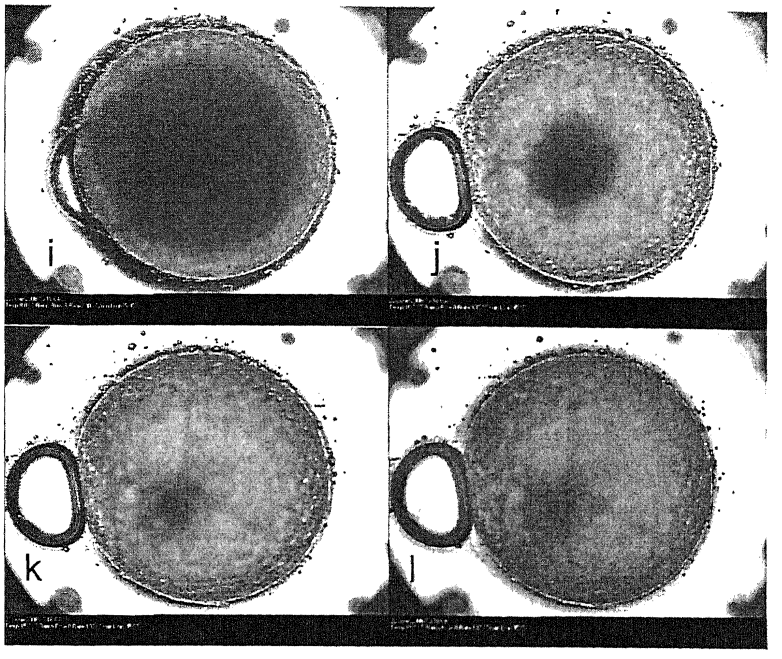

Fig. 7.16 a-l. 4M Methanol + 0.2M Glucose in KCl buffer was used as a freezing medium. Oocytes were frozen using optimal cryopreservation protocol, thawing rate was 130°C/min.

7.3. Discussion

7.3.1. Effect of cryopreservation on ATP content in stage III zebrafish oocytes.

The ATP content in intact stage III zebrafish oocytes determined in the present study ($6.52*10^{-12} - 9.49*10^{-12}$ mol per oocyte) is several thousands times lower than the ATP content, in common carp (*Cyprinus carpio*) mature oocytes ($1.14 *10^{-9}$ mol per oocyte) (Boulekbache et al., 1989). This phenomenon can partially be explained by the larger size (~1mm diameter) of mature carp oocyte. It is also known, that mature oocytes acquire extremely high energetic potential in order to provide sufficient amount of energy for events of fertilisation and embryo development. This energy potential is manifested in elevated levels of ATP in the cytoplasm of mature oocytes (Boulekbache et al., 1989). This might be the possible explanation for large differences between the results of present study and data on common carp oocytes.

The present study showed, that freezing of zebrafish oocytes to sub-zero temperatures dramatically affects the ATP content in these cells. Significant loss of ATP synthesizing ability by the cells occurs at temperature between -10°C and -20°C; at this stage of cooling the ATP content in oocytes decreased nearly 10 fold. Our assumption (confirmed by cryomicroscopic observations) is that this rapid fall is associated with occurrence of intracellular ice formation (IIF) in the oocytes. Below -20°C, no further substantial loss of ATP was observed until the samples were plunged into liquid nitrogen. After the plunge oocytes were left almost ATP-free, containing only very low amounts (10^{-14} – 10^{-15} M) of adenosine 5'-triphosphate. The results from the present study suggest, that although 4M Methanol + 0.2M Glucose in KCl buffer was the optimum cryoprotective medium for preservation of membrane structure of oocytes (confirmed by Trypan blue staining), it failed to preserve cell energy systems at -196°C. The ATP level in oocytes which were frozen to -196°C by optimised cryopreservation protocol with the use of controlled slow cooling and CPAs (methanol and glucose) was significantly lower, than ATP level in the oocytes, frozen by CPA-free LN plunge. The assumed reason for this is: whilst in both cases oocytes suffered lethal injuries and died when the temperature decreased below certain point (approximately -25 to -30°C) due to intracellular ice formation; in the case of controlled slow cooling, the time period between cell death and ATP determination was longer because of the slow cooling rate, and therefore more ATP molecules were lost.

Further investigations are required to improve the limited successes with stage III zebrafish oocytes cryopreservation showed by the present study.

Results of some authors have shown similar effect of cryopreservation on ATP content in the cells to those obtained in the present study. Kopeika et al. (1997) reported a clear pattern of ATP depletion in cryopreserved common carp (*Cyprinus carpio*) sperm, which is similar to our data. Results obtained from Billard et al. (2004) show very wide inter-specimen deviation between

ATP levels in frozen sperm from different males of sturgeon: ATP content in thawed sturgeon sperm ranged from 0.5 to 5 nmoles/10^8 spzoa, while ATP content in fresh sperm is about 6.5 nmoles/10^8 spzoa. Thus, depending on individual male, the ATP content in the thawed sperm can vary from nearly intact level to very low values. However, Jones et al. (2004) reported that no significant differences were found in ATP content in MII (i.e. mature) human oocytes after cryopreservation and 6h culture, although mitochondrial potential in such cells was clearly depleted. Summarising the data obtained so far, it can be concluded that the effect of cryopreservation on cellular ATP content is strongly dependent on the type of cells, which is being cryopreserved. As the results of the present study show, with stage III zebrafish oocytes the pattern of ATP loss during cryopreservation is pronounced, which facilitates the use of ATP detection as a vital test.

