DEVELOPMENT OF *IN-VITRO* CULTURE AND CRYOPRESERVATION PROTOCOL FOR ZEBRAFISH (*Danio rerio)* OVARIAN TISSUE FRAGMENTS

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by

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ABSTRACT

Cryopreservation of fish ovarian tissue fragments can be a viable alternative to cryopreservation of oocytes and embryos. The ability to cryopreserve both maternal and paternal gametes would provide a reliable source of fish genetic material for scientific and aquaculture purposes. The main aim of the present study was to develop an *in-vitro* culture protocol and cryopreservation protocol for zebrafish ovarian tissue fragments. *In-vitro* culture protocol for the tissue fragments containing stage I and stage II follicles were developed and the growth assessment of follicles were evaluated using biomarkers. To develop the cryopreservation protocol using control slow cooling method, the effect on freezing medium, cryoprotectants and cooling rate on the tissue fragments were investigated. The *in-vitro* culture experiments showed that L-15 medium (pH 9) containing 100mIU/ml FSH along with 20% FBS was effective for tissue fragments containing stage I and II follicles to grow *in-vitro*. The growth of the ovarian follicle stages was confirmed by the level of expression of p450aromA and vtg1 gene. The optimal cryopreservation protocol for the ovarian tissue fragments was found as 2M methanol+ 20%FBS in 90% L-15 medium with the cooling rate of 4°C/min. Although the highest survival rate obtained for stage II follicles within the fragments was 68.2±1.9% and stage I follicles within the fragments was 55.4±2.3% using TB staining, it showed a significant decrease in their ATP levels. This is the first study carried out on the zebrafish ovarian tissue fragments. Study on cryopreservation of the ovarian tissue fragments and development of the *in-vitro* culture protocol and use of biomarkers for the ovarian tissue fragments were reported here for the first time. The outcomes of this study have provided useful information for future cryopreservation protocol development.

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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 submitted before for any degree or examination in any other University.

Name of candidate: Signature:

Date:

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CHAPTER 1 INTRODUCTION

1.1 Principles of Cryobiology

Cryobiology is the study of the effects of low temperatures on living systems. The significant application of cryobiology is the preservation of the structural and functional integrity of cells or tissues for longer period at reduced temperature. Cryopreservation is derived from the Greek word "*kryos*", which means "cold or frost". The term cryopreservation indicates storage of cells or tissue, usually in liquid nitrogen (LN_2) at temperatures close to -196°C. Achieving high viability of frozen tissue requires the appreciation of the complex physical–chemical events occurring during freezing and thawing (Bank and Brockbank, 1987). At low temperatures, any biochemical reactions may cause cell death and hence it is important to discover the physical and biological mechanisms related to the cell injury. The main goal of the cryopreservation procedure is to minimize tissue injury from low subzero temperatures (Shaw and Jones, 2003). In this chapter, the factors and mechanisms of cryoinjury and its prevention have been itemised.

1.1.1 Chilling injury

Chilling injury is defined as low temperature stress in the absence of freezing (Levitt, 1980). In general, chilling injury is the damage caused to cells when they are held at critical temperatures below the temperature at which the cells normally function. Chilling injury is one of the limiting factors for achieving the optimal cryopreservation (Morris and Watson, 1984). Physiological problems associated with chilling injury arise from thermotropic damage to cell membranes resulting in metabolic imbalances and changes in membrane fluidity. Cold -induced injuries are also associated with reduced rates of protein synthesis, production of free radicals, neuromuscular injuries, excessive thermoelastic stress, and changes in ion homeostasis and membrane potentials (Dollo *et al*. 2010). Chilling injury increases with exposure time at critical temperatures and, rapid cooling through the critical

temperature range can reduce chilling injury (Mazur, 1992). There are two types of chilling injury to cells: direct chilling injury and indirect chilling injury.

Direct chilling injury influence cryopreservation-causing injuries to cells and tissues (Hays *et al*., 2001). Direct chilling injury also termed 'cold shock' is expressed quickly upon reduction in temperature and is dependent on cooling rates (Morris and Watson, 1984). Most cell types are sensitive to cold shock provided they are cooled rapidly to sufficiently low temperatures. The cellular viability is dependent on the rate of cooling with more injury observed following 'rapid cooling' than 'slow cooling' (Morris, 1987). Direct chilling injury causes a phase transition from a liquid crystalline state to the gel state, and the lateral separation of membrane proteins (Levitt, 1980, Larcher, 2001). The cell membrane consists of lipids, lipo-proteins; these lipids undergo a liquid-to-gel phase transition in a range between 0°C and 20°C, the temperature range of maximum chilling injury. A study in boar sperm (Drobnis *et al*. 1993) suggested that this form of injury is associated with thermotropic phase transition where lipid phase is undergone liquid crystal to gel phase (Fig 1.1)

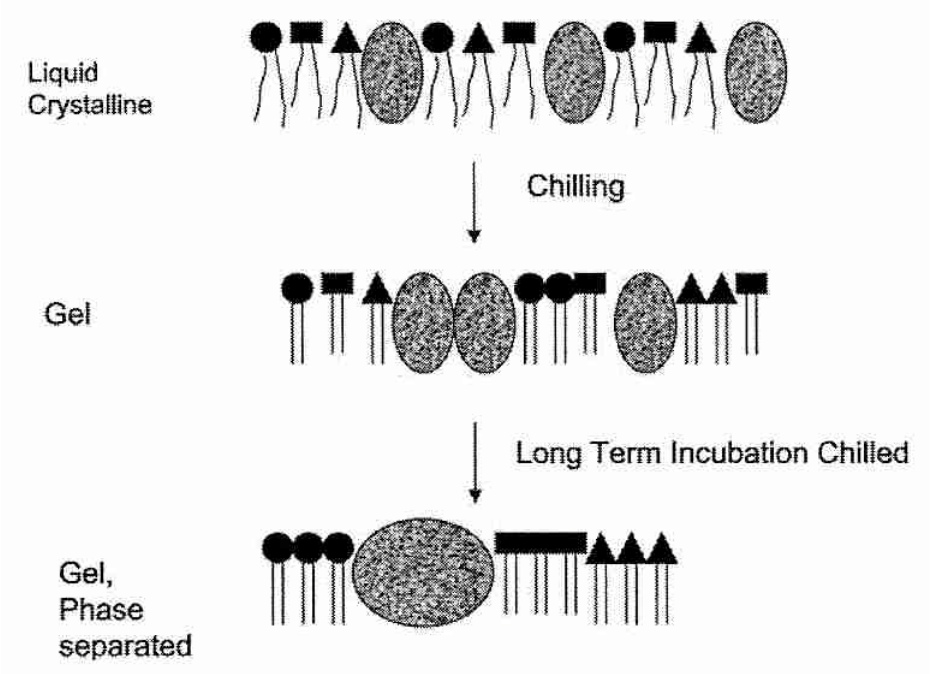


Figure 1.1 Lipid Phase transition in cell membranes undergoing lipid-to-gel phase transition (Benbest, 2012)

During chilling the lipid phase transition from the liquid crystalline to gel phase results in leakage of solutes across membranes (Watson and Morris, 1987), damages the cell structure, and reduces cell viability. Phase separation which is associated with the lipid phase transitions and damages the membranes by several mechanisms. Formation of 'packing faults' between lipid domains of different phases may disrupt membrane permeability (Pringle and Chapman, 1981; Jain, 1983), and the occurrence of concentrated lipids tend to form non-biolayer phases (Quinn, 1985) as well as aggregation of mobile, intrinsic proteins within the remaining liquid crystalline domains (Pringle and Chapman, 1981; Quinn, 1985). Studies have showed that due to rapid cooling of animal tissues, the mitochondria of the cells has become over permeable and resulted in damage of the cells (Rauen *et al*., 1999). Sufficient force to cause damage to liposomes develops for temperature decrease of 10-20°C, larger temperature producing greater tension. The faster cooling rate produce increased tension for a given temperature reduction, therefore rapid cooling cause cold shock (Liu 2000).

Indirect chilling injury is independent of the rate of cooling and usually associated with extended periods of exposure to low temperature (Morris and Watson, 1984). The injury occurs from a few degrees below zero to 10-15°C. Indirect chilling injury appears to be caused by thermotropic damage to cell membranes causing metabolic imbalance and loss of selective membrane permeability (Denlinger and Lee, 2010). At low temperature, the lateral segregation of proteins and phospholipids within membranes may be affected by decreasing the rate of enzyme activity (Morris and Clarke, 1987). Sequences of events occur in cellular membranes in response to low temperature exposure (Fig 1.2). As temperature decreases, the membrane viscosity increases. A phase separation occurs on further reduction in temperature. This phase separation depends on cell types and the extent of temperature reduction. Although many cell-types can adapt phase changes by modifying the lipid composition of their membranes, cells are usually trapped in a permanent phase change or transition after an extended period of exposure to low temperature. Following phase transition, many biological properties of the membrane are altered that leads to the alteration of the activities of membrane

proteins and membrane-associated enzymes (Cossins, 1983). In addition; it also affects the cytoskeleton system. It is a major problem for storage of chilled human tissues and organs for transplantation (Taylor *et al*., 2007). Mortality caused by the indirect chilling injury can be prevented by brief warming (Chen and Denlinger, 1992). Chilling injury may also result from oxidative stress during cold storage (Rojas and Leopold, 1996).

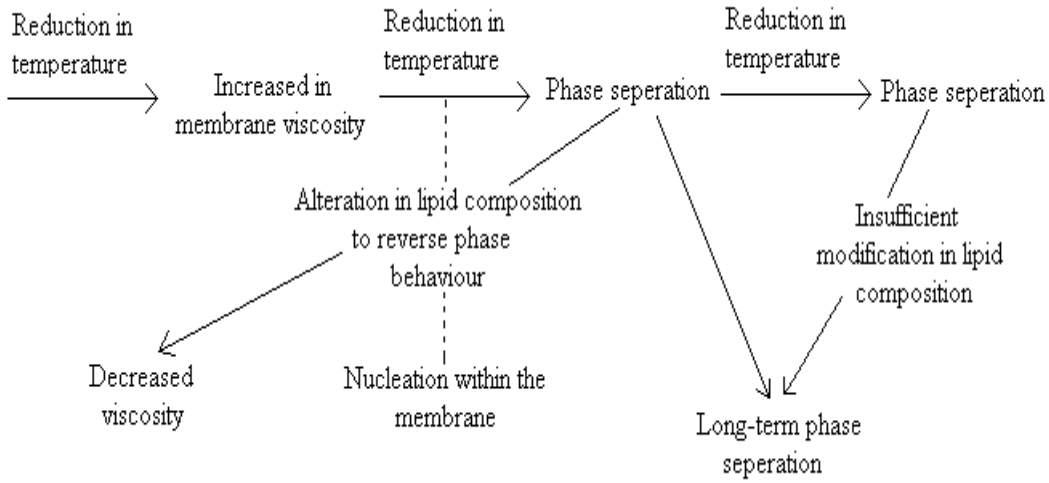


Figure 1.2 Flow diagram of membrane events during a reduction in temperature, indicating long-term responses (Morris and Clarke, 1987)

1.1.2 Freezing injury

Freezing injury of biological materials occurs at temperature below freezing point. Freezing is the conversion of liquid water to crystalline ice, although liquid water is vital for the living cells, solidification of water to ice can be lethal to the cells. The stress to which cells are exposed during freezing results from three main factors (Grout and Morris, 1987).

(i) The mechanical effects of extracellular ice crystals at cell surfaces, especially in tissues with cellular interconnections

(ii) Alterations in physical properties of solution outside the cell, including the concentration of solutes which results from the nucleation of a proportion of extracellular water

(iii) Intracellular freezing if it occurs.

During the freezing process, although the thermodynamic freezing point is -0.5°C; the cells and the surrounding environment do not undergo freezing at this temperature due to supercooling and the depression of the freezing point by the protective solutes (Mazur *et al*., 1981). Extracellular ice formation occurs at temperatures between -5°C to -15°C. As the temperature decreases and the ice phase grow, the extracellular solution becomes increasingly concentrated in solutes and a chemical potential imbalance between the biomaterial and the unfrozen external solution. If cooling rate is slow, the cells are able to lose water rapidly by exosmosis to concentrate the intracellular solutes sufficiently to eliminate supercooling and maintain the chemical potential of intracellular water in equilibrium with extracellular water, this result in cell dehydration and prevention of intracellular ice formation (IIF) (Pitt and Steponkus, 1989). But when cooling rate is too rapid, the rate at which the chemical potential of water in the extracellular solution decreases is much faster than the rate at which water can diffuse out of the cell, result in IIF (Muldrew and McGann, 1994). The cells undergo shrinkage during cooling (Fig 1.3). Extracellular freezing induces conditions that allow osmotically induce loss of water from cells during slow freezing. This correlates with survival on thawing. Rapidly cooled cells do not shrink, they forms intracellular ice and are dead on thawing.

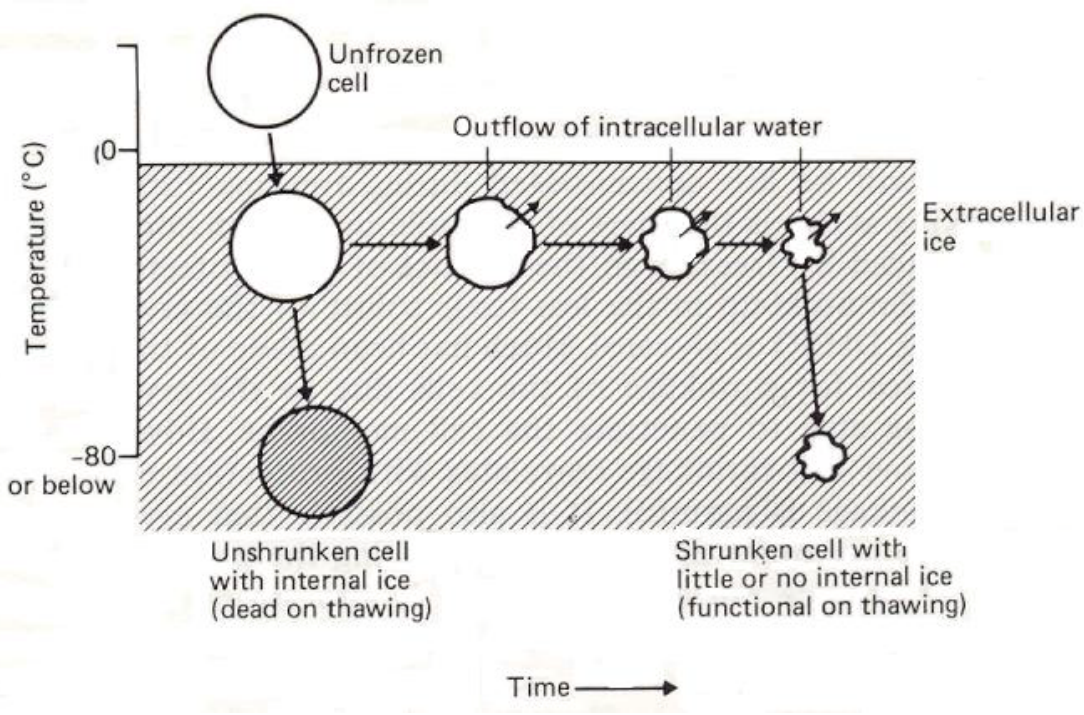


Figure 1.3: Shrinkage during cooling (Ashwood-smith and Farrant ,1980)

1.1.2.1 Extracellular ice formation

Cells can be injured by the formation of extracellular ice crystals disturbing their membranes. Although the exact mechanism of cell damage during extracellular freezing is not fully studied, several theories explains the cause of damage.

Increase in the concentration of extracellular electrolytes Lovelock (1953) suggested that the main cause of freezing injury associated with 'solution effects' was the high salt concentration in cells due to ultra-low freezing rate. Lovelock (1957) reported that hypertonic salt solution caused denaturation of lipoproteins, and that this process could induce haemolysis in red blood cells.

Reduction in cell volume Meryman and his colleagues had proposed the 'minimum cell volume theory' reporting the cellular damage associated with freezing and thawing (Meryman *et al.* 1977; Clegg *et al*. 1982). The theory proposed that the salt concentration was indirectly responsible for the damage, with high extracellular osmolarity causing cell to be shrunken beyond their limit and thus the cells been destroyed.

Mechanical effect of ice The cells are damaged by the mechanical interaction between the growing ice phase and cells between ice crystals causing haemolysis in erythrocytes (Pegg and Diaper, 1990). The mechanism of damage during slow cooling are related to the interaction between the cells in the unfrozen fraction and ice and characterised to ice shearing forces or cell deformation (Mazur *et al*. 1981, 1983, 1989)

In addition, certain physical factors including pressure changes, may be involved in the destruction of cells (Schneider and Mazur, 1987; Ashwood-Smith *et al*. 1988).

1.1.2.2 Intracellular ice formation

It is an implicit assumption that the formation of ice inside the cell is inevitably lethal. Many studies have reported that IIF during freezing correlates with the death of the cells. The mechanisms by which IIF can occur are: homogeneous nucleation, heterogeneous nucleation, or seeding by the extracellular ice (Franks, 1985). The homogeneous nucleation temperature of a 1 μm droplet of pure water is -39°C, and increases by approximately 2°C for each 10-fold increase in droplet diameter (Wood and Walton, 1970). Solutes depress the homogeneous nucleation temperature by 3.3°C for each unit increase in solution osmolality (Rasmussen *et al*.1975). Thus, the expected range of the homogeneous nucleation temperature in cells is -38°C to -44°C (Rall *et al*. 1983). Heterogeneous nucleation relies on the presence of intracellular nucleating agents and IIF at -31 to -38°C in many cell types (Mazur, 1977, Franks et al. 1983). Unfortunately, the molecular basis of seeding is not well understood. Mazur (1977) proposed that IIF occurs as a consequence of ice crystal growth through aqueous channels in the plasma membrane, which relies on

the extracellular ice crystals having a sufficiently small radius of curvature. Bronshteyn (Pitt *et al*. 1992) has suggested that epitaxy is a possible explanation to seeding. Epitaxy is the growth of a crystal from either a supercooled liquid or a vapour onto the surface, which may be another crystal (Cherov, 1984).

1.1.3 CRYOPROTECTANTS

Cryoprotectants (CPAs) are the chemicals that are used to protect cells from injuries during cryopreservation and long-term storage in liquid nitrogen. Cryoprotectants adapt to the eutectic properties of a solution so that the amount of ice formed and the concentration of salt are reduced. CPAs help to prevent ice nucleation within the cells (Yang *et al*.2009). Many compounds act as cryoprotectants to protect cells against freezing damage, however they can be toxic and cause damage to the cells (Fahy *et al*.1990). It is important to understand the molecular mechanisms of the toxicity of cryoprotectant agents in order to minimise their toxicity. A cryoprotectant concentration of about 5% to 15% is normally required to permit survival of the isolated cells after freezing and thawing from liquid nitrogen temperature. Cryoprotectants are of two types. Permeating and non permeating (Borini *et al*.2006). Permeating cryoprotectants enter into the cells in response to water molecules moving out of cells due to extracellular ice formation. Non-permeating cryoprotectants remain outside the cells and exert osmotic gradient that causes the intracellular water to migrate to the extracellular environment (Kopeika *et al.* 2005).

1.1.3.1 Permeating cryoprotectants

Permeating cryoprotectants are chemicals that are able to diffuse through the plasma membrane. The most common permeating cryoprotectants are methanol, dimethyl sulfoxide (DMSO), ethylene glycol (EG), propylene glycol (PG), and glycerol. These low-molecular-weight cryoprotectants have the ability to reduce the concentration of damaging solutes, increase the unfrozen fraction, and reduce

volume excursions during freezing and thawing (Mazur, 2004). The permeating cryoprotectants produce a considerable freezing point depression resulting in the prevention of intracellular ice formation (Shepard *et al.* 1976). These CPAs penetrate into the cells and displaces certain amount of water and forms hydrogen bonds with proteins, RNA and DNA. The hydrogen bonding increases the cytoplasm stability by keeping their structure functional and influence of ice. DMSO is the most widely used cryoprotectant in the cryopreservation of a wide range of cell types, tissues and organs. DMSO can increase the concentration of calcium ions in cytoplasm, causing a variety of metabolic responses such as depolymerisation and cytoskeleton assembly (Yamamoto, 1989). Methanol has shown to be the least toxic cryoprotectant for zebrafish embryos (Zhang *et al*.1993), oocytes (Plachinta *et al*. 2004), isolated ovarian follicles (Zampolla *et al*. 2008, Tsai *et al*. 2009), and sperms of several fish species (Lahnsteiner *et al*. 1997). EG and PG also promotes the small granular crystallisation and amorphous solidification due to their high viscosity at low temperatures, lessening the quantity of unbound water within the cells and reducing intracellular ice formation (Wu and Lee, 1996)

1.1.3.2 Non-permeating cryoprotectants

Non-permeating cryoprotectants are the chemicals that do not penetrate through the plasma membrane. They are the long chain polymers with high molecular weight (>50,000), they are soluble in water and have large osmotic coefficient. The common non-permeating cryoprotectants include polyethylene glycol (PEG), saccharides (sucrose, trehalose, lactose etc) and polyvinylpyrolidone (PVP). The effect of non-permeating cryoprotectants is based on the osmotic dehydration of the cell prior to cooling, which results in reduced intracellular ice formation during freezing. (Meryman, 1971). The non-permeating cryoprotectants also enhance vitrification of the solutions, stabilize proteins and membranes and prevent progressive ice formation (Fahy *et al*. 1984, Takahashi *et al*. 1986, Fahy 1986).

Saccharides are mostly used as an extracellular cryoprotectant, often in combination with permeating cryoprotectants to maximize the cryoprotection. Sugars afford their

protection by stabilizing lipid membranes and proteins due to direct interactions with polar residues through hydrogen bonding and their excellent glass forming properties (Crowe et al. 1993, Crowe *et al*. 1994). Therefore sugars offer a unique prospect for high temperature storage of mammalian cells (Crowe *et al*.1992). Sucrose is widely used as an extracellular cryoprotectant, often in combination with permeating cryoprotectants. It induces dehydration and osmotic shrinkage of the cell, lowering the risk of intracellular ice crystallization. The combination of intracellular and extracellular cryoprotectants such as DMSO with trehalose has shown to provide high survival, fertilization and embryonic development rates in mouse oocytes (Eroglu *et al*. 2009). Trehalose has been used as an effective cryoprotectant in many oocytes vitrification studies (Dinnyes *et al*. 2000, Li *et al*. 2002). Studies conducted by Szteins group also demonstrated that hybrid mouse spermatozoa viability when frozen with sugars (lactose 80%, raffinose 80%, trehalose 79%) was better than when frozen with glycols (glycerol 11%) (Sztein, Noble et al. 2001).