The results from the present study showed that cellular ATP levels in freeze-thawed zebrafish oocytes were very low. Possible explanations include: the cells may have consumed all of their ATP stock in coping with stress associated with cryopreservation, or freezing resulted in irreversible lethal damage to energy-production systems of the cell, as a result the stock of ATP can not be restored. If the former took place, the cell would be able to recover from the cryoinjury, rebuild its ATP stock and repair damaged components. In the latter case, the cell would be seriously and irreversibly damaged and could not be revived.

7.3.2. Effect of the "recovering" medium on ATP content in cryopreserved oocytes

In order to determine which of the above events had caused the ATP loss in cryopreserved oocytes, studies were carried out using a specially designed "recovering" medium. If energy system of cryopreserved oocytes could be recovered, the incubation of cryopreserved oocytes in such medium would result in increased levels of ATP; otherwise, the ATP levels of cryopreserved oocytes would remain the same as in the freeze-thawed oocytes incubated in Hank's solution.

In biological and especially in biomedical literature, a number of articles can be found with different protocols aimed at restoring ATP levels in ischemia-damaged heart and brain cells. Substances such as glutamine (Van Way et al., 2003), ribose and adenosine (De Jong et al., 1991) were successfully used to restore the ATP amounts in damaged cells to near-normal levels

The results from this set of experiments showed, that irreversible damage of cell energy system took place during cryopreservation of zebrafish oocytes, because ATP levels of cryopreserved oocytes were not increased after 1 hour incubation in "recovering" medium.

7.3.3. Cryomicroscopic observations of stage III zebrafish oocytes.

The results from this study showed, that in majority of cases, when stage III zebrafish oocytes were frozen by controlled slow cooling, EIF immediately induced IIF, temperature of EIF & IIF in 4M Methanol + 0.2 Glucose in KCl buffer is -13.5°C ± 1.2 (n = 6) when cooling rate of 0.3°C/min was used. These observations were similar to those obtained with zebrafish embryos at different stages, where IIF occurred at temperatures between -13°C and -21°C (Kopeika et al., 2006). Differential scanning calorimetry (DSC) studies also showed that in the intact zebrafish embryos IIF occurred approximately at -13°C, with addition of 2M Methanol lowering this temperature to -17°C (cooling rate 10°C/min) (Liu et al., 2000). The results obtained from the present study are also in agreement with data obtained by Muldrew & McGann (1990) and Liu et al. (2000), showing that the temperature of IIF is not dependent on the cooling rate, although the percentage of cells which undergone IIF and the type of forming intracellular ice are dependent on cooling rate.

In ~50% of cases ice formation occurred before the stage of ice seeding.

Therefore, the mechanism of intracellular ice initiation in zebrafish oocytes is surface-catalysed heterogeneous nucleation (SCN).

This is somewhat different from the data obtained with zebrafish embryos. In the case of embryos, EIF and IIF also occurred simultaneously, however, as it was observed, IIF did not always occur first in those embryos which had the contact with the extracellular ice. Moreover, IIF in embryos did not always develop from a single point (Kopeika et al., 2006), suggesting that the mechanism of ice nucleation in zebrafish embryos is not SCN and therefore, is different from that in oocytes. This phenomenon might be explained by the complex structure of embryos, which differs from that of oocytes. Zebrafish embryo contains blastoderm (blastocytes), which is separated by yolk syncitial layer (YSL) from the yolk mass, and has osmotic and other properties which are very different from the yolk. In zebrafish embryos, IIF normally starts from the blastoderm and then is transferred to the yolk (Kopeika et al., 2006).

Coincidence of EIF and IIF in zebrafish oocytes is very similar to the observations obtained with oocytes of other aquatic species: in oocytes of starfish EIF and IIF also occurred almost simultaneously, within 3°C interval, at relatively high temperatures (-4°C to -6°C) despite starfish oocytes have high membrane permeability and can be easily dehydrated and saturated with high concentrations of CPAs (Kaseoglu et al., 2001).

The average temperature of homogeneous volume-catalysed nucleation (VCN) (Fig. 7.11 – Fig. 7.14) of cytoplasm in stage III oocytes preincubated with 4M Methanol + 0.2 Glucose in KCl buffer, as determined by experiments with silicone oil, is -17.4°C ± 2.6 (n = 6). But in practice VCN can hardly take place (unless high, >10°C/min cooling rates are used (Karlsson et al., 1996; Rall et al., 1983)) because in case with zebrafish oocytes, as it was shown, the presence of extracellular ice normally initiates IIF through SCN mechanism long before VCN can take place.

Coincidence of EIF and IIF in a concentrated cryoprotective medium containing 4M Methanol + 0.2 Glucose indicates, that methanol even at high concentrations fails to prevent heterogeneous surface-catalysed nucleation by external ice. Studies with glycerol and DMSO (Rall et al., 1983) have shown

that these cryoprotective agents have the ability to prevent heterogeneous nucleation by external ice (SCN), With high concentrations of these cryoprotectants inside the cells the initiation of IIF occurred mainly by volume-catalysed nucleation. It is possible that methanol, unlike other CPAs, does not possess this property of SCN-inhibition.

Although in the vast majority of the cases IIF in oocytes coincided with EIF (Fig. 7.5 – Fig. 7.8), in some rare cases EIF did not initiate IIF immediately, and the IIF was a gradual process (Fig. 7.9). It is possible that in these cases supercooling of cytoplasm was avoided and IIF was probably not accompanied by dramatic deteriorating of oocytes viability (Trad et al., 1999).

Experiments with two slow cooling rates (0.3°C/min and 1°C/min) showed no noticeable differences between patterns of ice formation and temperatures of ice initiation. Percentage of cells which underwent IIF in both cases was 100%. However, other researchers (Muldrew & McGann, 1990) have demonstrated that the percentage of cells which undergo IIF was normally strongly dependent on cooling rate.

During slow-cooling stage no substantial changes in structure of ice were observed. However, at the final, "plunge" (-130°C/min) stage, certain processes of recrystallisation and reshaping of ice crystals were observed. The formation of large number of cracks in the ice also took place, which indicated the presence of mechanical tensions in the frozen phase and can negatively affect the integrity of oocyte structures.

During the thawing stage, processes of recrystallisation were seen: cracks in the ice mass disappeared at temperatures between -60°C and -50°C. Formation of gas bubbles also took place in many cases. Extracellular ice melted before intracellular ice and melting of intracellular ice had a radial pattern.

It was clear from the present study, that after the cycle of freeze-thawing, the cytoplasm of stage III zebrafish oocytes experienced dramatic

morphological changes: from opaque to transparent and granular. Similar effects were also reported for mammalian oocytes (Nagashima et al., 1994; Martino et al., 1996) and fish oocytes (Isayeva et al., 2004; Lubzens et al., 2005). The possible mechanism for this phenomenon is the fusion of yolk granules into one translucent mass, induced by low temperature exposure, formation of intracellular ice, or osmotic stress.

The experiments also showed, that Hank's-based cryoprotective solution provided ice crystallisation patterns which were essentially the same as in experiments with KCl -based cryoprotective medium. This is not unexpected, because the protective action of KCl buffer lies not in ice formation-altering properties, but in the membrane-protective properties at low temperatures. Hank's and KCl buffer have similar osmolality (246 mOsm and 250 mOsm respectively), which means that the cryoprotective solutions of same concentrations made in these media will have similar freezing points and similar dynamics of formation of ice phase.

It can be concluded from these studies that even the optimised freezing protocol has a number of significant disadvantages. In many cases ice initiation in the sample happened before ice seeding procedure. Even when ice seeding was successfully induced, it failed to prevent IIF. The protocol also failed to prevent instant and lethal IIF ("flashing"), which took place in nearly 100% of cases. Results obtained from the present study also suggest that IIF (rather than solution effects or toxicity of CPAs) is the main factor causing cryodamage and mortality of cryopreserved zebrafish oocytes. Future cryopreservation protocols need to be developed either to circumvent IIF, or to ensure formation of "safe", non-lethal forms of intracellular ice.

7.4. Summary

The ATP assay experiments have shown, that the ATP test is a reliable and sensitive test for determining viability of zebrafish oocytes. Although in the present study the ATP test was only used with vitellogenic oocytes, it can be used as a vital test for all stages of oocyte development. According to the ATP test, stage III oocytes suffer severe and irreversible damage during freezing to liquid nitrogen temperature, even with the optimal slow cooling cryopreservation protocol: the ATP content in the oocytes frozen to -196°C via controlled slow cooling was $2.38\pm1.04 * 10^{-15}$ Mol / cell, compared to $6.52\pm1.56 * 10^{-12}$ Mol / cell in intact oocytes. Incubation of freeze-thawed oocytes in specially formulated "recovering" medium, containing glucose, ADP and adenosine, didn't result in increase of ATP level in the oocytes, indicating that freezing to cryogenic temperatures leads to total death of cells.

Cryomicroscopic observations confirmed, that high mortality of oocytes, drastic fall of their ATP level, membrane integrity and developmental potential after freezing to -196°C are associated with intracellular ice formation (IIF) which even the optimised slow cooling protocol failed to prevent. IIF occurred simultaneously with EIF in nearly 100% of cases, and presumably, lethal hexagonal type of ice formed. Further investigations in controlled slow cooling techniques are required to enhance the limited success achieved in the present study.