1.1.3.3 Cryoprotectants toxicity

Whilst cryoprotectants protect living cells form cryoinjury, it can be damaging to cells when used at high concentrations (Fahy, 1986). Cryoprotectant toxicity could be caused by denaturation of proteins, the hydration damage hypothesis (Arakawa, 1990). The dehydration damage hypothesis states that toxic cryoprotectants cause dehydration damage by binding to water molecules thereby prevent the water molecules from properly hydrating proteins and other macromolecules (Clegg *et al*. 1982). Cryoprotectant toxicity is cell type dependent. For instance, methanol is found to be the least toxic on zebrafish embryos (Zhang *et al.*1993) but they are toxic on oyster embryos (Chao *et al.* 1994). Studies on marine gillbread seabream oocytes showed that methanol caused protein damage if used in high concentrations (Lubzens, Gattegno *et al*. 2006). Hoetelmans *et al.* (2001) has reported that methanol effect the cell membranes by interacting with phospholipids and destabilizing the lipid bilayer of membranes. They interact with the polar head group of lipids in bilayers due to their low hydrophobicity. The non polar regions of the alcohol creates gap between lipid chains in the membrane interior and introduce

instability within bilayer (Patra *et al.* 2006).DMSO has also been reported to cause membrane labialisation and denaturation of proteins (Parkes *et al*. 1953). Methylation of polyols (glycerol, ethylene glycol, propylene glycol etc.) increases the glass-forming ability while it increases toxicity due to increased hydrogen bonding strength of the hydroxyl groups (Maria *et al*. 1990).

1.1.4 Approaches used in cryopreservation

Cryopreservation allows nearly indefinite storage of biological material without deteriorating the quality over several thousands of years (Mazur, 1985). In other words, by usage of cryopreservation the biological clock can be halted for an unlimited time (Kuwayama, 2007). In 1949, Chris Polge and his team cryopreserved fowl sperm for the first time. Since then cryopreservation has become a wider practise for the storage and transportation of cells used in fertility treatments, cell therapies, drug screening and cell banking. Cryopreservation involves the process of cell freezing at extremely low temperature. The successful cryopreservation involves the controlling of formation of ice at both intracellular and extracellular levels during the freezing and thawing process preventing cell injury.

There are two approaches in cryopreservation: controlled slow cooling and vitrification

1.1.4.1 Controlled slow cooling

The controlled slow cooling procedure is characterised by the addition of molar concentration of cryoprotectant to the cell suspension and by the use of controlled freezing to the storage temperature (Zampolla, 2009). It is a common procedure to cryopreserve many different cell and tissue types. In the controlled slow cooling procedure, the cryoprotectants are added to the solution bathing the biological samples, and are cooled at optimal cooling rates that prevent slow cooling damage and allow sufficient dehydration of the cells to prevent intracellular ice formation (IIF) (Mazur *et al*. 1972). To design the optimal controlled slow cooling procedure, several factors need to be considered: cooling rate, ice seeding, thawing

rate and removal of cryoprotectants including the freezing medium, the type and concentration of cryoprotectant.

1.1.4.1.1 Cooling rate

During freezing, the ice nucleates first in the extracellular space causing an osmotic rise across the membrane, i.e between the intracellular isotonic solution and the freeze-concentrated extracellular solution (Mazur, 1963). Depending on whether the cooling rate is `low' or `high', the intracellular water moves across the cell membrane and joins the extracellular ice phase, or freezes and forms ice inside the cell, respectively (Devireddy *et al*. 2000). Both intracellular ice formation and long exposure to high solutes can lyse cells; thus cooling rates which are either too high or too low can lyse cells. Hence a major factor of the cell survival after freezing to low temperature determines is the rate at which they are cooled. The cooling rates are cell type specific; the cell survival plotted vs the cooling rate has an inverted U shape (Mazur *et al*. 1972). Therefore the optimal cooling rate can be defined as that cooling rate which minimizes both the slow cooling and IIF injury (Fig 1.4).

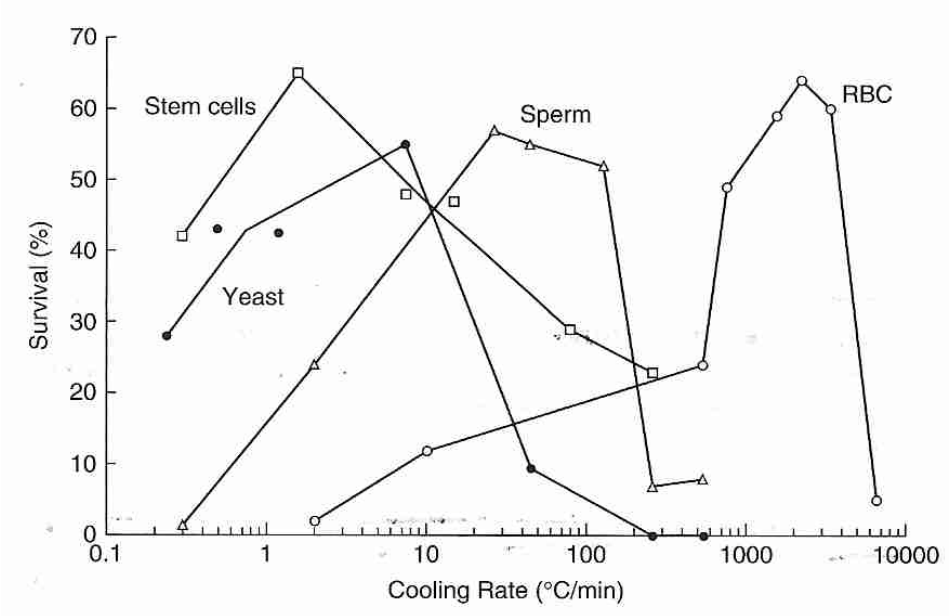


Figure 1.4: Survival of stem cells, yeast, mouse embryos, hamster cells and human red cells as function of cooling rate (Best. 2012)

Studies on mammalian oocytes and embryos have showed that the optimal cooling rates are in the range of 0.3°C/min - 0.5°C/min (Picton *et al*. 2003, Bass *et al*. 2004, Whittingham *et al*. 1972). The optimal rate for the cryopreservation of zebrafish embryos and oocytes are considered to be in the range of 0.07 °C/min - 0.5°C/min (Zhang *et al*.1989, Zhang *et al*. 1993, Guan *et al*. 2008, Tsai *et al*. 2009). The cooling rate of mammalian ovarian tissue cryopreservation are considered to be similar to that of the mammalian oocytes and embryos ranged from 0.3°C/min to 0.5°C/min (Newton *et al*. 1996, Borges *et al*.2009, Schmidt *et al* 2003, Wood *et al*. 1997).

1.1.4.1.2 Ice seeding

Ice seeding is the process that induces ice nucleation by touching the solution containing biological samples using pre-cooled forceps. Ice seeding is a vital procedure during the freezing of oocytes, embryos, ovarian tissues because it improves the dehydration of the cells by inducing extracellular ice crystallization and prevents intracellular ice crystallization. Studies on mammals have reported that ice-seeding temperature significantly affects the intracellular ice formation and cells viability during freezing (Trad *et al*.1998, Zhang *et al*. 2011). Nakamura et al. has also reported that on frozen storage of yeast the ice seeding temperature affects the cell viability significantly (Nakamura *et al*. 2009). Hence these studies signify the dependence of intracellular ice formation behaviour of cells on the extracellular ice seeding temperature.

Ice seeding temperatures depend on the concentration of the cryoprotectants. Zhang (1994) reported that for the addition of 1M cryoprotectant into the freezing medium reduces the seeding temperature by 2.5°C. Therefore, 1M cryoprotectant concentration requires the seeding temperature at -5°C, 2M at -7.5°C, 3M at -10°C and 4M at -12.5°C. This rule is based on the necessity to allow certain degree of supercooling for seeding (Zhang, 1994). The most common ice seeding temperature for 1.5M cryoprotectant concentration is at -7°C (Picton *et al*. 2003, Stachecki and Willadsen 2000, Carroll *et al*. 1993). The optimum seeding temperature on slow cooling whole cow ovaries is reported to be at -5°C for 10% (v/v) DMSO and 15%

FCS (Zhang *et al*. 2011). Trad *et al*. (1999) reported the optimal -4.5 °C seeding temperature for freezing human oocytes in 1.5M PG solution. The optimal seeding temperature for the studies on freezing the late stage zebrafish oocytes was considered to be -12.5°C for 4M methanol (Plachinta *et al*. 2007, Guan *et al*. 2008, Tsai *et al*. 2009).

1.1.4.1.3 Thawing rate

During thawing, additional damage to the cells is caused by the recrystallization process. Recrystallization refers to the growth of large ice crystals by the conversion of small ice crystals. The recrystallization process exerts an additional interfacial tension on the entrapped proteins and causes damage to the cells (Cao *et al*. 2003). Rapid thawing improves survival (Farrant, 1980); the major benefit of rapid thawing is the avoidance of recrystallization. On the other hand slow thawing is more damaging because of the rise in the total exposure time at subzero temperatures ice (Mazur, 2004).

Zhang *et al*. (1993) reported that fast thawing was more effective than slow thawing for zebrafish embryos cryopreservation. Guan et al. (2008) also reported that fast thawing (>300°C/min) was more effective than slow thawing for zebrafish oocytes cryopreservation. But some studies on mouse cryopreservation (Van den Abbeel *et al*. 1994) and carp embryos (Zhang *et al* 1989) have shown that fast thawing is more damaging due to the osmotic shock induction.

1.1.4.1.4 Removal of cryoprotectants

During the removal of cryoprotectants, the cells initially swells due to the influx of the extracellular water and then shrinks to isosmotic volume as cryoprotectants and water leaves the cells (Si *et al*. 2006). Osmotic stress is an important factor which results in cell damage. The osmotic tolerance of cells varies among different cells and tissues and extreme volume changes can result in cell death (Agca *et al*. 2005, Ball *et al*. 2001, Guthrie *et al*. 2002, and Walters *et al*. 2005). Removal of cryoprotectants by step-wise dilution decreases the osmotic

pressure. In step-wise dilution the cells are exposed to a range of gradually decreased cryoprotectant solutions which reduces the post-hyperosmotic stress and improves the membrane integrity (Wessel and Ball, 2004).

Several studies on human spermatozoa cryopreservation have shown that multi-step removal of cryoprotectant are more effective when compared to the single step removal (Gao *et al*.1995, Gao *et al*. 1997, Gilmore *et al*. 1997). Guan *et al*.(2008) and Tsai *et al*.(2009) also showed that the four-step removal of the cryoprotectants is more effective on the zebrafish oocytes reducing the post-hyperosmotic stress to the fish oocytes.

1.1.4.2 Vitrification

Vitrification is defined as 'the solidification of a solution brought about not by crystallization but by an extreme elevation in viscosity during cooling' (Fahy *et al*. 1984). *Vitri* is the Greek word for glass vitrification and was first described in 1860; and was successfully used on red blood cells in 1937 by Basile J. Luyet. But it was not until late 20^{th} century when Rall and Fahy described vitrification as a possible alternative over slow cooling showing that this technique was fully recognised (Rall *et al* 1987, Rall and Fahy, 1985). It is a method where rapid freezing transforms the cells from a liquid state to a glassy solid state without ice formation. This method eliminates the structural damage and cell injury related to intracellular ice formation. In the vitrification process, high concentrations of cryoprotectants and ultra-rapid cooling rates are used. The cells are incubated in cryoprotectants and then directly plunged into liquid nitrogen or nitrogen slush. For successful cryopreservation by vitrification, the cryoprotectant must permeate the cell membrane and be concentrated intracellularly to avoid lethal intracellular ice formation (Day *et al*.1995). This technique is cost effective when compared to slow cooling. Vitrification has been successfully performed in mouse embryos and oocytes (Rall and Fahy, 1985, Brockbank *et al*.2000, Wood *et al*. 1993. Shaw *et al*. 1991), sheep embryos and oocytes (Schiewe *et al*. 1991, Bogliolo *et al*. 2007), human oocytes (Lucena *et al*. 2006, Kuwayama *et al*, 2005). Studies on the cryopreservation of zebrafish oocytes using vitrification was not successful as they

did not survive after warming due to severe damage (Guan *et al*. 2010). Although vitrification is considered effective, some of the disadvantages of this technique are the use of drastically high concentration of cryoprotectant which leads the cells to undergo toxic and osmotic injury; there is also the potential risk of the liquid nitrogen induced disease transmission due to the open tools used in the process which leads to contamination; vitrified samples are also prone to cracks due to the slight changes in storage temperature.

1.2 Applications of cryopreservation technology

Cryopreservation allows virtually indefinite storage of biological material without deterioration over a time scale of at least several thousands of years (Mazur, 1985). It is a method of preserving tissues or cells at very low temperature. Cryopreservation has important role to play in the fields of aquaculture, biomedicine and conservation.

Due to the rapid expansion of the aquaculture industry, there is an increased demand on seed production from farmed fish stocks. Hence the fish stocks are maintained by the cryopreservation of the fish genetic material which is imperative to secure and sustain the long term development of fish production. Although successful cryopreservation of eggs and embryos is still elusive, fish sperm banks play an important role in aquaculture and conservation management (McAndrew, 1993). Cryopreservation also has important role to play in artificial propagation of diverse aquatic organism. In recent years there have been a number of live-born domestic animals resulting from artificial insemination. The first recorded offspring produced by artificial insemination using the cryopreserved semen are: cows (Stewart, 1951), sheep (Salmon and Lightfoot, 1967), Pigs (Hess *et al.* 1957), Horses (Banker and Gandier, 1957). A greater role for the use of cryopreserved semens in live stock production is seen recently in most domestic industry (Curry, 2000). Conservation of wild species was considered twenty years ago (Veprintsev and Rott, 1980); since then collection of genetic material has been established. Genetic resource banks are set up for collecting the biological material for germplasm used in animal breeding program (Holt and Pickard, 1999). Live offspring have been produced by the transfer

of frozen or vitrified embryos in mice (Wood *et al.* 2001), rats (Stein *et al.*1993). The ability to maintain the genetic diversity using cryopreserved gametes is important in conservation. During the last decade, cryopreservation has become one of the most effective tools for the reproduction management.

The most significant application of cryopreservation is in the field of biomedicine. Cryopreservation methods have been developed for most blood cells. There is also growing clinical interest in cryopreserved lymphocytes for treatment of blood stem cell transplantation (Fuller *et al*.2004). The most significant advance is in the field of reproductive medicine for infertility treatment. The first live birth from cryopreserved human spermatozoa was reported in 1950 (Bunge *et al.*1954), later in 1983 the first live birth from a cryopreserved human embryo was reported (Trounson and Mohr, 1983), then in 1986 the first live birth from a cryopreserved human oocyte was recorded (Chen, 1986), in 1987 the records revealed that at least 63 babies had been born from cryopreserved human embryos (Mandelbaum *et al.* 1987); in 1988 the first attempt to freeze immature oocytes were made (Mandelbaum *et al.* 1988) and in 2004 the first live birth following the cryopreservation of ovarian tissue was reported (Donnez *et al.* 2004).

1.3 Status of fish species cryopreservation

Due to environment factors and human activities, over a third of fish species are threatened or endangered. Cryopreservation of gametes of fish species plays an important role in preserving the genetic heritage of these species and the development of cryobanks allows storage of the genetic materials for unlimited periods. Cryopreservation of fish reproductive materials also has important applications in biomedicine and aquaculture. Due to the small sized genome in zebrafish it is easier for sequencing and is an ideal model for studying human diseases. Cryopreservation of fresh water fish species like salmonids, sturgeons and carps are well established (Lansteiner, 2000; Magyary *et al.* 1996); but it is only in the last decade that the research has been focussed on marine fish species. Fish sperm cryopreservation of many species has been achieved, but cryopreservation of fish oocytes and embryos has not been successful (Zhang *et al*. 1995, Hagedorn *et al*.

1996). Maternal genome cryopreservation is important as it preserves the mitochondrial DNA and mRNAs that determine the early stages of embryonic development (Tsai *et al*. 2010).

1.3.1 Cryopreservation of fish sperm

Cryopreservation of fish sperm has been successful in nearly more than 200 species (Kopeika *et al*. 2007, Tiersch *et al*. 2007). Blaxter (1953) successfully cryopreserved Atlantic herring spermatozoa which was the first report for teleost gametes. Thereafter successful cryopreservation of sperm has been reported in carp, salmonids, catfish, cichlids, medakas, white-fish, pike, milkfish, grouper, cod, and zebrafish amongst many other species (Scott and Baynes 1980, Harvey and Ashwood-Smith 1982, Stoss and Donaldson 1983, Babiak *et al*. 1995, Suquet *et al*. 2000, Van der Straten *et al*. 2006, Bokor *et al*. 2007). Fish sperm cryopreservation can make an important contribution in the germ storage of all transgenic lines. The major benefits of sperm cryopreservation include: synchronization of gamete availability, sperm economy, simplification of broodstock management, transport of gametes from different farms and germplasm storage for genetic selection programs or conservation of species. All these benefit the aquaculture industry (Cabrita *et al*.2010). Compared with fresh water species, cryopreservation of marine species is more successful as a high percentage of marine water species spermatozoa survive cryopreservation. The fertilization rate of the cryopreserved sperm of marine species is comparable to mammalian species (Tsvetkova *et al*. 1996). For the successful cryopreservation of spermatozoa, the controlled slow cooling approach has been used. Studies on common carp had showed the fertilization and hatching rate of 95% using the frozen-thawed sperm (Magyary *et al*.1996). Oetome et al (1996) has also reported that the African catfish fresh and cryopreserved semen resulted in no significant difference in hatching rate of 82.25% and 78.9% respectively. In most marine fish sperm cryopreservation the extenders used are saline or sugar solutions, DMSO as a successful cryoprotectant for sperm cryopreservation; with cooling rates from 8°C to 99°C /min with high thawing rates (Suquet *et al*.2000).

1.3.2 Cryopreservation of fish embryos

Whilst cryopreservation of fish spermatozoa has been successful in many species, maternal genome cryopreservation has still been a challenge over the last two decades. Unlike the mammalian embryos; fish embryo cryopreservation is not successful due to the multicompartmental biological systems, high chilling sensitivity, low membrane permeability and their large size (Zhang and Rawson, 1995). The fish embryos are larger in size compared to those of the mammalian embryos. The size of the fertilized fish egg is greater than 1mm in diameter. The large size of fish embryos results in a very low surface area to volume ratio and reduce the rate at which cryoprotectant can move into and out of embryos during cryopreservation (Mazur, 1984). Furthermore, the embryo's multicompartmental system which is made up of the outer chorionic membrane and inner vitelline membrane makes the diffusion of water and cryoprotectants across the membranes difficult (Kopeika *et al*. 2005). Studies on zebrafish embryos have shown that the water permeability of the plasma membrane at different developmental stages remained relatively stable (Tsai *et al*. 2012). The studies conducted by Zhang and Rawson (1998) showed that the permeability to methanol increased during embryo development. Hagedorn *et al*. (1996) has reported that the yolk's syncytial layer is responsible for the low cryoprotectant permeability of zebrafish embryos. The plasma membrane and yolk syncytial layer are supposed to be the main permeability barriers to water and cryoprotectant movements (Hagedorn *et al*. 1996, Zhang and Rawson 1996). Stage dependent chilling sensitivity of zebrafish embryos have shown to be a major obstacle for successful embryo cryopreservation (Zhang and Rawson 1995). It has been reported that the later stages are less sensitive to chilling than earlier stages and chilling sensitivity increases with decreased temperature (Zhang and Rawson 1995).

1.3.3 Cryopreservation of fish oocytes

Maternal genome cryopreservation is important as it preserves the mitochondrial DNA and mRNAs that determine the early stages of embryonic

development. Cryopreservation of early stage fish oocytes offers several advantages due to their smaller size, low water content and the absence of fully formed chorion (Zhang *et al.* 2005). Although cryopreservation of zebrafish oocytes has been studied (Isayeva *et al*. 2004; Plachinta *et al*. 2004; Zhang *et al*. 2005; Guan *et al*. 2008; Tsai *et al*. 2009), successful oocytes cryopreservation is still to be achieved. Some of the factors that limit successful zebrafish oocytes cryopreservation are their lower surface area which limits the rate of water transport within the oocytes, their sensitivity to chilling injury, and their low permeability of membranes (Tsai *et al.*2009; Zampolla *et al.* 2009).

Studies on zebrafish oocytes showed that they are susceptible to chilling (Isayeva *et al.*2004) and chilling sensitivity in zebrafish oocytes may be due to the lipid phase transition of the oocytes membrane (Plachinta *et al.* 2004) during chilling. The phase transition in zebrafish oocytes showed that chilling injury occurs when oocytes are exposed to temperature between 12 -22°C above the water freezing temperature (Pearl and Arav 2000). Studies on zebrafish oocytes has also shown that early stage oocytes are less sensitive to chilling when compared to the late stage oocytes (Isayeva *et al.* 2004, Plachinta *et al.* 2004, Tsai *et al.* 2009). Zebrafish oocytes are larger in size when compared to the mammals; they contain large amount of yolk, consisting of vitellogenesis which leads to the formation of embryos. Yolk has been demonstrated to cause high chilling sensitivity to zebrafish oocytes (Pearl and Arav 2000). Late stage oocytes are much more difficult to cryopreserve as they are much larger and have low volumes to surface ratios, they have also been shown to have low membrane permeability and highly sensitive to chilling (Isayeva *et al.* 2004, Isayeva *et al*. 2005).

Information on permeability of fish oocytes membrane is important in the successful cryopreservation. Zebrafish oocyte membrane permeability was first reported by Zhang *et al*. (2005). The study showed that the permeability of immature oocyte membrane to water and cryoprotectants is lower when compared to mammalian oocytes and higher than those of fish embryos. This is in line with the study reported by Seki *et al.* (2007). They also reported that immature oocytes are more suitable than mature oocytes for cryopreservation.