CHAPTER 8. Conclusions

8.1. Reiteration of aims

Although cryopreservation of oocytes of many species, especially mammalian, has become a routine practice (Isachenko et al., 2000; Agca, 2000; Picton et al., 2002), cryopreservation of fish oocytes has not been studied until recently (Pearl & Arav, 2000; Isayeva et al., 2004; Zhang et al., 2005). The main difficulties associated with cryopreservation of zebrafish oocytes are: (1) their large size and low surface-to-volume ratio, compromising diffusional transport of water and cryoprotectants throughout the cytoplasm; (2) the presence of highly chilling sensitive material – yolk (3) high vulnerability of genetic material of mature oocytes. The aim of the present investigation was to use zebrafish as a model system to explore the development of a successful controlled slow cooling protocol for cryopreservation of fish oocytes of different stages, and to identify the limiting factors associated with fish oocytes cryopreservation

In order to achieve this aim, five main areas of investigation were carried out: (1) developing suitable viability assessment methods for zebrafish oocytes; (2) studies on toxicity of different cryoprotectants to zebrafish oocytes at different developmental stages; (3) studies on controlled slow cooling of zebrafish oocytes; (4) studies on effect of cryoprotective supplements and other factors on post-thaw survival of zebrafish oocytes; and (5) assessment of quality of the cryopreserved zebrafish oocytes using ATP test and cryomicroscopic observations.

8.2. Review of the main findings

8.2.1. Vital tests for zebrafish oocytes

Several fluorescent and non-fluorescent dyes were tested in order to develop suitable vital tests for zebrafish oocytes, including: Ethidium bromide, Propidium iodide, Calcein blue, cFDA and Rhodamine 123 assays, Trypan blue (TB) and Thiazolyl blue (MTT) assay. *In vitro* maturation of oocytes followed by germinal vesicle breakdown (GVBD) assay was also tested. Two

vital tests were found suitable for zebrafish oocytes: Trypan blue (TB) staining and GVBD test. TB test was the fastest assay and can be applied to oocytes of all developmental stages. GVBD test is precise functional viability assessment method, but it can only be used for stage III oocytes. Although MTT test also distinguished the differences between positive and negative controls, it was found not to be sensitive enough for adequate assessment of oocytes viability.

Enzymatic methods for disaggregation of cumulus and defolliculation of oocytes, using trypsine, hyaluronidase and collagenase were found highly damaging for the cells and therefore were not used in subsequent experiments.

8.2.2. Optimum cryoprotective medium for zebrafish oocytes

Toxicity of six penetrating and non-penetrating cryoprotectants were tested: methanol, dimethyl sulfoxide (DMSO), propylene glycol (PG), ethylene glycol (EG), glucose and sucrose. Methanol was found to be the least toxic cryoprotectant of all CPAs tested, with No Observed Effect Concentration (NOEC) for stage III oocytes being as high as >4M according to TB results and 2M according to GVBD results. Sensitivity of zebrafish oocytes to toxic effect of cryoprotectants increased with developmental stage, with stage V (mature) oocytes being the most sensitive, and stage III (vitellogenic) oocytes being the least sensitive. Based on these results, stage III oocytes and cryoprotectant methanol were mainly used for further studies on cryopreservation of these oocytes using controlled slow cooling.

8.2.3. Controlled slow cooling of zebrafish oocytes

Studies with controlled slow cooling of vitellogenic zebrafish oocytes to subzero temperatures indicated that: (1) TB- and GVBD-assessed viability of oocytes decreases with the decrease of final freezing temperature; (2) 0.3°C/min was the optimal cooling rate for stage III zebrafish oocytes (3) methanol was the best CPA of the four penetrating CPAs which were tested (DMSO, PG, EG, Methanol); (4) 4M concentration of Methanol was the optimal concentration for purposes of cryopreservation. However, even with

the optimal cryopreservation protocol, none of the oocytes survived below -20°C according to the GVBD test.

8.2.4. Further studies on cryopreservation of zebrafish oocytes using controlled slow cooling

A number of additional approaches were tested in order to cryopreserve zebrafish oocytes: the use of sodium-free medium, the use of additional cryoprotective supplements, varying of conditions such as ice-seeding temperature, liquid nitrogen plunge temperature and the pH of the freezing medium.

It was shown, that the use of sodium-free medium (KCl buffer) provided significantly higher TB-assessed survival at -50°C, than that obtained with Hank's medium, which proved that KCl buffer had membrane-protective effect at subzero temperatures. However, GVBD results showed mild deteriorating effect of KCl buffer on oocytes.

Experiments also showed that changing the pH of cryoprotective medium from 7.4 to more acidic or alkaline areas didn't have any positive effect on oocytes cryosurvival. Ice-seeding temperatures -12.5°C and -10°C were found to be the optimal. Liquid nitrogen plunge temperature of -40°C was found to be the optimal.

The addition of membrane-stabilising and antioxidant supplements to the cryoprotective medium didn't have any beneficial effect on zebrafish oocytes quality. However, addition of Glucose at 0.2M concentration, or poly-(ethylene glycol)-200 at 5% concentration has a positive effect on post-thaw TB-assessed survival of stage III zebrafish oocytes.

The optimised protocol for cryopreservation of stage III zebrafish oocytes so far, evaluated by post-thaw TB-assessed ratio of oocytes with intact plasmalemma is summarised below:

30 min incubation of stage III zebrafish oocytes in freezing medium containing 4M Methanol + 0.2M Glucose in KCl buffer (pH 7.4) at room temperature; loading the oocytes into 0.5 ml plastic straws; controlled slow freezing as follows: cooling from 20°C to -12.5°C at 2°C/min; manual seeding at -12.5°C and hold for 5 min; slow freezing from -12.5°C to -40°C at 0.3°C/min; samples are then plunged into LN (-196°C). Samples should be fast thawed (~200°C/min) in a 27°C water bath and washed twice in cryoprotectant-free KCl-buffer. Oocytes should then be transferred to Hank's solution or to another incubation medium.

However, even with this optimum protocol, TB-assessed oocytes survival (ratio of oocytes with intact plasmalemma) did not exceed 20%, and no oocytes survived to below -20°C according to GVBD test. Therefore, successful cryopreservation of fish oocytes still remains elusive.

8.2.5. Further assessments of zebrafish oocytes quality after cryopreservation using controlled slow cooling

Further assessments of the quality of cryopreserved zebrafish oocytes were carried out using ATP test and cryomicroscope observations.

ATP test showed that cryopreservation resulted in significant and irreversible depletion of ATP content in the cells. After freezing to liquid nitrogen temperature (-196°C) ATP content in the cells decreases to very low levels. The loss of cellular ATP mainly occurred between -10°C and -20°C. Cryomicroscopic observations suggested, that loss of ATP in the cells is associated with intracellular ice formation (IIF), which normally occurs at temperature range between -10°C and -20°C. Post-thaw ATP content in the oocytes frozen following the optimal cryopreservation protocol was not higher than that of oocytes directly plunged into the liquid nitrogen in cryoprotectant-free medium, indicating that the "optimised" protocol is not effective. Freeze-induced depletion of ATP was proven to be irreversible, which suggested that oocytes experienced lethal damages during the course of freezing.

Cryomicroscope observations indicated, that the high mortality of oocytes, drastic fall of their ATP level, membrane integrity and developmental potential after freezing to -196°C are associated with intracellular ice formation (IIF) which even the optimised so far slow cooling protocol failed to prevent. IIF is induced by, and occurs simultaneously with, extracellular ice formation in vast majority of cases (>90%), which presumably results in build-up of lethal hexagonal type of ice. The mechanism of this intracellular ice initiation is assumed to be surface-catalysed nucleation (SCN) by external ice.

8.3. Conclusions

Fish oocytes cryopreservation has not been studied systematically before. The aim of the present investigation was to develop successful cryopreservation protocol for fish oocytes using zebrafish as a model system. Although this aim was not achieved, considerable progress has been made in understanding the conditions required for fish oocytes cryopreservation and this will undoubtedly assist the successful protocol design in the future.

There are four key areas where progress has been made in developing a method for cryopreservation of fish oocytes: (1) development of suitable viability assessment methods for fish oocytes, (2) identifying effective cryoprotectants, (3) determining optimum oocytes developmental stage for cryopreservation, and (4) identifying important features that should inform the development of a potential method for future zebrafish oocyte cryopreservation.

8.3.1. Viability assessment methods for fish oocytes

One of the major obstacles in fish oocytes handling was the lack of reliable vital tests for this type of cells. Whilst for mature fish oocytes, fertilisation rate and hatching rate have been used for assessment of viability, for immature fish oocytes there have been no suitable vital tests. The only test reported by Pearl & Arav (2000) using cFDA has been proven to provide ambiguous results. It was therefore very important that suitable viability assessment methods were developed before oocytes cryopreservation studies. In the present study, three suitable vital tests were developed: Trypan Blue staining, *in vitro* maturation followed by GVBD observation, and ATP

detection. TB test is quick and approximate method for "rough" evaluation of oocytes viability. It is suitable for oocytes of stages III, IV and V, however it estimates the integrity of plasmatic membrane rather than the physiological status of the whole cell, which can lead to misinterpretations. GVBD test is very reliable and adequate method for viability assessment, posing functional evaluation of oocyte viability and developmental potential; however serious disadvantage of this test is that it is suitable for stage III oocytes only. ATP test is precise and trustworthy vital test, indicating energy level of the oocytes and therefore their viability with high precision. Unfortunately, ATP test is invasive, i.e. the destruction of tested oocytes is inevitable. Also, the certain disadvantage of ATP test is its expensiveness, and also considerable amount of time required for preparation of samples and measurements.

The design of other vital tests for fish oocytes, in addition to three methods listed above is desirable. Application of RNA markers, alongside with biochemical methods would contribute to development of additional viability tests for fish oocytes.

8.3.2. Optimum cryoprotective medium for zebrafish oocytes

The toxic effect of six most commonly used CPAs, methanol, dimethyl sulfoxide (DMSO), propylene glycol (PG), ethylene glycol (EG), glucose and sucrose was determined. The No Observed Effect Concentrations for these CPAs after 30 min incubation were established. The least toxic CPAs were indicated: Methanol and Propylene Glycol.