1.3.4 Cryopreservation of ovarian tissue

Ovarian tissue cryopreservation can be a viable alternative to cryopreservation of oocytes or embryos (Borges *et al*. 2009, Newton *et al*. 1999, Schmidt *et al*. 2003, Wood *et al*. 1997). Ovarian tissue cryopreservation has attracted much scientific and public attention due to its potential use in human infertility treatment, in safeguarding the reproductive potential of the endangered species and in genome banking of genetically important lab animal strains (Agca. 2000). Cryopreservation of ovarian tissues is advantageous over the oocytes as they can be cultured and cryopreserved in small pieces which are rich in primary follicles. Cryopreservation of structurally intact tissues is more beneficial to cells, since it can retain all the tissues potential. Early studies on ovarian tissue cryopreservation were also performed in mouse (Parkes *et al*. 1953, Parkes 1957), and has been proven to be effective on other species such as sheep (Cecconi *et al*. 2004), cattle (Celestino *et al*. 2008), goat (Rodrigues *et al*. 2004) and pig (Borges *et al*.2009). Studies on mammals have shown that ovarian tissue cryopreservation enables the storage of large number of oocytes within primordial follicles. Unlike fully grown oocytes, oocytes in primordial follicles tolerated cryopreservation better (Lucci *et al*. 2004). Studies on human adult ovaries (Fabbri *et al*. 2003, Matrinez-Madrid *et al.* 2004) showed that in frozen tissues the histological morphology of ovarian follicles and surrounding tissue remained intact. The studies also indicate that in cryopreserved ovarian tissues, the ovarian follicles remain in their natural three-dimensional structure where they may be protected from physical stress and damage. In these studies the percentage of viable follicles, stromal cells and vasculature was similar to the fresh tissues before freezing (Matrinez-Madrid *et al.* 2004). The level of DNA replication and activity of anti-apoptosis in cryopreserved strips of adult human ovarian tissues was also shown to be similar to those in the fresh ovarian tissues (Fabbri *et al*. 2003). Frozen ovine ovarian tissue recovered and grew after transplantation and also after *in- vitro* culture (Onions *et al*. 2007). Similar results were also obtained with the human foetal ovarian tissues (Zhang *et al.* 1995).

Although several studies has been undertaken on zebrafish oocyte cryopreservation at different stages (Guan *et al*.2008, Zampolla *et al*. 2008, Tsai *et al*. 2009); they resulted in compromised viability. Hence cryopreservation of

zebrafish ovarian tissue provides a promising alternative for zebrafish oocytes cryopreservation. Studies on cryopreservation of fish ovarian tissues would need to be accompanied by the development of *in- vitro* culture method of these tissues as zebrafish ovarian tissue cryopreservation and *in-vitro* culture method has not been studied systematically although some studies have been carried out on culturing of isolated zebrafish oocytes (Wang and Ge, 2003).

1.4 *In-vitro* culture of isolated oocytes and ovarian tissues

The oocytes *in-vitro* culture system would help to establish a complete framework for further studies help develop oocytes from the primary follicles *in-vitro*. Successful protocols for maturation of oocytes are important, as it is necessary for ensuring successful fertilization, zygote formation, and attainment of blastocyst stage, embryo growth and development. In some species the efficiency of *in-vitro* maturation is still very low; hence many studies are being focussed on the new combinations of media supplements to enhance successful *in-vitro* maturation (Kempisty *et al*. 2011). The culture media plays an important role in the development of *in- vitro* culture system; since it can influence the success of the oocyte maturation, fertilization and development of the embryos (Gliedt *et al*.1996). The culture media are supplemented with different types of serum, such as bovine fetal serum (FBS), serum of cows in estrous (ECS), bovine serum albumin (BSA). Studies on bovine oocytes have shown that the use of fetal calf serum in the culture medium have stimulated the *in-vitro* maturation and fertilization (Blanco *et al.* 2011). Other supplements that play an important role in the process of maturation, are sodium pyruvate (Arlotto *et al.* 1996), sodium lactate, glutamine (Fukui, 1990), glucose and sodium bicarbonate (Younis *et al.* 1989) and EGTA (Blanco and Simonetti, 2000). The *in-vitro* maturation can be improved by the addition of some growth hormones such as follicle stimulating hormones (FSH) or human chorionic gonadotrophin (hCG), but FSH or hCG does not improve development to the blastocyst stage (Blanco et al. 2011). Since early1980s, progress has been made in establishing the optimal conditions for *in-vitro* oocytes maturation, fertilization and culture of resulting embryos. These *in-vitro* culture systems have contributed

significantly to the utilization of the cells and tissues after thawing and have made it possible to evaluate protocols designed to cryopreseve these biomaterials more effectively (Agca, 2000). Successful *in-vitro* procedures can be classified on the basis of the characteristics being assessed-physical integrity, metabolic activity, mechanical activity, mitotic activity and *in-vivo* function (Seki *et al*. 2008).

In-vitro culture of early follicles provides an alternative for generating mature oocytes. Although current systems are unable to effectively reproduce coordinated growth and differentiation of somatic and germinal compartments (Cecconi, 2002), several protocols have been developed, yielding varying results in rodents and large animals (Santos *et al.* 2010). *In-vitro* culture of preantral follicles from sheep (Cecconi *et al.* 1999, 2004), goats (Silva *et al.* 2006), cows (Telfer *et al.*2000) and primates (Wandji *et al.* 2001) has been performed successfully. The cultured early stage follicles grow at an accelerated rate, taking shorter time to reach the maturation stage (Cecconi *et al*. 1999, Telfer *et al*. 2008, McLaughlin and Telfer, 2010). In recent years, attention has been given to the possibility of obtaining mature oocytes from the culture of frozen-thawed preantral follicles (Santos *et al*. 2010). In fish, including rainbow trout (Jalabert 1976, Nagahama *et al*. 1980), goldfish (Jalabert 1976), yellow perch (Goetz & Theofan 1979), amago salmon (Nagahama *et al*. 1980), medaka (Iwamatsu *et al.* 1987), and zebrafish (Selman *et al*. 1993, 1994), 17a,20b-dihydroxy-4-pregnen-3-one (DHP) is effective as a maturation-inducing hormone for *in vitro* maturation of fully grown oocytes at the GV stage (stage III). Only a few studies have examined the ability of matured oocytes to be fertilized and to develop till hatching (Seki *et al*.2008). In zebrafish, Li *et al*. (1993) reported an *in vitro* maturation method for zebrafish oocytes at stage III in which oocytes could be matured and fertilized, and the fertilized eggs could develop to term. But this *in vitro* maturation method has been reproduced neither by other researchers nor by themselves, and its use has not become widespread (Seki *et al.*2008*).*

1.4.1 Mammals

Mammalian ovaries contain thousands of oocytes which contain both primordial and growing follicles that are in various developmental stages. The normal follicular developments of oocytes are capable of maturation, fertilization and embryonic development. But the largest portion of them are primordial follicles; hence throughout the female reproductive lifespan only a small portion of these follicles will produce oocytes which are capable to undergo successful maturation and ovulation and the rest undergo atresia (Ksiazkiewicz, 2006). So during *in-vitro* culture, the early events in folliculogenesis can be regulated by local growth factors and the ovarian steroid hormones like progesterone, estrogens and androgens (Blanco *et al*. 2011). Although *in-vitro* maturation of fully grown oocytes has been successful in ruminants, *in-vitro* maturation in human assisted reproduction is still experimental as its efficiency is low and only a small number of pregnancies and live births have been reported (Picton *et al*. 2003).

1.4.2 Fish

In teleosts, two different gonadotropins, follicle-stimulating hormone (FSH) and luteinizing hormone (LH) were identified (Kwok *et al.* 2005). The pituitary secretes FSH and LH which acts upon the gonads, stimulating their growth, production of eggs or sperms and synthesis of gonadal hormones (Moles *et al*. 2008). The growth stage is controlled by FSH and the maturation stage by LH (Nagahama, 1994). Unlike in mammals, only a little is known about the physiological roles of FSH and LH in teleosts.

It has been reported that FBS enhances cell growth (Frazer *et al*. 1999). FBS has been previously used in different fish cell culture experiments at different concentrations and it has been shown to increase the cellular growth rate (Goswami *et al*.2010, Kumar *et al.* 2001) but it has not been used in zebrafish cultures until now. Seki *et al.* (2008) reported that BSA was proven effective for the cytoplasmic maturation of zebrafish oocytes as of mammalian oocytes.

Pang and Ge (2002) reported that hCG significantly promoted the maturation of zebrafish stage III oocytes. Tsai *et al.* (2010) also reported that *in-vitro* culture with

hCG treatment increased the follicular diameter from isolated stage II follicles to stage III follicles. It is also known that FSH acts in early folliculogenesis and is essential to an adequate development up to the vitellogenesis (Kwok *et al*. 2005). The presence of FSH receptor in granulosa cells suggests that FSH can promote follicular development and growth (Magalhaes *et al.* 2009). An *in-vivo* study on salmonoids has shown that, FSH is important in promoting follicle growth in the ovary (Tyler 1991). Treatment with FSH significantly increased the follicular diameter in most of mammalian *in-vitro* cultures (Rosetto *et al*. 2009, Rajarajan *et al*. 2006). Since fish gonadotropins are not easily available, hormones from mammalian sources have been commonly used as the alternative in various studies in fish (Kwok *et al*. 2005).

1.5 Zebrafish (*Danio rerio*) as a model

Zebrafish (*Danio reiro*) are the tropical fresh water fishes. They have been studied widely since 1930's in fisheries research and are considered the ideal model organism because they are small; can develop rapidly, and can be raised easily in isolation (Rugh, 1948). They also have short generation time and breed throughout the year. There are high levels of genetic and physiological similarities between the zebrafish and mammals (Zon and Peterson, 2005). The zebrafish has become an important model for understanding human development, diseases and toxicology (Menke *et al.* 2011). The sequencing of its genome and the ease to perform gene modifications promotes the use of the organism for the identification and determination of the functions of genes identified in the human genome leading to the creation of numerous zebrafish models for studying human diseases such as cancer, heart disease, Alzheimer's, Parkinson's, and muscular dystrophy (Lieschke and Currie, 2007, Menke *et al.* 2011, Whitfield, 2002, Zon and Peterson 2005). Its advantages in the genetic and neuroanatomical analysis of larval behaviour have also been described (Westerfield, 2000). Zebrafish are naturally social animals that show preference for the presence of conspecifics and therefore an excellent model to probe

the genetics of social behaviour (Norton and Cuif, 2010). It is also used in the drug discovery and development (Flemming, 2007).

1.5.1 General information on zebrafish

Zebrafish (*Danio reiro*) (Fig 1.5) belongs to the Cyprindae family and lives in rivers of northern India, northern Pakistan, Nepal, and Bhutan in South Asia. The size of the fish rarely exceeds from 4-5cm in length and has cylindrical body with 7-9 dark blue horizontal stripes on silver, which run into caudal and anal fins, and an olive green back. Males are slender and torpedo in shape and golden sheen on their belly, ventral fin, pelvic fins and pectoral fins. Females are more silvery and they have larger abdomen, particularly prior to spawning. The fish are capable of withstanding wide ranges of temperature (15.5°C-43.3°C and pH (6.6-9.2) (Axelrod and Schultz, 1955). Although zebrafish reach sexual maturity in 10-12 weeks, the breeding fish should be between 7 and 18 months of age for maximum embryo production (Suwa and Yamashita, 2007). Zebrafish have asynchronous ovaries, containing follicles of all stages of development and the eggs are spawned throughout the year. Females spawn irregularly every 4-7 days in mixed populations (Niimi and Laham, 1974). Zebrafish show a photoperiodic response which allows the time of spawning to be controlled under laboratory conditions (Legault, 1958); however maximal embryo viability is observed when sexually isolated females are permitted to breed in 10-day intervals (Niimi and Laham, 1974, Selman et al. 1993). Interestingly, the wild species of zebrafish are primarily annual breeders; the spawning occurs just prior to the onset on monsoon (Spence *et al.* 2008).

Figure 1.5: Adult female zebrafish (Picture adapted from http://gomestic.com/pets/zebra-danio)

1.5.2 Developmental stages of zebrafish oocytes

The zebrafish ovary is a dynamic organ in which the follicles undergo asynchronous development (Ge, 2005) Zebrafish follicles possess a large oocyte surrounded by the zona radiata. (vitelline envelope) and a follicular layer consisting of an inner layer of granulosa cells separated by a basement membrane from an outer theca cell layer (Peng and Clelland, 2009) (Fig 1.6). Follicle development in the adult zebrafish is broadly divided into five stages (Fig 1.7): stage I (primary growth), II (cortical alveolus or pre-vitellogenic), III (vitellogenic), IV (maturation), and V (mature egg) (Selman *et al*.1993, Ge, 2005, Lubzens *et al*. 2010). The entire process of folliculogenesis from primary stage to the post-vitellogenic stage takes about 10 days (Wang and Ge, 2004).

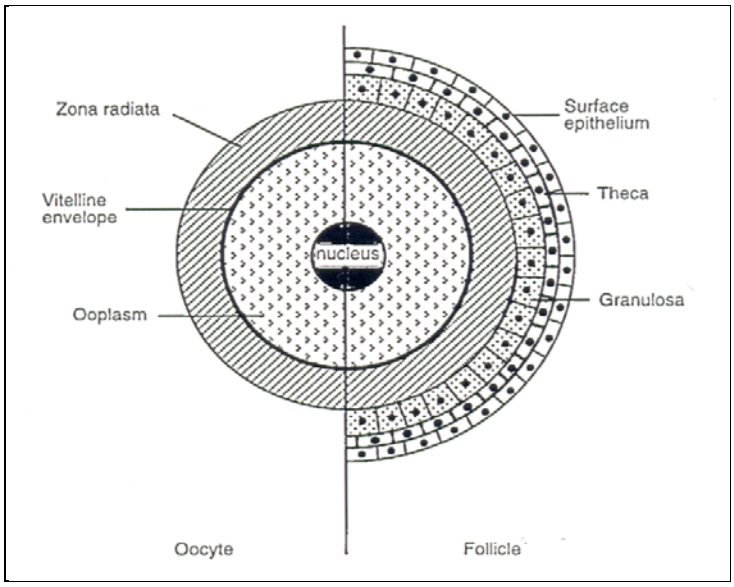


Figure 1.6: Diagrammatic representation of the distinction between an oocyte and an intact ovarian follicle

(Picture adapted from: Reviews in fish biology and Fisheries 6, 291 (1996)

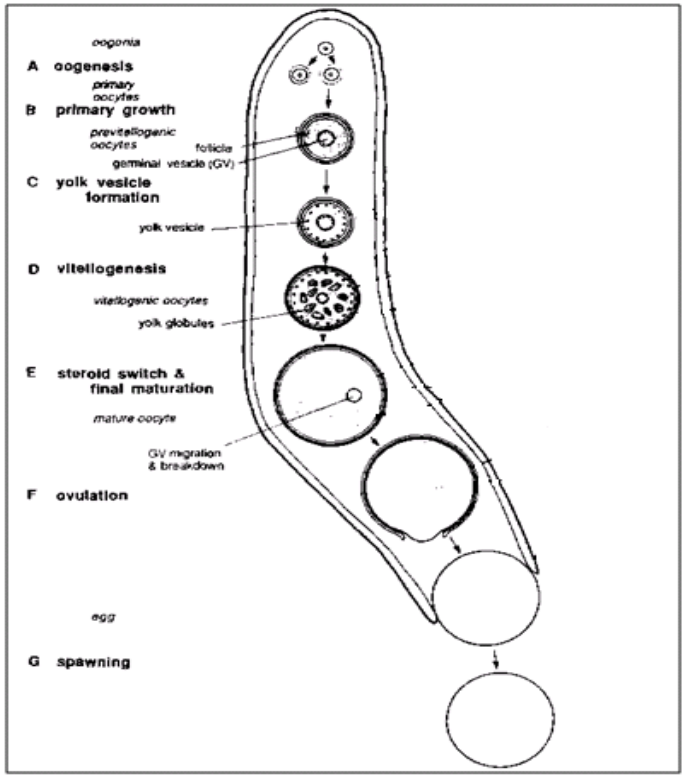


Figure 1.7: Maturation of fish oocyte in the ovary

(Picture adapted from: http://www.nzdl.org/gsdlmod?e=808a.4.7)

In zebrafish the early meiotic oocytes are found in nests, the smallest germ cells or newly formed oocytes are spherical in shape and the size is approximately 20 µm in diameter.

Stage I (the primary growth stage) (Fig 1.8): The diameter of the follicle is about 7-140 µm. The oocytes enter the early prophase stage and form the follicles. The primary growth stage is divided into further two stage IA (diameter of oocyte is 7-20 µm) also known as the pre follicle phase. At this stage the oocyte is located within a nest of oocytes, and the nuclei in the oocytes are large to the amount of cytoplasm. The chromosomes become more visible due to condensation. In stage IB (diameter of follicle is 20-140 µm) at this stage the oocyte leaves the nest and becomes part of the follicle (Selman *et al*. 1993). The chromosomes begin to extend and enter the deplotene stage. As the follicle develops, the nucleus of the oocyte increases in size; and during this stage the oocytes are surrounded by the layers of somatic tissue forming the theca and granulose cells to support the oocytes growth.

Stage II (cortical alveolus stage) (Fig 1.8): The diameter of the follicle is approximately 140 µm- 340 µm), at this stage the opacity of the oocytes becomes visible near the germinal vesicle due to the increase in size of follicles. The germinal vesicle increases and becomes irregular in shape, and the nucleoli become abundant due to proliferation of the cortical alveoli. The cortical alveoli initially form a ring around the periphery of the oocyte and then accumulate inward to the nucleus. Lipid droplets occur later on in this stage; and there is a space appearing between the granulosa layer and the oocyte surface (Wallace and Selman, 1980).

Stage III (vitellogenesis) (Fig 1.8): Size of the follicle is approximately 340-690 µm in diameter, the follicles become opaque at this stage. The oocytes increase in size due to addition of yolk, primarily due to the secretion of vitellogenin which is a female specific yolk precursor protein. Vitellogenesis regulation involves the interaction of the anterior pituitary in the brain, follicle cells, liver and eggs. The anterior pituitary of the fish produces hormones known as gonadotropins and they are released into circulation. These hormones stimulate the theca and granulosa cells to produce estrogen which stimulates the liver to produce vitellogenin protein. Vitellogenin is secreted into the blood and is taken up by the oocyte. As the

vitellogenesis proceeds, most of the oocyte cytoplasm becomes occupied by yolk bodies. At the end of stage III the follicles become capable of responding to endogenous hormones and move on to stage IV where maturation takes place (Selman *et al.* 1993).

Stage IV (oocyte maturation) (Fig 1.8): The follicle diameter increases between 690-730 µm. The germinal vesicle moves to the oocytes periphery and the nuclear envelope, it also marks the beginning of the first meiotic division. In this stage the chromosomes move to the second meiotic metaphase and the oocytes develop into eggs. During oocyte maturation, yolk bodies lose their crystalline bodies, and the membrane surrounding the nucleus disappears in a process known as germinal vesicle breakdown (GVBD). The maturation process lasts for about 4 hours; and during this stage the nucleus of the egg migrates from the centre of the egg to the periphery. After ovulation the remaining parts of the follicle become the post ovulatary follicle and the envelope stays back with the egg (Selman *et al.* 1993).

Stage V (mature egg) (Fig 1.8): The size of the egg ranges from 730-750 µm; they are homogeneous, finely granular and weakly basophilic. After an oocyte is released into the lumen of the ovary, the cells of theca and granulosa layer remain in ovarian stroma as the postovulatory follicle (Wallace and Selman, 1981, Selman *et al*. 1993).

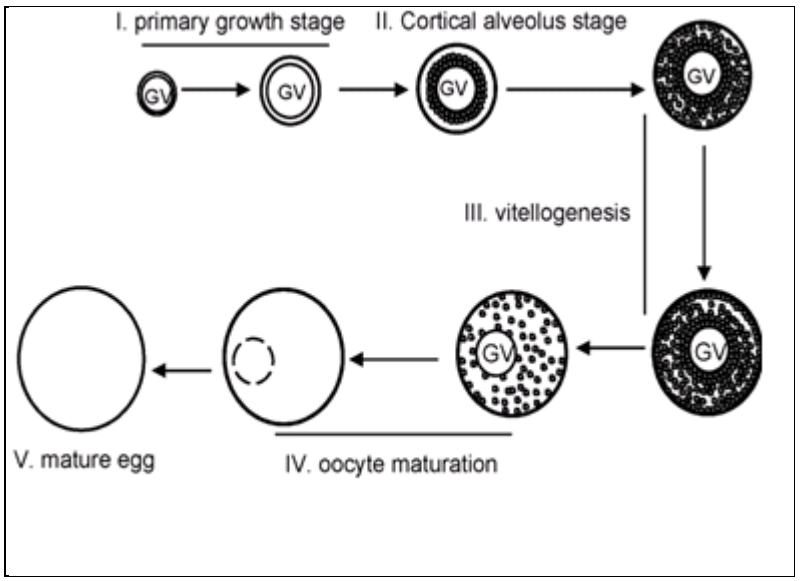


Figure 1.8: Stages of follicle development in zebrafish. Adapted from Clelland and Peng (2009)

1.6 Viability assessment assays before and after *in-vitro* culture studies

Viability assay is the most important step before undertaking further studies in identifying the ovarian tissue fragments of good quality. Viability assays enables the percentage of viable cells to be determined before and after *in-vitro* culture or cryopreservation. There are different types of viability assays depending on their mode of action: plasma membrane integrity assays, mitochondrial activity assays and functional assays. A range of vital stains have been used to assess the viability of zebrafish oocytes; these include trypan blue (TB) staining, fluorescein diacetate (FDA) and propidium iodide (PI) staining. Functional assay such as germinal vesicle breakdown assay (GVBD) assesses the developmental capability of the maturing oocytes (Plachinta et al. 2004, Selman et al. 1994, Zampolla et al. 2006).

Trypan blue (TB) staining is widely used to assess the membrane integrity of cells. TB stains the nuclei and cytoplasm of the dead cells. The cells that do not take up the stain are considered to be viable. However prolonged exposure to TB stain may be inaccurate because the dye penetrates into the cells and the number of blue stained cells increases with the increase in time (Hudson and Hay, 1980). TB stain assesses membrane damage as oppose to whole cell physiological status. However, this stain is applicable to all oocytes developmental stages. TB staining has been successfully used in many studies; including cryopreserved porcine oocytes (Didion *et al.* 1990), bovine oocytes (Fouladi *et al.* 1998), and zebrafish oocytes (Plachynta *et al.*2007, Guan *et al.* 2008, Tsai *et al.*2009).