8.3.3. Optimum oocytes developmental stage for cryopreservation

Stage III of oocyte development (vitellogenesis) was found to be the optimum stage for cryopreservation, as vitellogenic oocytes are less sensitive than oocytes of other stages to toxic effect of cryoprotectants, and unlike stage V (mature) oocytes, do not develop spontaneous cortical reaction due to incubation with CPAs or chilling. Genetic material of stage III oocytes is not so susceptible to damages in the course of freezing as that in mature and maturing oocytes. Moreover, stage III oocytes have higher membrane permeability for water and solutes, which facilitates procedure of their saturation with CPAs and dehydratation during slow cooling. Basing on this,

all consequent experiments were conducted on stage III (vitellogenic) oocytes.

8.3.4. Optimum protocol for controlled slow cooling of zebrafish oocytes

Several important facts were discovered in the controlled slow cooling studies:

0.3°C/min cooling rate was the optimal cooling rate for stage III zebrafish oocytes. Optimum ice-seeding temperature was -12.5°C, optimum liquid nitrogen plunge temperature -40°C, and optimum pH of a freezing medium was 7.4.

Methanol in 4M concentration was found to be the optimal cryoprotectant for the purposes of cryopreservation of zebrafish oocytes.

The study also showed that the use of sodium-free KCl buffer supplemented by 0.2M Glucose had significantly increased post-thaw TB-assessed survival of stage III zebrafish oocytes.

The optimal protocol for cryopreservation of stage III zebrafish oocytes so far is: 30 min incubation of stage III zebrafish oocytes in freezing medium containing 4M Methanol + 0.2M Glucose in KCl buffer (pH 7.4) at room temperature; loading into 0.5 ml plastic straws; controlled slow cooling from 20°C to -12.5°C at 2°C/min; manual seeding at -12.5°C and hold for 5 min; slow freezing from -12.5°C to -40°C at 0.3°C/min; samples are then plunged into LN (-196°C). Samples should be fast thawed (~200°C/min) in a 27°C water bath and washed twice in KCl-buffer. Oocytes should then be transferred to Hank's solution or to another incubation medium. But even with this protocol, TB-assessed ratio of oocytes with intact plasmalemma did not exceed 20%, no oocytes survived cooling to LN temperature according to GVBD test, and ATP content in cryopreserved oocytes was disappearingly low when compared to intact oocytes. Based on this, it can be stated, that the task of successful preservation of vitality and maturation ability of fish oocytes at the temperature of liquid nitrogen still remains elusive.

8.4 Future work

As it was demonstrated above, even the optimised cryopreservation protocol failed to prevent intracellular ice formation in zebrafish oocytes, with lethal consequences for the cells. Stability of the plasma membrane of oocytes is not the main limiting factor in their cryopreservation. Future investigations into cryopreservation of fish oocytes should be focused on the prevention of IIF and protection of the cytoplasm and yolk of oocytes from the deteriorating impact of intracellular ice crystals and associated solution effects. Attempt to apply two-stage fast exponential cooling programmes for fish oocytes should also be considered (Gordienko & Pushkar, 1994). The use of antifreeze glycoproteins (AFGP) from plasma of Arctic fish, which were successfully used for cryopreservation of mammalian oocytes, should also be considered, either as a normal constituent of cryoprotective media, or administered by intracytoplasmic microinjection (Rubinsky et al., 1991; Feeney, 1974; Wu et al., 1998).

A major obstacle to fish oocyte cryopreservation is its large size, obstructing uniform distribution of penetrating cryoprotectants throughout the oocyte volume, and limiting the effectiveness of action of macromolecular, non-penetrating cryoprotectants. One approach to enhancing the protective action of non- or poorly penetrating CPAs on fish oocyte would be to introduce these substances into the cell by microinjection (Kopeika et al., 2006). However, this method would have serious limitations for straightline commercial use. Other ways for permeabilisation of plasmatic membrane of oocytes for macromolecular substances can be ultrasonication, electroporation, and exposure of oocytes to small amounts of antibiotics and detergents (Hibino et al., 1993; Bart et al., 2001; Driessen et al., 1995; Iserovich et al., 1997). Alongside with these conventional methods of permeabilisation, increasing the oolemma permeability to water should be considered via upregulation of water-channel proteins – aquaporins, either, as explored in embryos, by expression of foreign mRNA (Hagedorn et al., 2002), or by such substances as okadaic acid, antidiuretic hormone, vasopressin or cAMP analogues (Johansson et al., 1998; Lee et al., 1997).

A possible non-invasive approach to accumulating macromolecular cryoprotectant material in oocyte using natural pathways could be "vitellogenic CPA accumulation technique". As it was repeatedly mentioned before (Introduction, Section 1.4.2), stage III fish oocyte naturally absorbs large amounts of protein vitellogenin from blood plasma via receptor-mediated pinocytosis (Selman et al., 1993), after which the vitellogenin is evenly distributed throughout the cytoplasm, cleaved into smaller yolk proteins lipovitelline and phosvitine and accumulated in yolk vesicles (Wallace & Selman, 1981; Wallace & Jared, 1976; Riggio et al., 2003; Ayzenshtadt, 1984, 1986). Numerous studies demonstrate that vitellogenesis in fish, amphibian, bird and reptile oocytes can occur outside the ovary, *in vitro*, when oocytes are incubated in specially designed growth medium containing above all, vitellogenin and growth-inducing hormones (estrogens, insulin) (Wallace & Misulovin, 1978). Successful absorption of vitellogenin by fish oocytes has been reported (Campbell & Jalabert, 1979; Ayzenshtadt, 1984). Moreover, some of the experiments showed that oocyte vitellogenic pinocytosis sometimes has fairly low specificity, and alongside with vitellogenin, oocytes can absorb another macromolecular substances of completely different nature, when they are placed in *in vitro* growth medium, or injected into bloodstream of female animal (Ayzenshtadt, 1984). Such substances include iron dextran (absorbed by amphibian oocytes after injection into female bloodstream; Wallace & Selman, 1981), electron-dense markers (absorbed by fish oocytes during *in vitro* culture; Wegmann & Gotting, 1971), plant enzyme horseradish peroxidase (absorbed by reptile oocytes after abdominal injection of female, Ayzenshtadt, and also by insect oocytes, Raikhel & Dhadialla, 1992), bovine albumin (absorbed by amphibian oocytes alongside vitellogenin during *in vitro* culture, Ayzenshtadt, 1986) and even DNA fragments (Ayzenshtadt, 1984). Therefore, it would be interesting to explore if *Danio rerio* oocytes, at the stage of early vitellogenesis, when placed into the incubation medium supplemented with vitellogenin (which is commercially available and can also be electrophoretically extracted from zebrafish oocytes (Heesen & Engels (1973)), growth factors and the macromolecular CPA (which could be, for example, AFGPs or PEOs of high molecular weight) could absorb CPAs alongside with vitellogenin, or CPAs covalently bound to

vitellogenin. Such covalent crosslinkers as divinylsulfonyl hexane, diamide, tartyl diazide or others could be used. Successful uptake of covalently-modified vitellogenin by amphibian oocytes was demonstrated by Danilchik & Gerhart (1987). Uptake of macromolecular CPA can be monitored by using fluorescently-, radio-, or spin-labelled forms of CPA (Danilchik & Gerhart, 1987). The uniform distribution of cryoprotectant throughout the cytosol could be achieved, if necessary, by manipulations with coated vesicles, or by introducing the oligosaccharide markers of cytoplasmic localisation to the molecule of macromolecular CPA. Also, if necessary, covalent modification of molecule of macromolecular CPA can be added, in order to protect it from the lysis by intracellular proteolytic enzymes. Introduction of normally extracellular CPAs inside the oocyte cytoplasm is expected to enhance its cold-protective effect on cells multi-fold, especially for such potent and specific ice-inhibitors as AFGPs.

The application of additional cryoprotective supplements to a freezing medium, besides those which have been tested in the present study, is suggested, as well as studies on the application of "cocktails" of KCl buffer with Hank's medium in order to balance the membrane-protective effect of KCl buffer with the safety of Hank's medium to cytoplasm.

As it was mentioned in Chapter 6, Section 6.1.2, lysosomal membrane cryoinjury and leakage of proteolytic enzymes into cytoplasm is one of the most damaging factors in post-thaw death of cryopreserved cells (Belous & Grischenko, 1994). Alongside stabilisation of lysosomal membranes by membrane-protective substances, another possible approach might be to neutralise the action of lysosomal enzymes, either by neutralisation of lysosomal pH (e.g., with such substances as bafilomycin A1 and desipramine, or by weak penetrating alkali trimethylamine), or by anti-proteolytic agents, such as ionised zinc at milli-molar concentration (which inhibits extralysosomal hydrolysis) or solutions of chlorophyll and ϵ-aminobutyric acid (Shrode et al., 1997; Jadot et al., 1984).

Supplementation of a freezing medium with quinine, which inhibits Gardos effect, and gadolinium ions, which block stretch-activated membrane channels and is used during manipulations with amphibian eggs, may help to prevent post-thawing lysis of fish oocytes (Reichstein & Rothstein, 1981;

Choe & Sackin, 1997; Yang, X.C., Sachs, 1989). Inhibitors of phospholipases, such as quinacrine (anti-ischaemic cerebroprotective drug), 4-bromophenacyl bromide or manoalide can be applied (Lu et al., 2001; Lombardo & Dennis, 1985), as cold-induced activation of phospholipases contributes to cryodamage (Belous & Grischenko, 1994; Introduction, Section 1.2.2.2). For prevention of spontaneous cortical reaction during manipulations with oocytes, especially during work with mature stage V eggs, treatment of oocytes with such substances as local anaesthetics and heparine can be practiced (Picard & Doree, 1982).

As glucose was demonstrated to be effective cryoprotective supplement to a freezing medium (Chapter 6, Section 6.2.2.2; Section 6.3.2), different specific ways to increase the rate of glucose penetration inside the cells should be considered, such as treatment of oocytes with insulin, anisomycin or cAMP-analogues (e.g., 8-bromo-cAMP) (Roger et al., 1999; King et al., 1993). Also, it was shown that hyperosmotic and temperature stresses activate glucose membrane transport, however, these factors inevitably accompany biological samples during cryopreservation.