Fluorescein diacetate (FDA) and propidium iodide (PI) staining assess both the metabolic activities and membrane integrity. Zampolla et al. (2006) had reported that this double staining was more sensitive than trypan blue staining and can be applied to zebrafish oocytes at all stages. FDA has been found suitable for most animal cells viability (Widholm, 1972). Rotman and Papermaster (1965) demonstrated that the intracellular retention of fluorescein is dependent on the integrity of the cell membrane. The non-polar fluorescein-diacetate molecules enter the cell and are hydrolyzed by cellular esterases to produce the polar compound fluorescein. This results in producing bright-green fluorescence inside the cell. In viable cells, the

fluorescein is unable to pass through the intact membrane, accumulating in the cytoplasm of the cell, whilst damaged cells show a distinct loss of fluorescein through the cell membrane (Zampolla *et al*.2006). Propidium iodide (PI) is a fluorescent dye that passess through the damaged cell membranes and intercalates with DNA and RNA to form a bright red fluorescent complex seen in the nuclei of dead cells (Edidin, 1970, Krishan, 1975). Since the dye is excluded by intact cell membranes, PI is an effective stain to identify non viable cells. The combination of FDA and PI has been used to determine viability and mammalian cells (Harrison and Vickers, 1990, Jones and Senft, 1985).

Functional assays such as germinal vesicle breakdown assay (GVBD) are based on the developmental capability of the maturing oocytes. The limitation of these methods is their stage specific applicability (Plachinta et al. 2004). Adenosine triphosphate (ATP) assay is a method for assessment of the level of ATP in the cytoplasm of the oocytes. ATP is the main energy carrier in all living cells, generated during exergonic reactions (chemical reaction which releases energy) which are used to drive endergonic (reactions requiring energy input to proceed) (Madigans *et al.* 2003). Quantification of ATP is exploited in the counting of cells in a bioluminescence assay with luciferase and its substrate D-luciferin (Campbell, 1988). In the presence of ATP and molecular oxygen from the air, Dluciferin is catalytically oxidized by the luciferase. ATP is not the energy source of the reaction; it converts D-luciferin which can be oxidized by the luciferase (Campbell, 1988). In the reaction, oxyluciferin is produced and ATP is dephosphorylated to adenosine monophosphate (AMP). The reaction leads to a light emission at 560 nm, giving a detectable signal, which increases linearly when the sample ATP concentration increases (Campbell, 1988). The plate is read with a luminometer, which precludes compound fluorescence or auto fluorescence interference (Olsen, 2009). ATP is present in all metabolically active cells, hence the quantification of ATP released from cells is related to the cell number. When the cells die, the concentration of ATP decreases rapidly, due to the endogenous ATP degrading enzymes (ATPases), and so only living cells are counted. ATP content is a vital substance for the oocytes survival and is an important parameter for evaluation of viability of the cells (Knoll-

Gellida and Babin, 2007). This assay has been used in zebrafish oocytes (Guan *et.al* 2008).

1.7 Study of biomarkers on fish

The development of biomarkers dates back to the late 1980s and is a characteristic that is objectively measured and evaluated as an indicator of biological processes. A biomarker can be defined as 'a xenobiotically induced variation in cellular or biochemical components, structures or functions that is measurable in a biological system' (Everaarts *et al.*1993). Biomarkers have been studied extentively in the field of ecotoxicology. A variety of changes observable or measurable at molecular, biochemical, cellular, or physiological levels in individuals have been studied as biomarkers for investigating the present or past exposure of the individuals to pollutants (Kaiser, 2001). The use of biomarkers such as plasma steroid hormones, vitellogenin (VTG), and gonad histology has advanced the understanding of fish reproductive toxicology in field and laboratory studies, as well as provided mechanistic alerts for other aquatic taxa (e.g., amphibians, echinoderms, and molluscs) that may share similar reproductive hormone systems (Hutchinson *et al.* 2006). VTG is a good example of a marker which provides an insight to the mode of estrogenecity that is vital to fish reproductive health (Hutchinson *et al.* 2006). Although the use of biomarkers to assess the level of toxicity in the aquatic ecosystem has been well studied little is known about their use as the growth assessment tools in fish (Cheung *et al*.2007). A major challenge in current fisheries research is to identify a suitable biomarker for indicating fish growth.

VTG (Vitellogenins) are the large multidomain apolipoproteins that are the precursors of the major egg yolk proteins in both vertebrates and most invertebrates. The term "vitellogenin" (vtg) was first used by Pan *et al*. (1969) to describe a female-specific protein in the hemolymph of the Cecropia moth. Vtg is specific to maturing females and hence the assessment of vtg gene expression is considered a useful approach in evaluating females in response to gonadal steroid changes

(Heppell *et al*. 1995). This protein is normally not detected in males or juveniles. In zebrafish, seven vtg genes were previously identified (Wang *et al*. 2005). The proteins fall into three main families represented by vtg 1, vtg 2 and vtg 3. While induction of *vtg-1* has been established as a biomarker of exposure to environmental estrogens in male fish, little is known about how its expression relates to the stages of oogenesis in females (Connolly *et al*.2012).

Cytochrome P450aromatase (Cyp19) is a member of the cytochrome P450 superfamily, it catalyses the synthesis of estrogens. In humans, Cyp 19 is extensively expressed in tissues including ovaries, placenta, adipose, and brain. In vertebrates, it is expressed in the gonads and brain (Simpson *et al.* 1994). In fish, *cyp19* is expressed in vitellogenic follicles during oogenesis, consistent with the function of estrogen in fish ovarian development (Tanaka *et al.* 1995; Fukada *et al.* 1996; Chang *et al*. 1997). Goldfish have at least two forms of *cyp19*, one expressed in ovaries and the other found in the brain (Tchoudakova and Callard 1998). In zebrafish they are divided into two forms: Cyp19a and Cyp19b respectively. Cyp19a is expressed mainly in the follicular cells lining the vitellogenesic oocytes in the ovary during vitellogenesis. *Cyp19b* is expressed abundantly in the brain, at the hypothalamus and ventral telencephalon, extending to the olfactory bulbs. The expression of duplicated *cyp19* genes at two different tissues highlights the evolutionary significance of maintaining two active genes on duplicated zebrafish chromosomes for specific functions in the ovary and the brain (Chiang *et al.* 2001).

1.7.1 Gene expression

Gene expression is the process in which the gene is transcribed into mRNA; and the mRNA is translated into protein. This process involves transcription and translation and plays a role in cell differentiation and morphogenesis of any organism (Fig 1.9). There are several methods to analyse gene expressions such as polymerase chain reaction (PCR), Northern blotting, SAGE (serial analysis of gene expression), DNA microarrays etc.

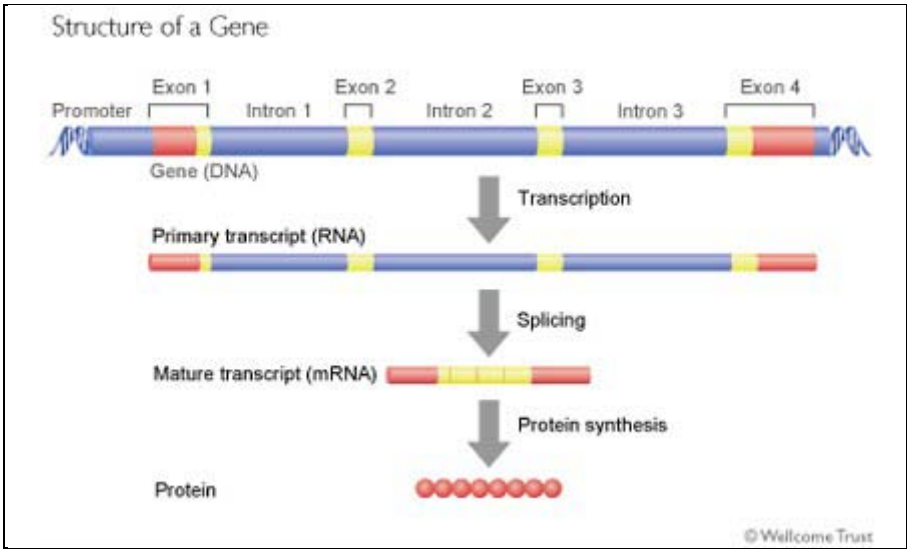


Figure 1.9: Simplified overview of gene structure and expression (adapted from http://genome.wellcome.ac.uk/doc_WTD020755.html)

1.7.1.1 Transcription

Transcription is the first step in gene expression, in which one fragment of DNA is transcribed to RNA to produce mRNA by the enzyme RNA polymerase. Major stages involved in the synthesis of m-RNA are transcription, RNA splicing (post transcription modification) and polyadenylation. Specific nucleotide sequence informs RNA polymerase at the beginning and end stage. The RNA polymerase attaches to the DNA at a specific site called the promoter region. RNA is composed of nucleotide bases, adenine, guanine, cytosine and uracil (U). When RNA polymerase transcribes the DNA, guanine pairs with cytosine and adenine pairs with uracil. RNA polymerase moves along the DNA until it reaches a terminator sequence. At that point, RNA polymerase releases the mRNA polymer and detaches from the DNA (Synder and Champness, 2007).

1.7.1.2 Translation

Protein synthesis is completed through a process called translation. After the DNA is transcribed into an mRNA during transcription, the mRNA is translated to produce a protein. In translation, mRNA along with transfer RNA (tRNA) and ribosomes work together to produce proteins. Translation is divided into three phases: initiatation (a special tRNA for initiation), elongation (addition of amino acids to the growing chain) and termination (release of the completed polypeptide) (Synder and Champness, 2007).

1.7.2 Gene expression analysis:

1.7.2.1 Polymerase chain Reaction (PCR)

Mullis *et al*. (1986) developed the process known as PCR. This is a technique which allows amplification of particular regions of DNA. The procedure involves three major steps which are denaturation of the DNA template, annealing and extension. In order to initiate the process of replication of DNA, two informing sequence codes denominated primers are required which promote the beginning and reversion of the reaction of the polymerase (RNA-pol) at particular locations of the genome (Montaldo *et al.*, 1998). The amplification of thousands of copies of a gene of interest is obtained by repeated cycles of synthesis and denaturation of the DNA using temperature changes. Since the primers are specific sequences to bond to a determined region of DNA, only the specific amplification of the desired sequence of DNA instead of amplifying the DNA in its totality is obtained (Burastowski, 1994; Koleske and Young, 1995; Stein *et al.* 1996).

1.7.2.1.1 PCR primer design

Good primer design is essential for successful PCR reactions; since this is the key to specific amplification with high yield. The main factors to be considered are: (1) primer length is generally accepted that the optimal length of PCR primers is 18-

22 bp; (2) primer melting temperature is defined as the temperature at which one half of the DNA duplex will dissociate to become single stranded and indicates the duplex stability. Primers melting temperatures should be in the range of 52-58 ^{o}C; (3) primer annealing temperature is the estimate of the DNA-DNA hybrid stability and critical in determining the annealing temperature.The primer annealing temperature should not be too high or too low. Primer annealing temperature can be calculated using formula Ta = (0.3 X Tm of primer) + (0.7 X Tm of product); (4) GC content - is the number of G's and C's in the primer as a percentage of the total bases. The GC content of the primer should be 40 – 60%; (5) Primary secondary structure-avoid secondary structure (self complimentarily) particularly in 3’ region to prevent primer dimer and (6) PCR product size should be more than 1000bp and not cross axon-intron junction.

1.7.2.1.2 RT PCR (Reverse transcriptase PCR)

RT PCR technique is used to study the gene expression; in this study RNA cannot be used as a template, and hence as the first step it is important to convert the RNA into cDNA. The procedure involves three main steps: denaturation, annealing and extension. Initially the denaturation of RNA secondary structure takes place, when the primer anneal to the RNA, the deoxynucleotide triphosphates (dNTPs), RNAse inhibitor, reverse transcriptase and RT buffer initiates their reaction. Finally the temperature is increased to inactive the enzyme. Oligo (dT) primers, random primers and gene specific primers are commonly used for the RT reaction. The successful amplification of DNA depends on good primer design and selection of taq enzyme, MgCl2 concentration and template concentration. The whole procedure is a cycle (Figure 1.10).

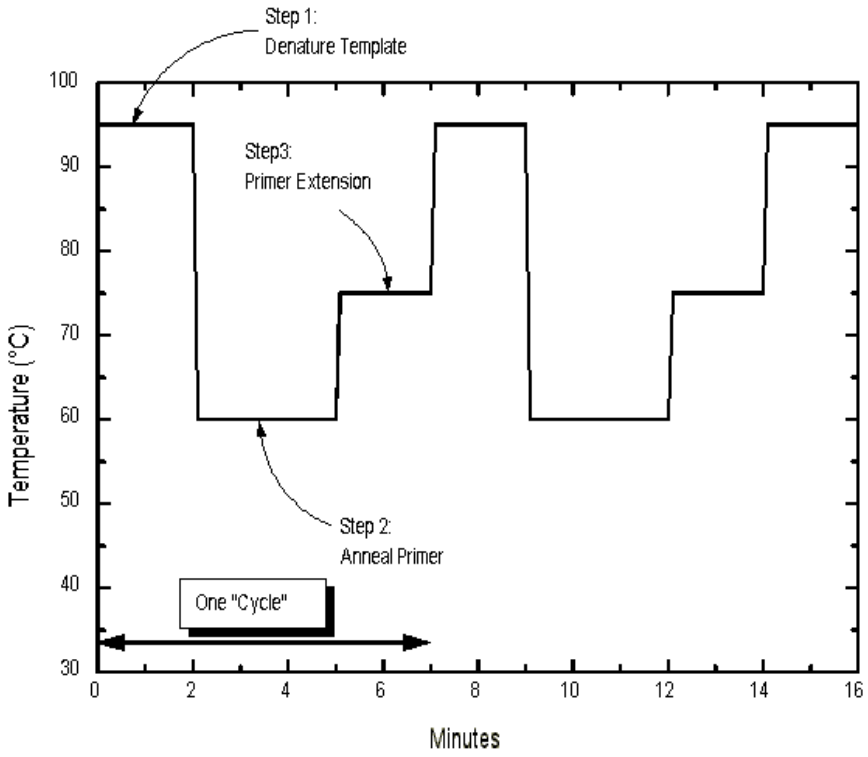


Figure 1.10: The profile of PCR temperature cycling. Step 1: the *initial* denaturation of template is accomplished at 95-100 °C. Step 2: annealing takes place at temperature ranging 50-60°C. Step 3: primer extension is usually performed at 72 °C. Step 1 to step 3 is one cycle and the process is usually repeated 35 times. (Adapted from http://www.mikeblaber.org/)

1.7.2.2 Real time PCR (Quantitative polymerase chain reaction-qPCR)

Real time PCR is the technique to detect and quantify the increase in the amount of DNA as it amplified in real time. Real time PCR allows quantification of small transcripts and changes in gene expression (Pfaffl, 2001; Bustin, 2000). This method is very sensitive and can detect even a single copy of a specific transcript (Palmer *et al.* 2003), and it requires only less templates as starting material for gene expression and produces relatively high throughput. Analysis of gene expression is a multi-step procedure that includes RNA extraction, reverse transcription, real time

PCR data acquisition and data analysis. Several different methods are available for determining the amount of PCR product present at the end of each cycle, but it depends on measuring the amount of fluorescent molecule that is associated with newly synthesized DNA copy. Some of the methods are DNA-binding dyes, molecular beacons, hybridization probes and hydrolysis probes (Wittwer *et al*. 1997).

1.7.2.2.1 Quantification method of real time PCR

The data can be analysed by two methods namely absolute quantification and relative quantification. In absolute quantification the PCR signal is converted to Ct values (cycle number) and the concentration is evaluated using a calibration curve. In this method, a known concentration of oligonucleotides is run along with the experimental sample, at the end of the run the calibration curve was obtained and the transcript concentration was calculated. Relative quantification, or comparative quantification, measures the relative change in mRNA expression levels. It determines the changes in steady state mRNA levels of a gene across multiple samples and expresses it relative to the levels of RNA. Relative quantification does not require a calibration curve or standards with known concentrations and the reference can be any transcript, as long as its sequence is known (Bustin, 2002). To achieve optimal relative expression results, appropriate normalization strategies are required to control for experimental error (Vandesompele *et al*. 2002; Pfaffl *et al*. 2004), and to ensure identical cycling performance during real-time PCR. These variations are introduced by various processes required to extract and process the RNA, during PCR set-up and by the cycling process. There are two mainly mathematical models available to calculate mean normalised gene expression from relative quantification assay which are (1) without efficiency correction using equation below (Eqs. 1 & 2) and (2) With kinetic PCR efficiency correction (Eqs 3) (Pfaffl 2004) Fig 1.11.

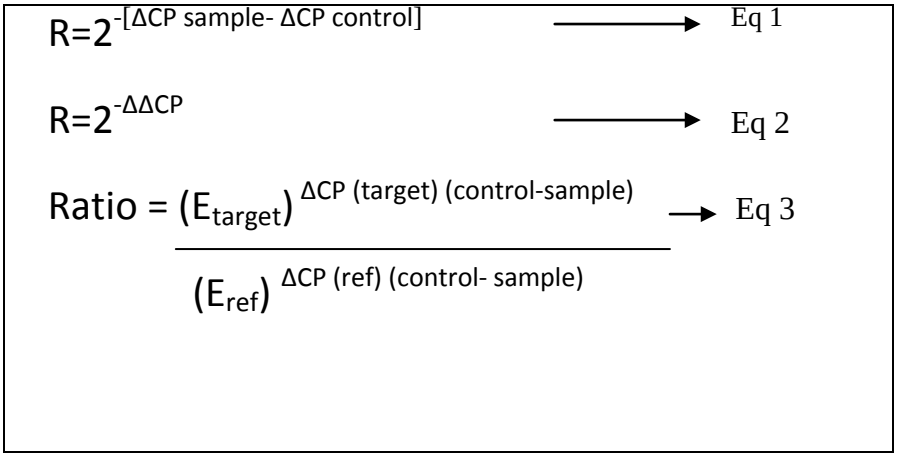


Fig 1.11 Equation to calculate relative quantification

1.8 Aim and rationale of the intended research project

Cryopreservation of fish reproductive tissue has important applications in aquaculture, conservation and biomedicine. The ability to cryopreserve both maternal and paternal gametes would provide a reliable source of fish genetic material for scientific and aquaculture purposes. Fish sperm cryopreservation of many species has been achieved, but cryopreservation of fish oocytes and embryos has not been successful (Zhang *et al*. 1995, Hagedorn *et al*. 1995). A number of studies on the cryopreservation of zebrafish oocytes have been carried out in our laboratory, the results showed that although ovarian follicle viability remained relatively high after cryopreservation and especially at early stages using vital stains, their ATP levels decreased dramatically after cryopreservation indicating damages to the energy system (Tsai *et al*. 2008). Ovarian tissue cryopreservation is a viable alternative to cryopreservation of oocytes or embryos in humans (Borges *et al*. 2009, Newton *et al*. 1999, Schmidt *et al*. 2003, Wood *et al*. 1997) and also proved to be effective on other species such as sheep, cattle and goat (Borges *et al*. 2009). Ovarian tissue cryopreservation has had much scientific and public attention due to their great potential use in human infertility treatment, in safeguarding the reproductive potential of the endangered species and in genome banking of genetically important lab animal strains (Agca. 2000). Cryopreservation of ovarian tissues has advantageous over the oocytes as they can be cultured and cryopreserved

in small pieces which are rich in primary follicles. Late stage oocytes are much more difficult to cryopreserve as they are much larger and have low volume to surface area ratios, they have also been shown to have low membrane permeability and highly sensitive to chilling (Isayeva *et al*. 2004, Isayeva *et al*, 2005). Hence studies on cryopreservation of fish ovarian tissues would need to be accompanied by the development of *in vitro* culture method of these tissues as although some studies have been carried out on culturing of isolated zebrafish oocytes (Ge *et al.* 2003), zebrafish ovarian tissue culture has not been studied systematically.

The present study focuses on:

(i) Studies on cryoprotectant toxicity to ovarian tissue fragments containing stage I and stage II follicles. Selection of cryoprotectants is an important step in designing the cryopreservation protocols; in the present study, as the first step for zebrafish ovarian tissue cryopreservation the impact of cryoprotectants on zebrafish ovarian tissue fragments was studied.

(ii) Development of *in-vitro* culture protocol for zebrafish ovarian tissue fragments and growth assessment of stage I and stage II ovarian follicles using biomarkers. *In vitro* culture of zebrafish ovarian tissue fragments are studied here for the first time although preliminary work had been done for isolated early stage zebrafish ovarian follicles in our laboratory. Although follicle growth can be assessed by measuring the diameter of the follicles, biomarkers would provide more important information on follicle development. The present study also focuses on the development of biomarker to identify the growth of early stage follicles before and after *in vitro* culture.

(iii) Development of cryopreservation protocols using controlled slow cooling for zebrafish ovarian tissue fragments. In the present study, cryopreservation of zebrafish ovarian tissue fragments containing stage I and stage II follicles are studied using controlled slow cooling. The effect of freezing medium, cooling rate, cryoprotectants, ovarian follicle developmental stage, different viability assessment methods were studied. The uses of disaccharides in the cryoprotectant mixture were also investigated.

CHAPTER 2 MATERIALS AND METHODS

2.1 Introduction

There were 3 main areas of study in the project: (i) cryoprotectant toxicity of ovarian tissue fragments containing stage I and stage II follicles; (ii) development of an *in-vitro* culture protocol for zebrafish ovarian tissue fragments and growth assessment of stage I and stage II ovarian follicles using biomarkers; and (iii) development of cryopreservation protocols using controlled slow cooling for zebrafish ovarian tissue fragments. All experimental work was carried out in the laboratories at the Institute of Research in the Applied Natural Sciences (LIRANS), University of Bedfordshire, UK.

2.2 General Methods

2.2.1 Maintenance of zebrafish (*Danio rerio)*

2.2.1.1 General Information

Adult zebrafish (12-14 weeks old) were obtained from Aquascape Ltd. (Birmingham, UK). They) were maintained in 40L fish tanks (30 x 30 x 60 cm). Adult zebrafish (both males and females) were kept in filtered and aerated 40L glass fish tanks (30 x 30 x 60 cm); at the maintained room temperature 27°C±2°C (pH 7.2-8) with a light/dark cycle of 12/12h. Approximately 40 fishes were kept in each tank with an in-tank filtration system. The water was replaced twice a week. Tap water aged for 2 days was used in the tanks in order to decrease the contents of chlorine; and also alternatively the tank was filled with deionised water by adding 2.5 g of salt per 10L water. The water was aerated and filtered using an electric pump connected to upright funnel contained filter floss in 1 L beaker immersed in 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.

2.2.1.2 Feeding

Fish were fed three times a day with 'Tetramin' (Tetra, Germany) dry fish flake food (ingredients: processed fish and fish derivatives, cereals, yeast, vegetable protein extracts, molluscs and crustaceans, oils and fats, derivatives of vegetable origin, algae, various sugars contains permitted colorants) and once in the afternoon with fresh brine shrimp (*Artemia salini)*. Brine shrimps cysts (ZM systems, UK) were prepared in sea-water- filled, aerated hatcheries (made up with 52.5g of sea salt (ZM systems, UK) in 1.5L distilled water maintained at 27±1° C.). During weekends and holidays, automatic fish feeders (Fish mate F14 aquarium fish feeder) were used to feed the fish.

2.2.1.3 Collection of ovarian tissue fragments containing stage I and stage II follicles

Cryopreservation of ovarian tissue is advantageous over the oocytes as they can be cultured and cryopreserved in small pieces which are rich in primary follicles. There are several studies undertaken on zebrafish oocytes cryopreservation at different stages (Guan *et al.* 2008, Zampolla *et al.* 2008, Tsai *et al.* 2009), they have resulted in compromised viability. Hence cryopreservation of zebrafish ovarian tissue fragments provides a promising alternative for zebrafish oocytes cryopreservation. Since the follicles within the ovarian tissue fragments remain in the natural three-dimensional structure (Fabbri *et al.* 2003). Studies on zebrafish oocytes has also shown that early stage oocytes are less sensitive to chilling when compared to the late stage oocytes (Isayeva *et al*. 2004, Plachinta *et al*. 2004, Tsai *et al*. 2009), hence tissue fragments containing early stage follicles (stage I and stage II) were used in the present study. Experiments were conducted on tissue fragments of 0.35-0.45mm in length and 2.3mm in thickness containing stage I and stage II ovarian follicles. To obtain the ovarian tissue fragments (Fig 2.1); the ovaries were

collected from adult female zebrafish which were anaesthetized with a lethal dose of tricaine (0.6mg/ml for 5-10mins), ovaries were removed after decapitation and were immersed immediately in 90% Leibovitz-15 (L-15) medium at pH 9. L-15 medium is widely used in fish ovarian follicles culture and for *in-vitro* maturation (Pang and Ge, 2002, Seki *et al*. 2008, Tsai *et al.* 2010). The ovarian tissue fragments containing stage I and stage II follicles were carefully dissected from the ovaries and were cut into thin slices (2.3mm) using syringe needles. The ovarian tissue pieces were flattened and stretched until stage I and stage II was clearly visible. The stage III ovarian follicles were separated by gentle pipetting of the ovaries. After dissection, the ovarian fragments were washed three times in L-15 medium and then were randomly distributed in wells of 6-well plates containing L-15 medium. Ovarian fragment dissections were carried out within 20 min at the room temperature.

Ovarian follicles within the tissue fragments were determined by light microscopy according to the criteria of the developmental stages described by Selman *et al*. (1993). Stage I ovarian follicles are transparent and the size ranges from 7-140µm; stage II ovarian follicles are translucent and are 140-340 µm.

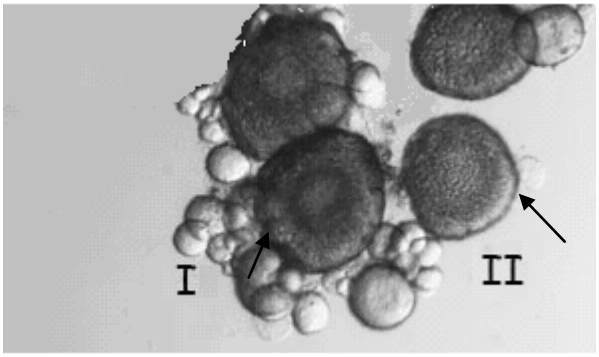


Figure 2.1: Ovarian tissue fragments containing stage I and stage II follicles

2.2.2 Chemicals

Information on the chemicals used in the present study is given in Table 2.1 of Appendix A. Fresh aqueous solutions were prepared in deionised water shortly before their use. If necessary, solutions were stored in the fridge (4°C) or freezer (-20°C).

Table 2.1 The chemicals used in the present study

Chemicals	Source	Product No.
Agarose	Bioline	BIO41025
Amphotericine B	Sigma	A2942
Albumin from bovine serum	Sigma	A8022
ATP assay kit	Sigma	FLAA213-579-1
BIOTAQ^{TM}DNA Polymerase	Bioline	BIO21040
Chronic gonadotropin, human (hCG)	Sigma	C1063
Dimethyl sulphoxide (DMSO)	Sigma	472301
dNTP mix	Bioline	BIO39053
EDTA	BDH	16079
Ethanol	Sigma	E7023
Ethylene glycol (EG)	BDH	10324
Ethidium bromide	Sigma	E1510
EZNA gel extraction kit	Omega Bio-tek	A112907W
Fluorescein diacetate (FDA)	Sigma	F7378
Follicle stimulating hormone (FSH)	Sigma	F4021
Fetal bovine serum (FBS)	Sigma	F6178
Gentamycin	Sigma	G1272
Hyperladder^{TM}V	Bioline	BIO33031
L-15 medium	Sigma	L4386
Methanol	Sigma	154903
Potassium chloride	Sigma	P3911
Potassium hydroxide	Aldrich	22147-3
Potassium iodide (PI)	Sigma	P4170
PBS tablet	Sigma	P4417

Propylene glycol	BDH	29673
Penicillin G	Sigma	P7794
Sensimix SYBR no-ROX kit	Bioline	QT650-05
Sucrose	Sigma	S5016
Trehalose	Sigma	T0167
Tris Buffer	Sigma	T5912
Tricaine	Sigma	A5040
Trypan blue (TB)	Sigma	T8154

2.3 Ovarian tissue fragments viability assessment

In the present study different arrays of viability assessment have been used for zebrafish ovarian tissue fragments: trypan blue (TB) staining, fluorescein diacetate (FDA) and propidium iodide (PI) staining, ATP (Adenosine 5´-triphosphate) assay.

2.3.1 Trypan blue assay

Trypan blue staining is a simple and widely used technique to assess the membrane integrity of cells. TB is a vital dye and does not penetrate the cells unless the membrane is damaged. To perform the TB assay, ovarian tissue fragments were incubated in 0.2% trypan blue for 3-5min at the room temperature and then washed with 90% L-15 medium. The stained follicles were considered non-viable and unstained follicles were considered viable. The follicles were observed under light microscope. And the viability of the follicles within the fragments was calculated as follows:

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

2.3.2 Fluorescein diacetate (FDA) and propidium iodide (PI) staining

FDA+PI staining assay is considered to be more sensitive to TB staining (Zampolla *et al.* 2006); this technique is used to assess both the physiological state and the membrane integrity of cells. In the FDA-PI assay, FDA stock solution was prepared by dissolving 5mg FDA in 1ml acetone and the PI stock was prepared by dissolving 1mg PI in 50ml Hank's solution. The FDA working solution was prepared freshly each time by adding 20µl of FDA stock in 5ml Hank's solution. To stain the ovarian tissue fragments, 0.1ml of FDA working solution and 30µl PI was added directly to the tissue fragments and the tissue fragments were incubated in the dark for 3-4 min at 22°C. The follicles were observed under an inverted fluorescence microscope (LEICA DM IL) with two filter cubes: 13 excitation filter: band pass (BP) 450-490nm; dichromatic mirror: 510; suppression filter: long pass (LP) 515 and N.2.1 filter excitation filters: BP: 515-560 nm; dichromatic mirror: 580; suppression filter: LP: 590. This filter arrangement does not permit both green and red fluorescing follicles to be seen simultaneously. The bright green fluorescing follicles was considered to be viable and the bright red stained follicles were considered as non-viable (Fig 2.2). And the viability of the follicles within the fragments was calculated as follows:

$$\text{Viability (\%)} = \frac{\text{Number of green stained cells}}{\text{Total number of cells (Green+ Red)}} \times 100$$

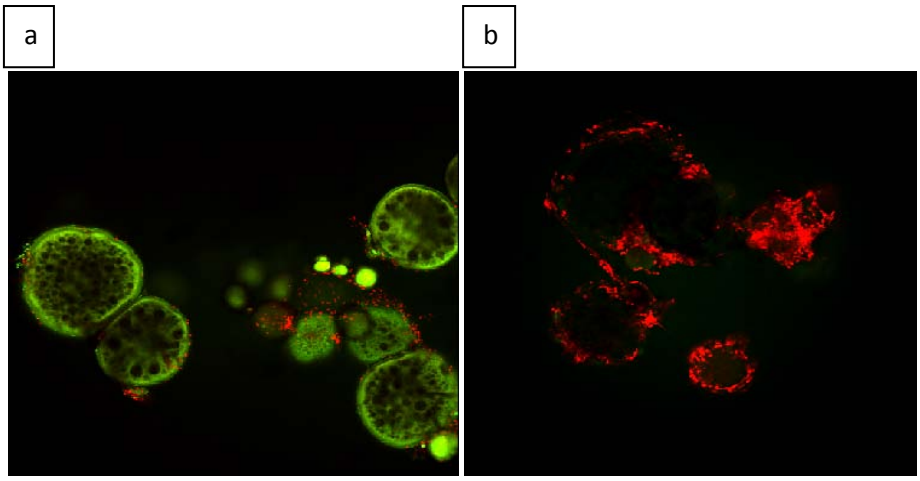


Figure 2.2: Stained ovarian fragments after FDA (a) +PI (b) staining. Follicles fluorescing bright green colour were considered to be viable, and follicles stained red were considered non-viable.

2.3.3 ATP (Adenosine 5´-triphosphate) assay

ATP assay is based on the level of the ATP content present in the cytoplasm of the oocytes to evaluate the viability of the oocytes. ATP is a vital substance for the normal development and reproductive potential of the oocytes. High ATP content is required for progression of immature oocytes through maturation stage, as meiosis and the associated processes consume large amounts of energy. It has been used in fish oocytes (Knoll-Gellida and Babin, 2007; Guan *et al*. 2008). The ATP content of the zebrafish ovarian follicles within the tissue fragment was evaluated by using ATP bioluminescent assay kit (FL-AA, Sigma).

Preparation of extract from the ovarian tissue fragments

Ovarian tissue fragments from control group and fragments exposed to different conditions were used for the preparation of extracts for ATP determination. For the extract preparation, three pieces ovarian tissue fragments in 1ml of ice cold 0.5M perchloric acid and 4mM EDTA were homogenised with a conical glass pestle. The

homogenate was centrifuged at 20,000g for 5min at 0.2°C in a refrigerated centrifuge. The supernatant was separated and neutralised to between pH6 and 7 with 2.5M KOH. The neutralised supernatant was centrifuged for 5min at 8000g and the new supernatant was collected. This extract was loaded into eppendorf tubes and stored at -20°C until ATP determination. For all experiments, three replicas were used for each treatment and experiments were repeated at least three times.

Preparation of FL-AA reagents

FL-AAM (ATP assay mix): The ATP assay mix contained: a lyophilized powder containing luciferase, luciferin, $MgSO_4$, DTT, EDTA, bovine serum albumin and tricine buffer salts, this mixture was dissolved in 5ml deionised water to make a stock solution with pH 7.8. The stock solution was stored at 5°C up to 2 weeks. Prior to the experiments, FL-AAM solution was diluted with FL-AAB (ATP assay mix dilution buffer) a 25-fold dilution was used for ATP concentrations of $2x10^{-10}$ to $2x10^{-7}$ mol/l.

FL-AAB (ATP assay mix dilution buffer): This mix contained lyophilizes powder containing $MgSO_4$, DTT, EDTA, bovine serum albumin and tricine buffer salts, this mixture was dissolved in 50ml of deionised water. This solution was stored at 5°C up to 2 weeks.

FL-AAS (ATP standard) - The ATP standard contained 1mg (2.0×10^{-6} mole) of ATP. The solutions were made in sterile deionised water. A number of solutions of decreasing ATP concentrations 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. Solutions were stable for 2 weeks when stored at -20°C or upto 24h at 5°C. A calibration curve was established from the fluorescence measurement emitted during reaction with FL-AAM.

Determination of ATP level

0.1ml of ATP assay mix solution was added to a vial, gently swirled and left to stand at room temperature for approximately 3 min. 0.1ml of the sample was added. The mixture was then swirled quickly and light emitted was immediately measured with a luminometer. To determine the amount of light produced, 0.1ml of deionised water was added to 0.01ml of ATP assau mix solution, the mixture was swirled and the luminescence was measured. The value obtained was subtracted from that of the samples. The final value was proportional to the amount of ATP in the sample. A calibration curve was established from different dilutions of FL-AAS, and this was then used to determine the ATP concentrations in the samples. ATP content of the ovarian fragments was measured using the luciferin-luciferase bioluminescence assay provided by a FL-AA kit. A luminometer (TD-20/20 luminometer-turner design) was used for all measurements. Light was measured and subtracted by running an appropriate blank. As directed by the manufacturer's instructions (Sigma –A1), a fresh calibration curve was constructed for each assay. The curve was constructed by plotting the log of relative luminescence intensity (RLI) against the log of ATP concentration (moles/l) using a serial dilution of an ATP standard. The least square method was used to convert ATP-induced light to moles of ATP according to the calibration plot prepared each day. The regression parameter (slope and intercepts) were used to convert test measurement to ATP:

$$\log_{10}(RLI_{measure}\text{-}RLI_{background}) = \text{slope} \times \log_{10}(ATP/M) + \text{intercept}$$

$$\log_{10}(ATP/M) = \log_{10}(RLI_{measure} - RLI_{background}) - \text{intercept}/\text{slope}$$

$$ATP/M = 10\text{^}((RLI_{measure}\text{-}RLI_{background}) - \text{intercept}/\text{slope}$$

ATP released from the fragments was monitored using the luciferin-luciferase bioluminescence assay in a luminometer (TD-20/20 Luminometer). 10sec integration period was used, with 2sec delay period before each integration period. The total amount of light produced by the sample was measured 5 times over a period of 10 minutes, in order to eliminate the quenching of luminescence with time. The mean

luminescence level of each sample was then calculated. For each treatment, three replicates were measured.

Statistical Analysis

Statistical analysis was carried out using SPSS (SPSS for windows version 16.0) and Microsoft Excel. The normality and homogeneity of the variance were tested. Comparisons were made by one-way ANOVA. Where difference was found, Tukey's post hoc test was carried out to establish which samples were significantly different from the control group. All data were expressed as mean ± SEM across the three replicates and *P* values of less than 0.05 were considered to be significant.

2.3.4 Confocal Microscopy

The samples were examined using a Leica TCS-SP/DM IRBE (Leica, Microsystems (UK) Ltd, Miltonkeynes, UK) confocal microscope equipped with Ar/Kr laser. The ovarian follicle growth within the fragments was assessed through bright field. Objectives (20X, 40X water immersion), pinhole, filters, gain and offset were kept constant throughout the experiment. The digital images were obtained with Leica TCS software and stored in TIFF format. The TIFF images were processed with the Leica LAS-AF Lite software. Atleast 10-15 fragments were analysed in three repeated experiments.

2.4 Studies on cryoprotectant toxicity to ovarian tissue fragment

The first step in any cryopreservation protocol is the exposure of the cells to cryoprotectant; this procedure can result in extreme fluctuations in volume, causing cell damage or making the cells more susceptible to damage during subsequent cooling. Hence, it is important to determine the impact of cryoprotectants on zebrafish tissue fragments as a first step in cryopreservation protocol. The optimum cryoprotectant (CPA) should have low toxicity and be able to permeate the cells

(Pegg, 2007). In order to identify the CPA in a suitable concentration for zebrafish ovarian tissue cryopreservation, studies on toxicities of five permeating cryoprotectants methanol, ethanol, dimethyl sulfoxide (DMSO), ethylene glycol (EG) and propylene glycol (PG) were carried out. Methanol has been shown to be the most effective cryoprotectant in zebrafish ovarian follicle studies in our laboratory (Zhang *et al*. 1996, Plachinta *et al*. 2004, Tsai *et al.* 2008, Zampolla *et al*. 2008). Ethanol has also been found to be an effective cryoprotectant for catfish (Muchlisin *et al*. 2009) sperm cryopreservation but it has not been used for zebrafish embryos or ovarian follicles cryopreservation before. DMSO is commonly used cryoprotectant in the cryopreservation of a wide range of cells types, tissues and organs. Both PG and EG have been widely used for mammalian oocytes and ovarian tissues.

2.4.1 Exposure of zebrafish ovarian tissue fragments to different cryoprotectants

The ovarian tissue fragments were placed in 90% L-15 medium at room temperature immediately after collection. Solutions of cryoprotectants were made up in 90% L-15 medium at a range of concentrations: 1M, 2M, 3M and 4M.

For cryoprotectant toxicity studies, 10-12 ovarian tissue fragments were placed into each well of the 6 well tissue culture plates. Ovarian tissue fragments were treated in 3ml cryoprotectant solution for 30min at room temperature. Control ovarian tissue fragments were incubated in 90% L-15 medium for 30min at room temperature. After incubation, the tissue fragments were washed twice in 90% L-15 medium and the viability of the ovarian tissue fragments were assessed immediately.

Ovarian tissue viability after cryoprotectant treatment was assessed using three different assays: TB staining, FDA+PI staining and ATP assay. Statistical analysis was carried out using SPSS (SPSS for windows version 16.0) and Microsoft Excel. The normality and homogeneity of the variance were tested. Comparisons were made by one-way ANOVA, where difference was found Tukey's post hoc test

was carried out to establish which samples were different. All data were expressed as mean ± SEM across the three replicates and *P* values of less than 0.05 were considered to be significant. Normalised ovarian follicles survival (Guan *et al*. 2008) was used to compare viability results obtained from the three different viability assessment methods following cryoprotectant exposure:

Normalised ovarian follicles survival (%) = (100/untreated control survival) x experimental survival

2.5 Development of *in-vitro* culture protocol for zebrafish ovarian tissue fragments

Development of *in vitro* culture protocol for zebrafish ovarian tissue fragments is important since cryopreserved early stage ovarian follicles would need to be matured *in vitro*. Studies on *in vitro* culture of ovarian tissue fragments has been reported here for the first time although preliminary work has been done for isolated early stage zebrafish ovarian follicles (Tsai *et al.* 2010). Although several procedures that can be applied on late stage of ovarian follicles have been reported (Seki *et al.* 2008) only a little is known about the early follicle development. Thus, the main aim of the present study was to develop an optimum *in- vitro* culture condition for zebrafish ovarian tissue fragments containing stage I and stage II follicles. The culture medium with growth supplements bovine serum albumin (BSA) and foetal bovine serum (FBS) provides better growth of ovarian follicles than when cultured in normal medium. And the use of growth hormones human chorionic gonadotropins (hCG) and follicle stimulating hormones (FSH) along with growth supplement enhance the culture system by stimulating proliferation and differentiation of granulose cells *in-vitro*. In these experiments, procedures for *in-vitro* culture of ovarian tissue fragments were developed. The effect of growth supplements BSA and FBS; and the effect of growth hormones hCG and FSH were

studied. The ovarian tissue fragments were cultured in 90% L-15 medium. In total, three replicates were used for each experiment with 3 tissue fragments (containing stage I and stage II follicles) in each replicate. The experiments were repeated at least three times.

2.5.1 Effect of growth supplements on the ovarian follicle growth competence within the tissue fragment

2.5.1.1 Effect of FBS on early stage ovarian follicle growth competence within the tissue fragment

Ovarian fragments were prepared for culture by washing three times in washing medium (0.01M PBS, 400µg/ml gentamycin, 200 U/ml penicillin and 2.5mg/ml amphotericine B). Ovarian fragments containing stage I and stage II follicles were placed in 1.5ml of 90% L-15 medium (pH 9) containing 10, 20, 25% FBS for 24h at 28° C in 6-well tissue culture plates. The culture medium was freshly made and filter sterilized. One piece of ovarian fragment was individually cultured. Control ovarian fragments were incubated in 90% L-15 medium (pH 9). After *in vitro* culture, ovarian follicle growth within the fragment was assessed by measuring the diameter in bright field with confocal microscope.

2.5.1.2 Effect of BSA on early stage ovarian follicle growth competence within the tissue fragment

Ovarian fragments were prepared for culture as described above. Ovarian fragments containing stage I and stage II follicles were placed in 1.5ml of 90% L-15 medium (pH 9) containing 0.5% BSA (Seki *et al*. 2008) for 24h at 28°C in 6-well tissue culture plates. The culture methods and assessment methods described in (see paragraph 2.5.1.1) were used in this experiment.

2.5.2 Effect of growth hormones on the ovarian follicle growth competence within the tissue fragment

2.5.2.1 Effect of hCG on early stage ovarian follicle growth competence within the tissue fragment

Ovarian fragments were prepared for culture as described above. Ovarian fragments containing stage I and stage II follicles were placed in 1.5ml of 90% L-15 medium (pH 9) containing 10IU/ml hCG for 24h at 28° C in 6-well tissue culture plates. The culture methods and assessment methods described in (see paragraph 2.5.1.1) were used in this experiment.

2.5.2.2 Effect of FSH on early stage ovarian follicle growth competence within the tissue fragment

Ovarian fragments were prepared for culture as described above. Ovarian fragments containing stage I and stage II follicles were placed in 1.5ml of 90% L-15 medium(pH 9) containing 10, 20, 30, 40, 60, 80, 100 and 120 mIU/ml FSH for 24h at 28° C in 6-well tissue culture plates. The culture methods and assessment methods described in (see paragraph 2.5.1.1) were used in this experiment.

2.5.3 Confocal microscopy

The cultured samples were examined using a Leica TCS-SP/DM IRBE (Leica, Microsystems (UK) Ltd, Miltonkeynes, UK) confocal microscope equipped with Ar/Kr laser (see paragraph 2.3.4).

2.6 Growth assessment of early stage follicles within the tissue fragments using biomarkers before and after *in-vitro* culture

Based on the results obtained from the *in-vitro* culture study in the present study, the tissue fragments containing stage I and stage II follicles could be cultured in 90% L-15 medium (pH 9) with 20% FBS and 100IU/ml FSH for 24 h at 28°C. The study demonstrated the follicle growth in size from stage I and stage II or stage II to stage III respectively. Although the follicle growth could be assessed by measuring the diameter of follicles using confocal microscopy (see paragraph 2.3.4), biomarkers would provide more vital information on the follicular development. The aim of the present study was to develop a biomarker to identify stage II and stage III ovarian follicles after *in vitro* culture. Studies on the levels of vtg1 and p450aromA genes in stage I, II, III ovarian follicles of zebrafish before and after culture were carried out using PCR. Vtg1 and p450aromA genes are known to be expressed in zebrafish ovary (Munck *et al.* 2006; Ings *et al*. 2006).

2.6.1 To study the p450aromA and vtg1 gene expression on early stage ovarian follicles

In the present study, gene expression studies were carried out in zebrafish ovarian follicles. Gene expression experiment was carried out for vtg1 gene and p450aromA gene at different ovarian follicle developmental stages (stage I, stage II and stage III follicles). The process includes RNA extraction, DNAse treatment, reverse transcription, polymerase chain reaction (PCR), and analysis of PCR product using agarose gel.