Wider investigations into the application of vitrification techniques are proposed: cryobiological experience suggests, that many objects which can not be successfully cryopreserved by controlled slow cooling may be cryopreserved by vitrification (Kaseoglu et al., 2001; Hamaratoglu et al., 2005; Robles et al., 2005; Chen & Tian, 2005; Kopeika et al., 2006). Use of metoxylated and aminated forms of CPAs (which improves their vitrifiability) is suggested, including the use of methanol and 2-amino-2-methyl-1-propanol - the best vitrifier of all researched amino-alcohols so far (Liebermann et al., 2002). The use of ice-suppressing AFGPs in combination with "vitellogenic CPA accumulation method" and vitrification technique is proposed.

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APPENDIX A

Table 1. The chemicals used in the present study

Chemical	Source	Product No.
Acetyl salicylic acid	Sigma	A5376
Adenosine	Sigma	A9251
ADP	Sigma	A2754
ATP analysis kit	Sigma	FL-AA
Bovine serum albumin (BSA)	Sigma	B4287
Butylated hydroxyanizole (BHA)	Sigma	B1253
$CaCl_2$	Aldrich	22,231-3
Calcein blue	Sigma	M1255
cFDA	Sigma	C4916
Choline chloride	Sigma	C1879
Collagenase type II	Sigma	C2674
Dexamethasone	Sigma	D4902
DHP (17α-Hydroxy-20β-dihydroprogesterone)	Sigma	P6285
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich	154938
EDTA	BDH	16079
Egg yolk emulsion	Fluka	17148
Ethidium bromide	Sigma	E8751
Ethylene glycol	BDH	10324
Foetal calf serum (FCS)	Sigma	S5394
Gentamicin	Sigma	G1272
Glucose	Sigma	G5767
HCl	Aldrich	31,894-9
$HClO_4$	Aldrich	24,425-2
HEPES	Sigma	H3375
Hyaluronidase	Sigma	H2126
K acetate	Sigma	P1190
KCl	Sigma	P3911

KH_2PO_4	BDH	10203
KOH	Aldrich	22,147-3
Leibovitz (L15) cell medium, pre-mixed powder	Sigma	L4386
Methanol	Sigma	M3641
$MgSO_4$	BDH	10151
Na_2HPO_4	Sigma	S9390
N-Acetyl-Cysteine	Sigma	A7250
NaCl	Aldrich	43,320-9
$NaHCO_3$	BDH	10247
Poloxamer-188 (Lutrol F68)	BASF	51633115
Polyethylene glycol-200 (PEG-200)	Aldrich	20,236-3
Polyethylene glycol-400 (PEG-400)	Fluka	81172
Propidium iodide	Sigma-Aldrich	P4170
Propylene glycol	BDH	29673
Rhodamine 123	Sigma	R8004
Sea salt	ZM Ltd	N/A
Sucrose	Sigma-Aldrich	55016
Taurine	Sigma	T0625
Thiazolyl blue (MTT)	Sigma	M5655
Tocopherol (vitamine E)	Sigma	T3251
Tricaine	Sigma	A5040
Trypan blue (TB)	Sigma	T8154
Trypsin	BDH	39041

APPENDIX B

The following co-authored papers and presentations were produced during the course of the research project:

Max Plachinta, Tiantian Zhang, David M Rawson; Toxicity of cryoprotectants to zebrafish (*Danio rerio*) oocytes; conference paper ; SLTB - NIBSC Joint Meeting 4-5th September (2003).

Tiantian Zhang, Max Plachinta, Anna Isayeva and David Rawson, Studies on zebrafish (*Danio rerio*) oocytes sensitivity to chilling and cryoprotectant toxicity (abstract), conference paper SL21A, CRYOBIOMOL 2003, University of Coimbra, P-3004-535, Coimbra, Portugal, 14-18 September (2003).

Tiantian Zhang, Max Plachinta, Anna Isayeva, and David Rawson; Studies on zebrafish (*Danio rerio*) oocytes sensitivity to chilling and cryoprotectant toxicity. Cryobiology, vol. 47, Number 3 (2003).

M. Plachinta, T. Zhang, D.M. Rawson; "Studies on Cryoprotectant Toxicity to Zebrafish (*Danio rerio*) Oocytes"; CryoLetters 25(6) 415-424 (2004).

Max Plachinta , Tiantian Zhang, David M. Rawson; Studies on the effect of certain supplements in cryoprotective medium on zebrafish (*Danio rerio*) oocytes quality after controlled slow cooling; poster presentation for Cryo2005 – 42nd Meeting of Society for Cryobiology; Minneapolis, Minnesota, USA, July 22 – 27, (2005).

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10th June 2005

To: Whom It May Concern

Dear Sir/Madam

Re: Maxim (Max) Plachinta/Maksym Plachynta

I confirm that the above-named is one and the same person.

The name he is registered with at this University is not the same as his passport but he is definitely one and the same person.

If you have any queries regarding this please contact me on 01582 489129.

Yours faithfully

J. R. Seymour

J.R. Seymour
Senior International Student Adviser