2.6.1.1 RNA extraction

Total RNA was extracted from ovarian follicles using trizol (Invitrogen, UK) method; using manufacturer's protocol. RNA was extracted from stage I, stage II and stage III ovarian follicles. 20-30 ovarian follicles were collected for each stage in separate effendorf tubes respectively. The steps involved in RNA extraction are detailed below:

1. 125µl of trizol was added into each tube.
2. The tubes were vortexed until the samples are digested completely.
3. 25µl of chloroform per 1ml trizol was added and was mixed vigorously. This gives rise to a pale pink colour solution.
4. Centrifuged the tubes at 12000g for 15min at 4°C, and the aqueous layer were transferred into the new effendorf tubes.
5. Isopropanol was added to the aqueous solution in a 1:1 ratio; and the tubes were vortexed.
6. The tubes were refrigerated for 20min at 4°C, after which, the excess isopropanol was removed.
7. 1ml of 75% ethanol was added to these tubes and was centrifuged at 7500g for 8 min at 4°C.
8. The excess ethanol was removed and the samples were air dried. The extracted RNA was dissolved in 50µl PCR water for DNAse treatment.

2.6.1.2 DNAse treatment

DNAse treatment was performed to remove any genomic DNA contamination in the extracted RNA. The steps involved in the DNAse treatment are detailed below:

1. 0.1 volume of DNAse buffer I(100mM Tris, 25mM $MgCl_2$, 1mM $CaCl_2$) and 1µl DNAse I enzyme was added to the extracted RNA sample and incubated at 37°C for 20min.
2. 0.1 volume of DNAse inactivation reagent was added to the sample-enzyme mixture and was incubated for 2min at room temperature.
3. Sample mixture was centrifuged at 13000rpm for 1.5min to pellet the inactivation reagent and genomic DNA.
4. RNA containing the supernatant was collected and stored at -80°C until further use.

2.6.1.3 Total RNA quantification

RNA quantification was carried out using Biophotometer^{TM}(Eppendorf, Germany). RNA was diluted 1:50 with PCR water (Sigma, UK) in the cuvette(Eppendorf, Germany) and the absorbance was read at 260 and 280nm. The Biophotometer^{TM} automatically calculated the RNA concentration in the original sample using the following equation:

(Absorbance at 260nm x RNA's co-efficient) X Dilution factor = µg/ml of RNA

Where the RNA coefficient= 40ng/µl and dilution factor= 50

The Biophotometer^{TM} also showed the purity of the RNA by calculating the absorbance ratio at 260nm and 280nm. The pure RNA sample was ranged from 1.7-2.1.

2.6.1.4 cDNA synthesis (Reverse transcription)

RNA was converted into cDNA using reverse transcriptase. Aliquots of total RNA(1µg) was transcribed using the precision qScript reverse transcription kit (Primer design Ltd, UK) according to the manufacturers protocol. The steps involved are detailed below:

1. 1 µg RNA was mixed with 1µl oligodT primers; and the mixture was made up to 10µl volume with RNAse/DNAse free water.
2. The RNA mixture was incubated at 65°C for 5min and chilled for 2min on ice.
3. To the mixture in step 2; nanoscript buffer (2 µl), dNTP mix (10 mM each -1 µl), DTT (100 mM – 2 µl), nanoscript reverse transcriptase (1 µl) was added and the final volume of 20 µl was made up using DNAse/RNAse free water.
4. The reaction mixture (step 3) was incubated at 55°C for 20min, followed by the termination of the reaction by inactivation enzyme at 75°C for 15min.
5. Thermocycler PCR machine (Techne, UK) was used in both incubation steps (in step 2 and step 4).

6. For conventional PCR, undiluted cDNA was used subsequently; and for real time PCR experiment, cDNA was diluted 1:2 in molecular biology grade water (Sigma, UK) and stored at -80°C.

2.6.1.5 Conventional polymerase chain reaction (PCR)

Polymerase chain reaction (PCR) was performed for each gene and internal control housekeeping genes. For each gene specialized primer pairs were designed and used for amplification and detection. Forward and reverse primers, annealing temperatures and product sizes are given in Table 2.2.

Table 2.2

Information of gene name, accession ID and primer sequences inclusing annealing temperature and product size

Gene name	Accession ID	Forward/reverse primer(5'-3')	Annealing temp. (°C)	Amplicon size (bp)
P450aromA	AF226620.1	F:CAGACTGGACTGGCTGCACAAGAA R:TGTCTGGAGCCGCGATCACCAT	67	221
Vtg1	NM_22.4	F:ACTACCAACTGGCTGCTTAC R:ACCATCGGCACAGATCTTC	63	100
EF1-α	NM_131263.1	F:CTGGAGGCCAGCTCAAACAT R:ATCAAGAAGAGTAGTACCGCTAGCATTAC	60	87

The PCR reactions consisted of NH_4 PCR buffer (Bioline, UK), 200µM dNTP (Bioline), 1.5mM $MgCl_2$ (Bioline), 2U BIOTAQ^{TM} DNA polymerase (Bioline), 0.5 µM each primer (see Table), 1µg RNA template and PCR water. The conditions for PCR were initial denaturation at 94°C for 5min, 40 cycles of amplification at 94°C for 30s, annealing temperature(see Table 2.3) for 30s and the extension step at 72°C for 10min. The PCR products were run on 2% agarose gels. PCR reactions was performed at the same time for samples from different ovarian follicle

developmental stages (stage I, II & III) along with control housekeeping gene EF1-α. Housekeeping genes are responsible for basic cellular function and expressed similar level across many types of cells.

Table 2.3

Gene name	Intial Denaturation	Amplification temperature	Additional extension
P450aromA	94°C for 5 min	40 cycles 94 for 30 seconds 67 for 30 seconds 72 for 30 seconds	72°C
Vtg1	94°C for 5 min	40 cycle 94 for 30 seconds 63 for 30 seconds 72 for 30 seconds	72°C
EF1-α	94°C for 5 min	40 cycle 94 for 30 seconds 60 for 30 seconds 72 for 30 seconds	72°C

2.6.1.6 Analysis of PCR product

The PCR product was analysed usin agarose gel electrophoresis and ethidium bromide stain. 2% agarose gel was prepared by dissolving 2% agarose powder in TAE buffer (Sigma, UK); the mixture was dissolved by warming gently. 0.5 µg/ml of ethidium bromide (Sigma, UK) was added to the warm agarose solution, the solution was mixed and poured into the gel cast with comb immersed to form wells to load the samples, and the gel cast was left to set for 20min. Samples were mixed with the gel loading buffer (Sigma, UK) and loaded into well along with Hyperladder^{TM} V (Bioline, UK). The Gel was run at constant voltage at 100V for 2hours and the gel was visualized using Genosmart UV gel documentation system (VWR, UK).

2.6.2 Studies of p450aromA and vtg1 gene expression on early stage ovarian follicles using quantitative qPCR

This experiment investigates the level of expression at different ovarian follicle development stages. In order to assess the gene expression level at stage I, II and III ovarian follicles quantitative analysis has been done. Quantification measures the relative change in mRNA expression levels. This assay can detect gene expression difference as least as 23% between the samples (Gentle *et al.* 2001).

2.6.2.1 RNA extraction and cDNA synthesis

RNA was extracted from ovarian follicles using trizol (Invitrogen, UK) method; using manufacturer's protocol. RNA was extracted from stage I, stage II and stage III ovarian follicles. 20-30 ovarian follicles were collected for each stage in separate effendorf tubes respectively. The protocol for RNA extraction and DNAse treatment was given in section 2.6.1.1-2.6.1.2.

RNA was then converted into cDNA using reverse transcriptase. RNA (1µg) was reverse transcribed using Precision nanoscript reverse transcription kit (Primerdesign, UK) according to manufacturer's protocol. The protocol involved denaturation and annealing (see section 2.6.1.4). cDNA was diluted in 1:2 with molecular biology grade water before use in real time PCR.

2.6.2.2 Generation of standards for Real time PCR

The standards for real time PCR of p450aromA and vtg1 along with housekeeping gene EF-1α were produced using conventional PCR. The primer sequences were given in the Table 2.1. PCR reaction was performed according to the procedure given in the section 2.6.1.5. The PCR product was run on 2% agarose gel and the DNA was isolated from the excised bands using EZNA gel extraction kit (Omega Bio-Tek) according to the manufacturer's protocol. The isolated DNA was quantified using BioPhotometer (Eppendorf, UK) at 260nm. The steps involved are detailed below:

1. The excised gel slice containing DNA fragment of interest is transferred into a 1.5 ml centrifuge tube, and added an equal volume of binding buffer and incubated at 55-60°C for 7 min or until the gel melts completely.
2. The solution was applied to the HiBind® DNA mini column containing 2 ml collection tubes and the whole assembly was centrifuged at 13000 x g for 1 min.
3. The HiBind® DNA mini column was washed with 300µl of binding buffer and the flow through was discarded.
4. The HiBind® DNA mini column was followed by two other washes using 700µl of SPW wash buffer and air dried by centrifugation at 13000 x g for 2 min.
5. The flow through was discarded and the empty HiBind® DNA mini column was centrifuged at 13000 x g for 2 min to dry the column.
6. The HiBind® DNA mini column was placed into a sterile centrifuge tube and the DNA was eluted with 30-50 µl of elution buffer.

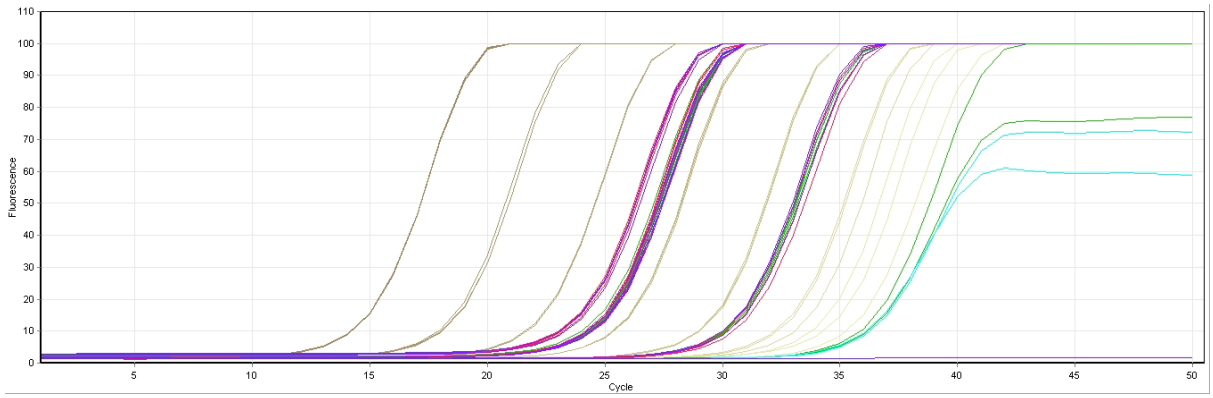
2.6.2.3 Quantification of p450aromA and vtg1 genes using real time PCR

Real time PCR was performed on RotorGene 6000 cycler (Corbett Research, UK) using a 72 well rotor to quantify expressions levels of p450aromA and vtg1. The reaction tube contained 7.5µl of sensimix 2X reaction buffer (contains heat activated DNA polymerase, Ultrapure dNTPs, $MgCl_2$, SYBR® Green I), 333nm of each primers (see Table 2.1) and 2 µl of cDNA sample diluted in PCR water (Sigma, UK). The reaction conditions were 1 cycle at 95°C for 10 min, followed by 50 cycles at 95°C for 10 seconds, at appropriate annealing temperature (see Table 2.1) for 15 sec and at 72°C for 15 sec. Data were acquired on FAM/SYBR channel at the end of each extension step. Melt cure was analysed to check the absence of mis-priming and amplification efficiency from a standard curve (R^2 should be close to 1). The relative gene expression data were obtained using RotorGene software (version 1.7, Corbett research) and Microsoft excel. Relative gene expression levels were calculated using relative quantification method.

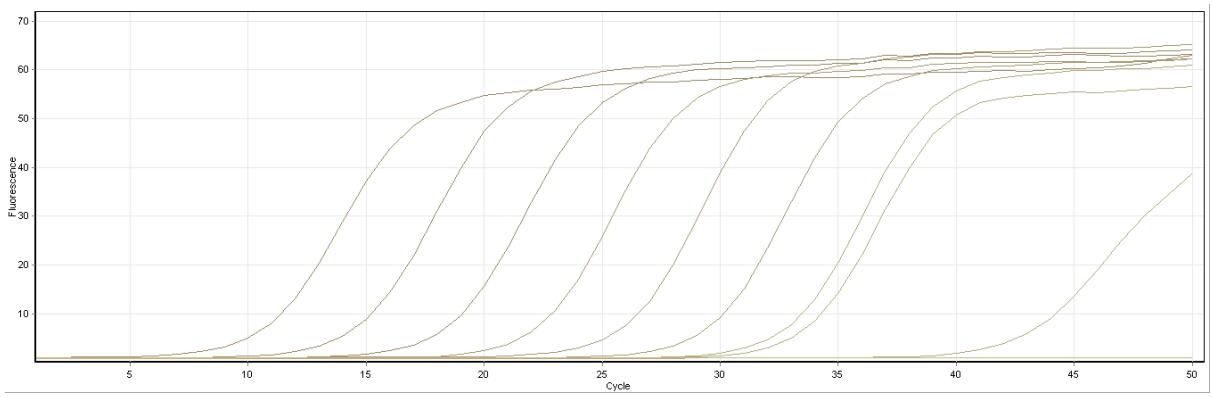
SYBR green I dye fluoresces when it is bound to the double strand DNA. At the end of each extension step, florescence data was collected (Fig 2.3a). Early PCR cycle shows very low florescence which is undetectable, as the cycle progresses the florescence level also increases until plateau is reached. The standards of 10-fold dilution of DNA showed equally spaced curves on the florescence graph (Fig 2.3b). The cycle number at which threshold level is reached can be used to create a standard curve from which sample data can be quantified (Fig 2.3c). The melting point of the PCR product is also determined using the melting curve. The melting point of the product is also determined at the end of amplification (Fig 2.3d).

Amplification curve (Fig 2.3a is mainly divided into four phases: (1) linear ground phase (2) early exponential phase (3) exponential phase and (4) plateau phase (Tichopad *et al.*2003). During linear ground phase, florescence emission does not increase above the background. At the early exponential phase, the amount of florescence starts to increase and reach threshold where it is significantly higher from the ground phase. The cycle number at this time is called the crossing point; this value is used to calculate gene expression (Heid *et al.* 1996). The PCR product doubles every cycle during the linear phase and reaches the optimal amplification period in the plateau stage. During the plateau stage the reactions starts lacking PCR components and the florescence intensity is no longer useful for data collection (Bustin, 2000)

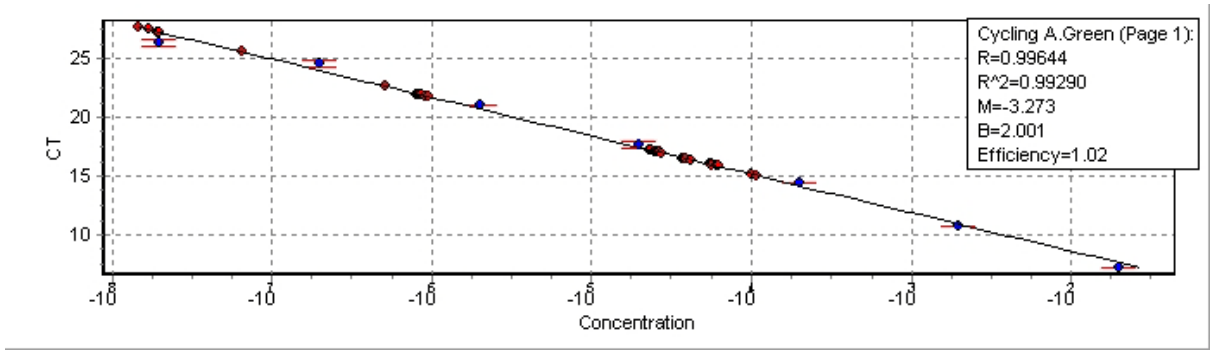
(a)


Fluorescence
Cycle

(b)


Fluorescence
Cycle

(c)


Cycling A.Green (Page 1):
R=0.99644
R^2=0.99290
M=-3.273
B=2.001
Efficiency=1.02
CT
Concentration

(d)

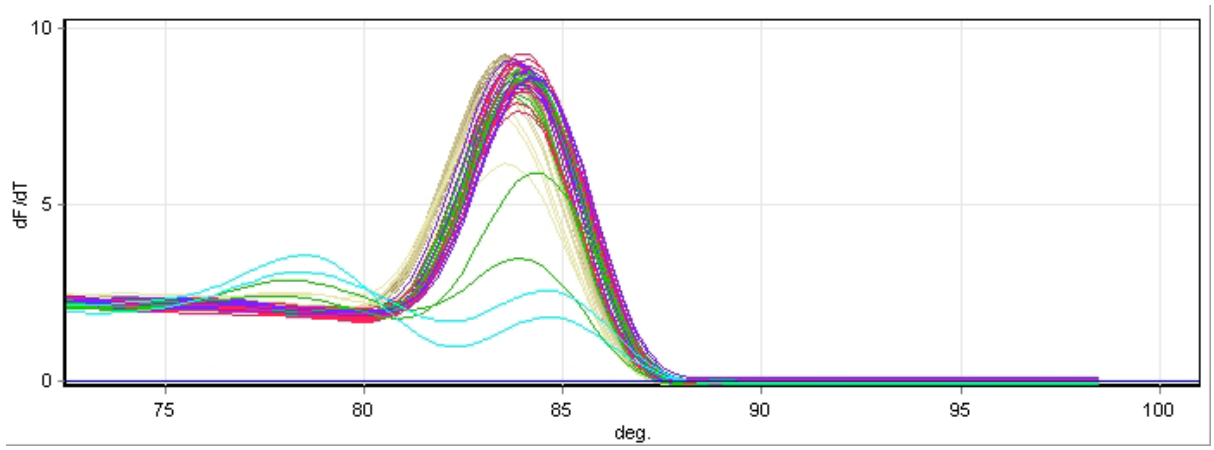


Fig 2.3 Graph produced during real time PCR experiments: (i) fluorescence measurement after each cycle (ii) standards of decreasing concentration crossing the threshold line with increasing cycle numbers (iii) standard curve produced from 10-fold dilution concentration of equally spaced standard concentration (iv) melting curve using real time PCR

2.6.2.4 Relative quantification

Relative gene expression levels were calculated using the two- standard curve quantification method with kinetic PCR efficiency correction (equation below) in the rotorgene software (Pfaffl 2003). Relative quantification is calculated based on the expression levels of the targer versus the housekeeping gene (Pfaffl, 2003). Calculations are based on the comparison of the distinct cycle determined by crossing points (CP) and threshold values (Ct) at a constant level of fluorescence. Kinetic PCR efficiency correction (see equation below) was used to quantify relative gene expression.

$$\text{Relative quantification} = \frac{(E_{target})^{\Delta CP\ (target)\ (control\text{-}sample)}}{(E_{ref})^{\Delta CP\ (ref)\ (control\text{-}sample)}}$$

Where E is the real time PCR efficiency and ΔCP is the crossing point difference between the unknown sample and the control sample.

Negative control (reverse transcriptase control and no template control) was used in all experiment and data was omitted where both controls showed gene expression.

2.7 Development of cryopreservation protocols for zebrafish ovarian fragments using controlled slow cooling

Several studies has been undertaken on zebrafish oocyte cryopreservation at different stages (Guan *et al*. 2008, Zampolla *et al*. 2008, Tsai *et al*. 2009); however they resulted in compromised viability. Hence cryopreservation of zebrafish ovarian tissue provided a promising alternative for zebrafish oocytes cryopreservation. Cryopreservation of ovarian tissue fragments is advantageous over oocytes as they can be cryopreserved in small pieces which are rich in primary follicles and the follicles within the fragments could stay intact in their natural three-dimensional structure. Investigations were carried out on cryopreservation of zebrafish ovarian tissue fragments using controlled slow cooling method. In this experiment, the effect of different freezing medium; the effect of different cooling rate and the effect of different cryoprotectants were studied. In total, three replicates were used for each experiment with 3-5 fragments in each replicate. The experiments were repeated at least three times.

2.7.1.1 Effect of different freezing medium

L-15 medium is widely used in fish culture and *in-vitro* studies. The amino acid supplemented medium has been widely used for the *in-vitro* growth of animal cells. KCl buffer has been used in cryopreservation of zebrafish oocytes (Guan *et al.* 2008, Tsai *et al*.2009). Therefore, KCl buffer and 90% L-15 medium

were used as freezing media in the present study. As methanol was found to be the most effective cryoprotectant in the previous experiment, the no observed effect concentrations (NOECs) of 2M methanol was used for these experiments. 2M methanol was made up in 90% L-15 medium or KCl buffer (KCl buffer composition: 55mM KCl, 55mM potassium acetate, 1mM $MgCl_2$, 2mM $CaCl_2$, 10mM HEPES). Ovarian tissue fragments were exposed to cryoprotectant solutions for 30 min at 22°C and then were loaded into 0.5ml plastic straws before placing in a programmable cooler (Planer KRYO 550). Ovarian tissue fragments incubated in cryoprotectant-free L-15 medium or KCl buffer were used as controls. The following cooling protocol was used: cooling at 2°C/min from 20°C to seeding temperature (-7.5°C for 2M methanol). Manual seeding and hold for 5 min, freezing from seeding temperature to -40°C at 2°C/min, from -40°C to -80°C at 10°C/min and hold for 10 min, samples were then plunged in liquid nitrogen at -196°C and held for at least 10min. Samples were thawed using a water bath at 28°C. Removal of cryoprotectant was conducted in four steps (1M methanol, 0.5M methanol and 0.25M methanol in 90% L-15 medium, 2.5 min for each step). Ovarian tissue fragments viability was assessed using trypan blue and FDA-PI staining after thawing.

2.7.1.2 Effect of cooling rate

In these experiments six different cooling rates were used: 0.3°C/min, 0.4°C/min, 1°C/min, 2°C/min, 4°C/min and 7°C/min. As 90% L-15 medium was shown effective in the previous experiments, it has been used in these experiments. Ovarian tissue fragments were exposed to 2 M methanol (made up in 90% L-15 medium) for 30 min at room temperature and then loaded into 0.5ml plastic straws before placing in a programmable cooler (Planar KRYO 550). Ovarian tissue fragments incubated in cryoprotectant-free 90% L-15 medium were used as controls. The following cooling protocols were used: cooling at 2°C/min from 20°C to seeding temperature (-7.5°C for 2M methanol), manual seeding and held for 15 min, freezing from seeding temperature to -40°C at 0.3°C/min, 0.4°C/min, 1°C/min, 2°C/min, 4°C/min and 7°C/min, from -40°C to -80°C at 10°C/min and hold for 10

min, samples were then plunged in liquid nitrogen at -196 °C and held for at least 10 min. Samples were thawed using a water bath at 28°C. Removal of cryoprotectant was conducted in four-step (1M methanol, 0.5M methanol and 0.25M methanol in 90% L-15 medium, 2.5 min for each step). Ovarian tissue fragments were incubated in 90% L-15 medium at room temperature for 10 min and 120 min after thawing. The ovarian follicles within the tissue fragment was assessed using trypan blue staining, FDA-PI staining, ATP assay and *in-vitro* culture after freeze thawing.

2.7.1.3 Effect of different cryoprotectants

Two cryoprotectants were used in these experiments: methanol and ethanol. The no observed effect concentrations (NOECs) for methanol and ethanol for stage I and stage II follicles within the follicles were 2M in the previous experiments, therefore 2M were used in the controlled slow cooling experiments. 2M methanol and 2M ethanol was made up in 90% L-15 medium. The ovarian tissue fragments were exposed to cryoprotectant solutions for 30 min at room temperature and then were loaded into 0.5ml plastic straws before placing in a programmable cooler. Ovarian tissue fragments incubated in cryoprotectant-free 90% L-15 medium were used as controls. The following cooling protocols were used: cooling at 2°C/min from 20°C to seeding temperature (-7.5°C for 2M), manual seeding and held for 15 min, freezing from seeding temperature to -40°C to -80°C at 10°C/min and hold for 10 min, samples were then plunged in liquid nitrogen at -196 °C and held for at least 10 min. Samples were thawed using a water bath at 28°C. Removal of cryoprotectant was conducted in four-step (1M methanol, 0.5M methanol and 0.25M methanol in 90% L-15 medium, 2.5 min for each step). Ovarian tissue fragments were incubated in 90% L-15 medium at room temperature for 10 min and 120 min after thawing. The ovarian follicles within the tissue fragment was assessed using trypan blue staining, FDA-PI staining, ATP assay and *in-vitro* culture after freeze thawing.

2.7.2 *In-vitro* culture after cryopreservation of ovarian tissue fragments

90% L-15 medium (pH 9) containing 100mIU/ml FSH and 20% FBS was found effective for culturing the ovarian tissue fragments containing stage I and stage II follicles in previous experiment. The tissue fragment were cultured under these conditions after freeze-thawing, the ovarian fragments were washed twice in 90% L-15 medium (pH 9) and were prepared for culture by washing three times in the washing medium (0.01M PBS, 400µg/ml gentamycin, 200 U/ml penicillin and 2.5mg/ml amphotericine B). One fragment were cultured in 1.5ml of 90% L-15 medium (pH 9) containing 100mIU/ml FSH and 20% FBS in 6 welled plates for 24 h. Ovarian follicle growth within the fragment was assessed by measuring the diameter with an ocular micrometer under microscope. Three replicates were used for each experiment. The experiments were repeated at least three times.

2.7.3 Study the effect of using non-permeating cryoprotectants on the ovarian tissue fragments

Research has demonstrated that sugars are useful in the stabilization of lipid membranes and proteins when cells are dehydrated (Crowe *et al*. 1998). In nature, a variety of organisms including arctic frogs, salamanders, insects, brine shrimps, bacteria, yeasts, fungi, some plant seeds tolerate extreme conditions due to accumulation of large amounts of intracellular sugars (Crowe *et al*. 1992, Potts, 1994). Cells contain free water and water that is bound to atoms and proteins and membrane phospholipids (Shaw and Jones, 2003). As cells are dehydrated during cryopreservation, a balance is maintained between the removal of free water that could form ice crystals without the excessive removal of bound water, resulting in loss of structural support to the proteins and lipids. It is thought that the sugars may serve as a replacement for bound water on the membranes to diminish injury from dehydration stresses (Wright *et al.* 2004).

2.7.3.1 Studies on cryoprotectant toxicity on ovarian tissue fragment using disaccharides

In order to identify the disaccharides in a suitable concentration for zebrafish ovarian tissue cryopreservation, studies on toxicities with sucrose and trehalose were carried out. As methanol was found to be the most effective cryoprotectant for cryopreservation in the previous experiments, 2M methanol was used for these experiments. Studies on toxicities were carried out in the following condition: 2M methanol along with sucrose or trehalose and 2M methanol along with 20%FBS and sucrose or trehalose. 20% FBS was used in this study since it was found to be effective for zebrafish ovarian tissue fragment culture in the previous experiment.The ovarian tissue fragments were placed in 90% L-15 medium at room temperature immediately after collection. Solutions of 2M methanol were made up in 90% L-15 medium at a range of concentrations of sucrose and trehalose: 0.1M, 0.2M and 0.5M.

For cryoprotectant toxicity studies, 10-15 ovarian tissue fragments were put into each well of the 6-well culture plate. The 90% L-15 medium was then removed and 3ml cryoprotectant solution made up with sugars was added. The ovarian fragments were incubated in this mixture for 30 min at room temperature. Control ovarian fragments were incubated in 90% L-15 medium without the CPA + sugar mixture under the same conditions. After incubation, the ovarian fragments were washed twice with 90% L-15 medium and the viability tests were conducted. Three viability assessment methods were used in this study: trypan blue staining, fluorescein diacetate (FDA) + propidium iodide (PI) staining and ATP assay.

2.7.3.2 Controlled slow cooling procedure for zebrafish ovarian tissue fragments with the addition of sucrose and trehalose

After the toxicity studies, investigations were carried out on cryopreserving the ovarian tissue fragments using controlled slow cooling. In this experiment the effect of freezing medium were studied. In total, three replicates were used for each experiment with 3-5 fragments in each replicate. The experiments were repeated at least three times.

2.7.3.2.1 Effect of different cryoprotective medium

As 2M methanol along with 0.1M Sucrose and 0.1M Trehalose was found to be the most effective cryoprotectant mixture in the previous experiment, this medium were used as freezing media in the present study. Ovarian tissue fragments were exposed to cryoprotectant solutions for 30 min at 22°C and then were loaded into 0.5ml plastic straws before placing in a programmable cooler (Planer KRYO 550). Ovarian tissue fragments incubated in cryoprotectant-free L-15 medium were used as controls. The following cooling protocol was used: cooling at 2°C/min from 20°C to seeding temperature (-7.5°C for 2M methanol). Manual seeding and hold for 5 min, freezing from seeding temperature to -40°C at 4°C/min, from -40°C to -80°C at 10°C/min and hold for 10 min, samples were then plunged in liquid nitrogen at -196°C and held for at least 10min. Samples were thawed using a water bath at 28°C. Removal of cryoprotectant was conducted 4 steps (1M methanol+0.1M sucrose or 0.1M trehalose, 0.5M methanol +0.05M sucrose or trehalose and 0.25M methanol or 0.025M sucrose or trehalose in 90% L-15 medium, 2.5 min for each step). Ovarian tissue fragments viability was assessed using trypan blue and FDA+PI staining after thawing.

2.7.3.2.2 Effect of different cooling rate

In these experiments two different cooling rates were used: 2°C/min and 4°C/min. The optimal cooling rates for the ovarian tissue fragments containing stage I and stage II follicles were found to 4°C/min from the previous experiment. Hence these cooling rates have been used in these experiments. Ovarian tissue fragments were exposed to 2 M methanol (made up in 90% L-15 medium) along with 0.1 M Sucrose or 0.1M Trehalose for 30 min at room temperature and then loaded into 0.5ml plastic straws before placing in a programmable cooler (Planar KRYO 550). Ovarian tissue fragments incubated in cryoprotectant-free 90% L-15 medium were used as controls. The following cooling protocols were used: cooling at 2°C/min from 20°C to seeding temperature (-7.5°C for 2M methanol), manual seeding and held for 15 min, freezing from seeding temperature to -40°C at 2°C/min

and 4°C/min, from -40°C to -80°C at 10°C/min and hold for 10 min, samples were then plunged in liquid nitrogen at -196 °C and held for at least 10 min. Samples were thawed using a water bath at 28°C. Removal of cryoprotectant was conducted in four-step (1M methanol+0.1M sucrose or 0.1M trehalose, 0.5M methanol +0.05M sucrose or trehalose and 0.25M methanol or 0.025M sucrose or trehalose in 90% L-15 medium, 2.5 min for each step). Ovarian tissue fragments were incubated in 90% L-15 medium at room temperature for 10 min and 120 min after thawing. The ovarian follicles within the tissue fragment was assessed using trypan blue staining, FDA-PI staining, ATP assay after freeze thawing.

2.8 Data analysis

2.8.1 Statistical analysis

In the study, nearly 10-15 ovarian tissue fragments were used in each treatment and each treatment was replicated for at least three times. Statistical analysis was carried out using SPSS (version 18.0). One-sample kolmogorov-smirnov test was used to make sure the data was normally distributed. One-way ANOVA was performed and homogeneous of variance was tested using Levene's test ($p > 0.05$). Where differences were found Tukey's post –hoc test was carried out in order to find out which groups differ. Independent sample student's t-tests were used if only two-groups were compared. All data were expressed as mean ±SEM across the three replicates and p values of less than 0.05 were considered to be significant.

CHAPTER 3 STUDIES ON CRYOPROTECTANT TOXICITY TO ZEBRAFISH (*Danio rerio)* OVARIAN TISSUE FRAGMENTS

3.1 Introduction

Cryopreservations of gametes of aquatic species play an important role in preserving the genetic heritage of these species and development of cryobanks allows storage of the genetic materials for unlimited periods. One of the important factors that lead to successful cryopreservation is the addition of cryoprotectants during the course of freezing. Cryoprotectants (CPAs) are the chemical substances characterised by their ability to reduce cryoinjury during freezing. They help to prevent ice nucleation within the cells (Yang *et al.*2009). However, cryoprotectants can be toxic to cells especially used at higher concentrations. They may cause osmotic stress to cells and disarrangements of lipid bilayer (Fahy 1986). Toxicity of the cryoprotectants is dependent on the type and concentration of the cryoprotectants, the temperature and duration of exposure (Fahy *et al.* 1984; Steponkus *et al*. 1991). Hence the ideal cryoprotectant should have low toxicity and be able to permeate the cells. As a first step in cryopreservation protocol, it is important to determine the impact of cryoprotectants on zebrafish tissue fragments.

In order to identify the CPA in a suitable concentration for zebrafish ovarian tissue cryopreservation, studies on toxicities of five permeating cryoprotectants methanol, ethanol, dimethyl sulfoxide (DMSO), ethylene glycol (EG) and propylene glycol (PG) were carried out. Methanol is a widely used cryoprotectant in fish gamete cryopreservation. Methanol has been found to protect cells during cryopreservation in zebrafish oocytes and embryo cryopreservation (Zhang *et al*. 1996, Plachinta *et al*. 2004, Tsai *et.al* 2008, Zampolla *et al*. 2008). It has been found that methanol was an effective cryoprotectant in zebrafish embryo cryopreservation because it has low toxicity (Zhang and Rawson 1995) and it protects the cells from chilling injury, due to possible depression of phase transition temperatures in the

lipid membranes (Zhang *et al*.2003).Ethanol has also been found to be an effective cryoprotectant for catfish (Muchlisin *et al*. 2009) sperm cryopreservation but it has not been used for zebrafish embryos or ovarian follicles cryopreservation before. The effect of ethanol and methanol on yeast during rapid cooling is a result of their ability to induce increased membrane permeability, allowing rapid water equilibrium during extracellular freezing and avoidance of intracellular ice crystal formation (Lewis *et al.* 1994). DMSO is commonly used cryoprotectant in the cryopreservation of a wide range of cells types, tissues and organs. The functions of DMSO in the cryopreservation solution, protects the cells from excessive dehydration during freezing process and to avoid intracellular ice formation (Lovelock and Bishop 1959). Both EG and PG have been widely used for mammalian oocytes and ovarian tissues cryopreservation. These compounds differ only by the presence of the additional methyl group in the PG molecule. These compounds promote small-granular crystallisation and amorphous solidification due to their high viscosity during freezing. They also reduce the quantity of unbound water within the cells, hence reducing the intracellular ice formation (Picton *et al*. 2002, Plachinta *et al.* 2004).

3.2 Results

3.2.1 Effect of cryoprotectant on viability of zebrafish ovarian follicles within the tissue fragments

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

The No Observed Effect Concentrations (NOECs) of methanol, ethanol, DMSO, PG and EG for ovarian tissue fragments at two different developmental stages (Stage I and Stage II) using three viability assessment methods are given in Table 3.1. For ATP assay, stage I and stage II follicles were assessed together since it is not possible to separate follicles at different stages embedded in tissue fragment. The results showed that the NOECs of the five tested cryoprotectants for stage I ovarian follicles are 2M for both methanol and ethanol and 1M for DMSO, PG and EG. The NOECs for stage II ovarian follicles are 1M for all five cryoprotectants. Both TB and FDA-PI staining provided same results.

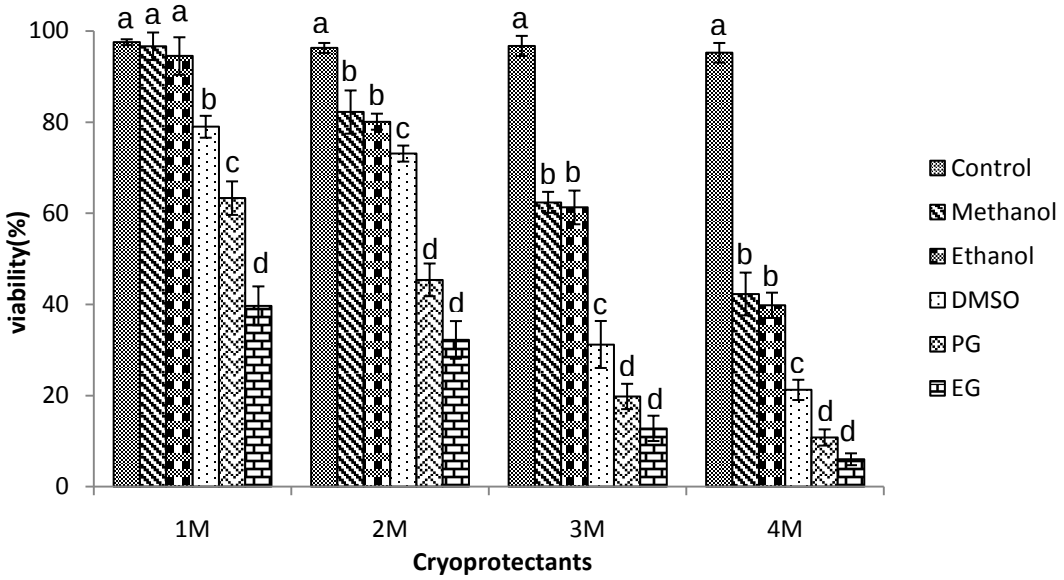
Table 3.1. No Observed Effect Concentrations (NOECs) of cryoprotectants obtained from TB, FDA+PI and ATP tests with ovarian tissue fragments

CPA	Stage I		Stage II		Stage I & II
	TB	FDA+PI	TB	FDA+PI	ATP
Methanol	2M	2M	1M	1M	1M
Ethanol	2M	2M	1M	1M	1M
DMSO	<1M	<1M	<1M	<1M	<1M
PG	<1M	<1M	<1M	<1M	-
EG	<1M	<1M	<1M	<1M	-

3.2.1 Effect of cryoprotectant on viability of zebrafish ovarian follicles within the tissue fragments

The effect of cryoprotectants at different concentrations on stage I and stage II ovarian follicles in tissue fragments are shown in Figure 3.1 and 3.2 respectively. The results indicated that methanol and ethanol were the least toxic cryoprotectants when compared with other tested cryoprotectants and the toxicity increased in the order of methanol/ethanol, DMSO, PG and EG. Both TB and FDA+PI tests showed that cryoprotectant toxicity increased with concentration.

(a)



(b)

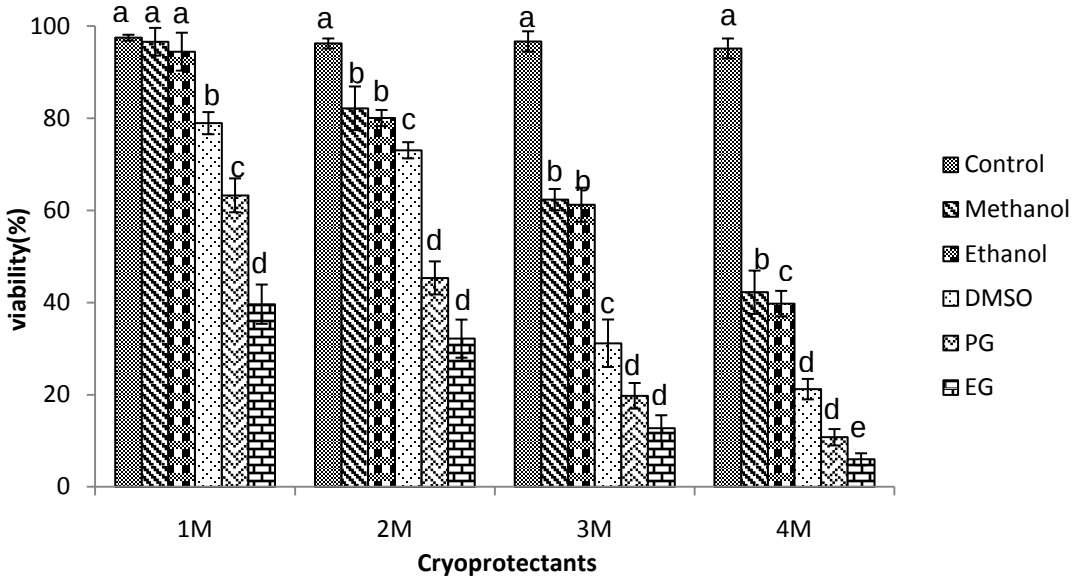


Figure 3.1. Viability of stage I zebrafish ovarian follicles in tissue fragments assessed with (a) Trypan blue staining and (b) Fluorescein diacetate + Propidium iodide staining. Stage II ovarian follicles were exposed to different concentrations of methanol, ethanol, DMSO, PG and EG for 30min at 22°C. Different letters indicate significant differences between control and treated groups (P<0.05).

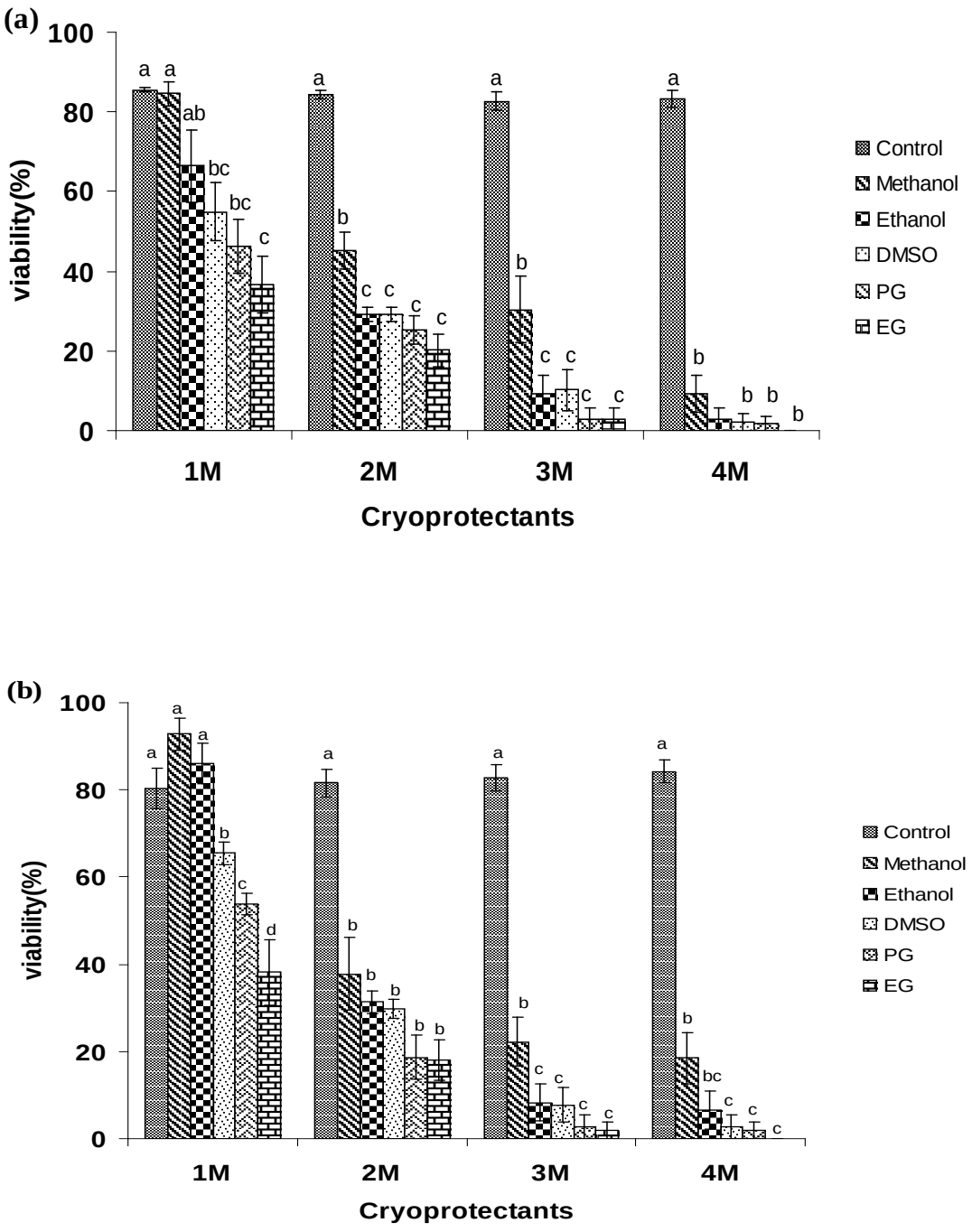
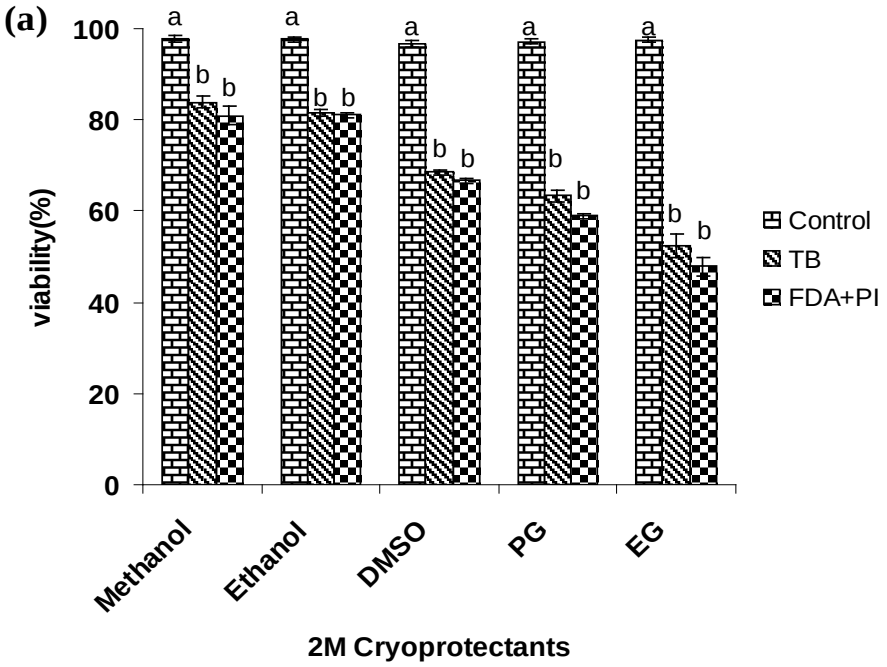


Figure 3.2. Viability of stage II zebrafish ovarian follicles in tissue fragments assessed with (a) Trypan blue staining and (b) Fluorescein diacetate + Propidium iodide staining. Stage II ovarian follicles were exposed to different concentrations of methanol, ethanol, DMSO, PG and EG for 30min at 22°C. Different letters indicate significant differences between control and treated groups (P<0.05).**3.2.3 Comparison of the three viability assessment methods**

The results of viabilities of stage I and stage II ovarian follicles after 2M cryoprotectants exposure assessed by TB and FDA+PI staining are shown in Figure 3.3. Both stage I and stage II ovarian follicle viabilities were significantly lower than those of controls assessed by either TB or FDA+PI staining.



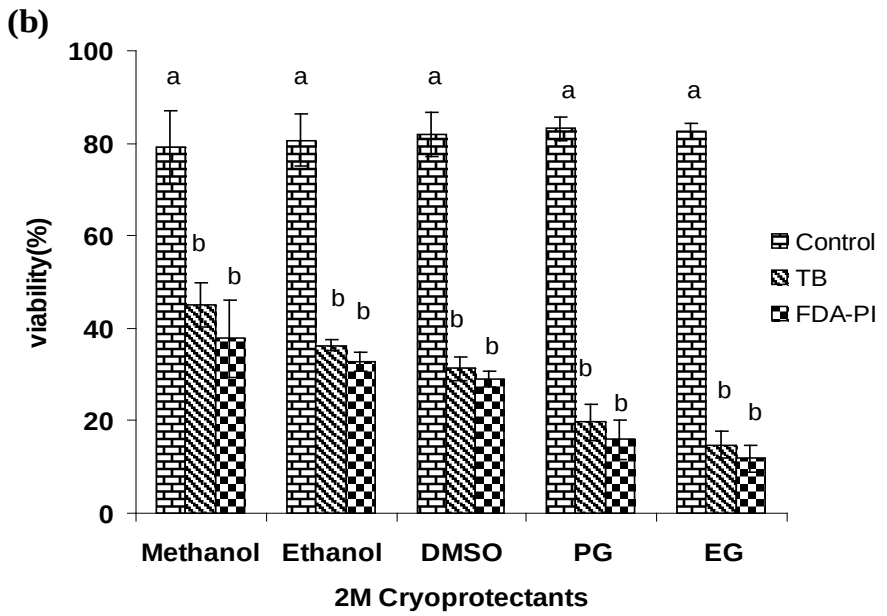
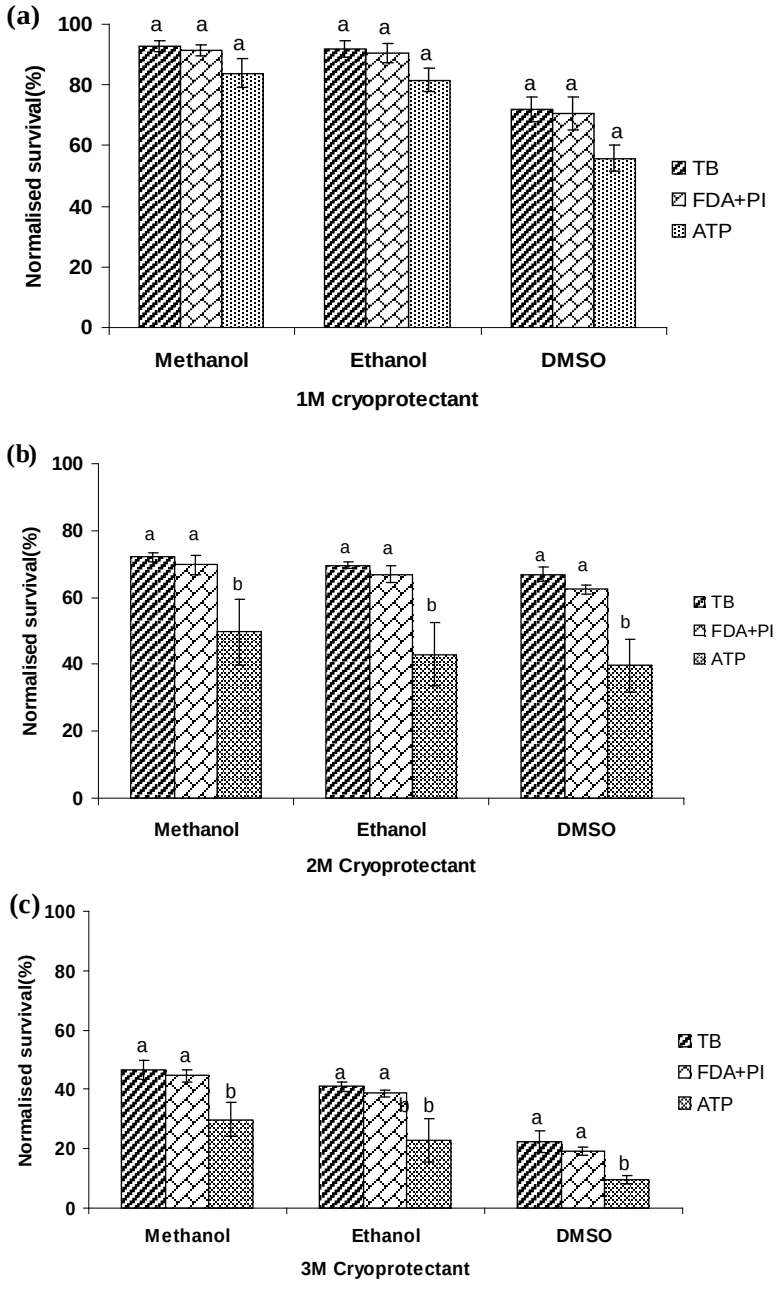


Figure 3.3 : Viability of stage I (a) and stage II (b) ovarian follicles after 2M cryoprotectants exposure assessed by Trypan blue (TB) or Fluorescein diactate + Propidium iodide (PI) staining. Ovarian tissue fragments of size 0.35mm- 0.45mm containing stage I and stage II zebrafish ovarian follicles were exposed to 2M cryoprotectants for 30min at 22°C. The average controls for stage I (a) and stage II (b) are 96.5± 0.64% and 79.5± 0.316% respectively. Different letters indicate differences between control and treated groups ($P<0.05$).

Viabilities of stage I and stage II ovarian follicles in ovarian fragments following 2M cryoprotectant exposure assessed by TB staining, FDA+PI staining and ATP assay of ovarian fragments are shown in Figure 3.4. Comparisons of the three viability assessment methods indicated that ATP is the most sensitive method when compared with TB and FDA+PI staining. For the ATP assay stage I and stage II follicles were assessed together since it is impossible to separate them in tissue fragments. All ovarian follicle viabilities was normalised with respect to that of the control incubated in 90% L-15(pH 9) medium for 30 min at 22°C.



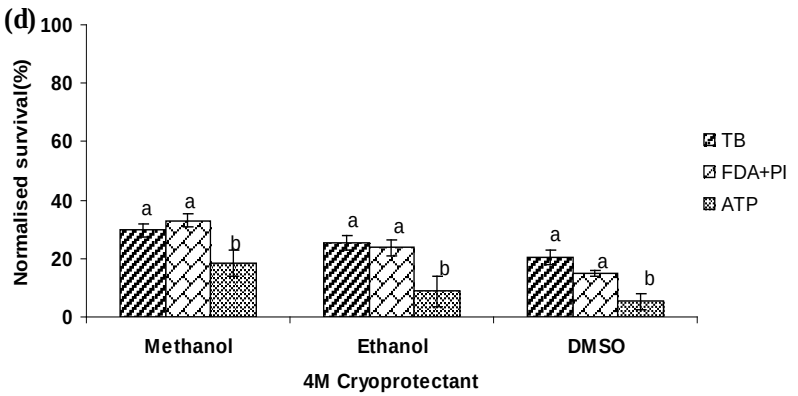


Figure 3.4 : Viability of stage I and stage II ovarian follicles following 1M (a), 2M (b), 3M (c) and 4M (d) cryoprotectant exposure assessed by Trypan blue(TB) staining, Fluorescein diacetate+Propidium iodide (PI) staining and ATP (Adenosine 5'-triphosphate) assay. Ovarian tissue fragments of size 0.35mm-0.45mm containing both stages I & II zebrafish ovarian follicles were exposed to methanol, ethanol and DMSO for 30min at 22°C. Ovarian fragment viabilities was normalised with respect to that of the controls incubated in 90% L-15 (pH 9) medium for 30 min at 22°C. Different letters indicate difference among the viability assessed by TB, FDA+PI and ATP tests of the treated groups (P<0.05).

3.3 Discussion

3.3.1 Effect of cryoprotectants

The results obtained from the present study showed that methanol and ethanol were the least toxic cryoprotectants when compared with other tested cryoprotectants. Cryoprotectant toxicity increased in the order of methanol/ethanol, DMSO, PG and EG. Methanol has been shown to be the least toxic cryoprotectant for isolated zebrafish ovarian follicles in our laboratory (Tsai *et al*. 2008; Zampolla *et al*. 2008); the results from the present study on methanol toxicity to zebrafish ovarian follicles in tissue fragments are in agreement with previous findings. Methanol penetrates the zebrafish ovarian follicles 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). Ethanol has been reported to be an effective cryoprotectant for catfish sperm cryopreservation (Muchlisin *et al*. 2009) and therefore used in the present study. The results showed that ethanol toxicity to follicles in ovarian tissue fragment was comparable to that of methanol. Ethanol is a small molecular weight cryoprotectant, which permeates the plasma membrane readily due to its low viscosity; it would render the interstitial and intracellular water hyperosmotic without posing an enormous osmotic stress to the cells (Meryman *et al*. 1970; Wang *et al.* 1992). It has also been reported that ethanol is relatively non-toxic making it a favourable cryoprotectant for cryopreservation of mammalian cardiac explants (Wang *et al*.1992).

As methanol has been shown to be the most effective cryoprotectant for zebrafish ovarian follicles cryopreservation in our laboratory, the use of ethanol will also be considered in assisting future freezing protocol design for follicles in ovarian fragments. Results obtained from the present study also showed that DMSO is more toxic than methanol, which is in agreement with a previous study on zebrafish ovarian follicles (Tsai *et al*. 2008). DMSO has also been reported to cause membrane labilisation and denaturation of proteins (Orvar *et al*. 2000). DMSO can also increase the concentration of calcium ions in cytoplasm, causing various chronic negative

metabolic responses such as cytoskeleton depolymerisation and reassembly (Yamamoto, 1989). The present study showed that EG and PG is highly toxic to zebrafish ovarian follicles embedded within ovarian tissue fragments. EG and PG decreases the polarity of the aqueous phase and the external phase, causing dehydration of the phospholipid bilayer and possible damage on fish spermatozoa (Leung, 1991). These are in agreement with results obtained from previous studies on zebrafish oocytes and isolated ovarian follicles (Plachinta *et al*. 2004; Tsai *et al.* 2008).

3.3.2 Effect of developmental stage

The results obtained from the present study indicated that the sensitivity of stage II ovarian follicles in tissue fragments were more sensitive than stage I follicles when exposed to different cryoprotectants. These results are in agreement with previous studies on isolated zebrafish ovarian follicles (Tsai *et al* 2008). The sensitivity of stage II follicles may be due to structural differences between stage I and II follicles. Stage I follicles are transparent and the nucleus of the follicle is visible. The follicle is surrounded by a single layer of squamous follicle cells. In stage II the follicles increase in size, the cortical alveoli proliferate, the follicle becomes opaque (Selman *et al*.1993) and the granulosa cells multiply to form an epithelial coating of cubic cells joined together by gap junctions (Babin *et al*.2007). The matured follicles are less permeable to solutes, due to the loss of membrane channel proteins while the immature follicles are much more permeable to water and solutes (Ecker and Smith, 1971). It has also been reported that, the presence of granulosa cells and the absence of microtubule spindles in early stage oocytes protect them against cryoprotects, while the spindle damage is high for late stage oocytes exposed to cryoprotectants (Van der Elst *et al*. 1992; Fabbri *et al.* 2001). However, the viability assessment methods used in the present study only inform on the impact of cryoprotectants on membrane integrity and metabolic activity.

3.3.3 Comparison of the three viability assessment methods

ATP assay has been considered to be a sensitive assay for cell viability (Tsai *et al* 2009). Although it has been used in assessing quality of isolated zebrafish ovarian follicles (Guan *et al*.2008, Zampolla *et al*. 2009, Tsai *et al*.2009), it has not been used in zebrafish ovarian tissues. The results from the present study showed that ATP assay is the most sensitive method for assessing the quality of the follicles in tissue fragments after cryoprotectant exposure when compared with TB and FDA+PI staining. ATP plays an essential role in gamete maturation and embryo development and high quality oocytes contain significantly higher levels of ATP (Bradbury *et al*. 2000, Tsai *et al*. 2009). The present study also showed that the sensitivity of TB and FDA+PI tests are comparable to each other although previous studies on isolated zebrafish ovarian follicles showed that FDA+PI staining was more sensitive than TB staining (Plachinta *et al*.2004, Tsai *et al*. 2008, Zampolla *et al*. 2008). TB is a vital dye and does not penetrate the cell unless the membrane is damaged, as it assesses only the membrane integrity (Isayeva *et al*. 2004, Plachinta *et al*. 2004, Tsai *et al*. 2008). FDA+PI staining assess both metabolic activities and membrane integrity. FDA+PI and TB staining are rapid tests that are useful in screening ovarian follicles after cryoprotectant treatment and after cryopreservation.

3.4 Summary

In this study, the effects of cryoprotectant toxicity to zebrafish ovarian tissue fragments have been reported for the first time. Three different assays were used to assess the ovarian follicles with the tissue fragments viability: trypan blue staining, FDA-PI staining, ATP assay. These assays indicated that cryoprotectant toxicity increased in the order of methanol/ ethanol, DMSO, PG and EG. The NOECs of the five tested cryoprotectants for stage I ovarian follicles are 2M for both methanol and ethanol and less than 1M for DMSO, PG and EG. The NOECs for stage I and II ovarian follicles are 1M for both methanol and ethanol and less than 1M for DMSO,

PG and EG. ATP assay is the most sensitive method for assessing the quality of the follicles in tissue fragments after cryoprotectant exposure. The results obtained in this study provided useful information for ovarian tissue fragment cryopreservation protocol design.

CHAPTER 4 DEVELOPMENT OF *IN-VITRO* CULTURE PROTOCOL FOR ZEBRAFISH OVARIAN TISSUE FRAGMENTS AND GROWTH ASSESSMENT OF STAGE I AND STAGE II OVARIAN FOLLICLES USING BIOMARKERS

4.1 Introduction

Development of *in-vitro culture* protocol for ovarian tissue fragments is important since cryopreserved ovarian tissue fragments would need to be matured *in vitro*. Studies on *in-vitro* culture of ovarian tissue fragments are reported here for the first time although preliminary work had been done for isolated early stage zebrafish ovarian follicles (Tsai *et al.*2010). Although several procedures that have been successfully applied on late stage of ovarian follicles have been reported (Seki *et al.*2008) little is known about the early follicle development. The main aim of the present study was to develop an optimum *in vitro* culture condition for zebrafish tissue fragments containing stage I and stage II follicles.

In this study, *in vitro* culture of ovarian tissue procedure was investigated using growth supplements- foetal bovine serum (FBS) and bovine serum albumin (BSA) and growth factors- human chorionic gonadotropin (hCG) and follicle stimulating albumin (FSH).

Serum and serum components may provide beneficial factors to the culture environment, including energy substrates, vitamins, amino acids, and growth factors (Bavister, 1995). However, a major biological role of serum is to search ions and small molecules in addition to providing factors in the culture medium (Maurer, 1992). FBS has rich content of growth factors and low content of gamma-globulin, which makes it become the great supplement in culture media for promoting cell growth. The immune system of the FBS is not mature and contains fewer molecules that could inhibit the growth of the cells. Other advantageous in using FBS as the media supplement includes: FBS is a mixture of most of the growth factors, protein, cholesterol required for cell proliferation, it is hence the universal growth

supplement effective in most cell types of human and animal cells (Gstraunthaler, 2003). It has been reported that growth medium foetal bovine serum (FBS) enhances cell growth (Frazer *et al.*1999). FBS has been used in cat fish cell culture experiments and has been shown to increase the cellular growth rate (Kumar *et al.*2001). BSA is added to cell culture media as a supplement to increase the growth and proliferation of cells. Serum albumin carries and delivers important nutrients to the cells. BSA binds toxins to avoid toxic effects, binds excessive proteins to act as a buffer and binds hormones and growth peptides to keep them stable. Albumin also binds free radicals to reduce damage to cells (Peters, 1995).

One of the commonly used substances for *in vitro* culture of early stage follicles is the follicle stimulating hormone (FSH). Follicle-stimulating hormone receptors are expressed in granulose cells from the primary follicle stage onwards (Rossetto *et al.* 2009). Gonadotropins hormones play a critical role in the growth and development of the vertebrate ovarian follicles. In teleosts fishes, the gonadotropic hormones (GtH I) functions to stimulate follicle production and secretion of estrogen and vitellogenin uptake by oocytes, and GtH II induces follicle cell production of steroid maturation-inducing substance (MIS) which acts directly on oocyte to induce maturation (York *et al.* 1993). A change in terminology replacing the two different gonadotropins GtH I and GtH II with follicle-stimulating hormone (FSH) and luteinizing hormone (LH) has been well justified (Kwok *et al.*2005). The pituitary secretes FSH, and LH which acts upon the gonads, stimulating their growth, production of eggs or sperms and synthesis of gonadal hormones (Moles *et al.* 2008). The growth stage is controlled by FSH and the maturation stage by LH (Nagahama *et al.* 1994). Unlike in mammals little is known about the physiological roles of FSH and LH in teleosts. It has been reported that FSH is important in promoting follicular development and growth in zebrafish (Tyler 1991) and also follicular development in most of mammalian cultures *in-vitro* (Rosetto *et al*. 2009). FSH is widely used in reproduction technologies, and for *in-vitro* maturation of immature oocytes in humans (Kreeger *et al.* 2006). The gonadotropins are the major hormones involved in initiating the majority of processes in the ovary including follicle cell growth and survival, steroidogenesis, oocyte maturation, and ovulation (Clelland and Peng, 2009). Studies in zebrafish have shown that ovarian activin expression is stimulated

by the gonadotropin analogue human chorionic gonadotropin (hCG) and goldfish pituitary extract, suggesting that activins are downstream mediators of gonadotropin signalling (Pang and Ge, 2002). Pang and Ge (2002) reported that hCG significantly promoted the maturation of zebrafish stage III oocytes.

Although follicle growth can be assessed by measuring the diameter of the follicles, biomarkers would provide more important information on follicle development. Development of the fish oocytes can be followed both at the morphological level by visual inspection of their size, colour and transparency (Selman and Wallace, 1986; Selman *et al*. 1993) and also at the molecular level by following changes in the protein and mRNA patterns accompanying their maturation (Bobe *et al.* 2004). The aim of the present study was to develop a biomarker to identify stage II and stage III ovarian follicles after *in vitro* culture.

Vitellogeninis (Vtgs) are yolk precursor proteins with a high molecular weight (250-600 kD) glycophospholipoprotein synthesised in the liver in response to the steroid hormone 17-β estradiol (E_2) (Ng and Idler, 1983; Wallace 1985; Specker and Sullivan, 1994). It is secreted into the bloodstream from where it is taken up by developing oocytes and cleaved into the two yolk proteins, lipovitellin and phosvitin (Wallace and Jared, 1969; Tyler *et al*. 2000). In oviparous vertebrates, the yolk is critical for embryonic development as it is rich in source of nutrients, including amino acids, phosphate, carbohydrates, lipids, and vitamins. In oviparous species the Vtgs they are divided into three –lipovitellin I (LVI), phosvitin (PV), and lipovitellin II (LVII)- in oocytes in vertebrates. Male fishes also possess the Vtg gene but expression is not normally found, due to the absence of lower levels of estrogens (Harries *et* al. 1997). It is only recently that sufficient N terminal sequence data for Vtg-derived egg yolk proteins in fishes have become available (Finn, 2007). Analysis of teleost Vtg genes has identified from one (La Fleur *et al*. 1995) to several distinct Vtg genes (Lee *et al*. 1994; Trichet *et al*. 2000; Wang *et al*. 2000). The zebrafish genome contains at least seven vtg genes (vtg 1-7) encoding heterogeneous vitellogenins with three disteinct types of Vtgs: type I (Vtg 1, 4-7), type II (Vtg 2) and, type III (Vtg 3) (Wang *et al*.2005). Expression of Vtgs is under hormone regulation and is stage, sex and tissue dependent. The Vtg genes expression in the zebrafish is found in liver, intestestines, and ovary in female fish, and testis

and muscle in E_2 treated male fish. However, not all three types of Vtgs are found in the same species because of incomplete sequence. Vitellogenesis is where the majority of growth occurs and is where the actions of the gonadotropins have been well established. FSH is suggested to be the key hormone regulating follicular growth and this is due to high levels of FSH receptor mRNA levels in ovarian follicles during vitellogenesis (Kwok et al., 2005). FSH stimulates production of 17β-estradiol (E2) in the follicle, which in turn stimulates the production of vitellogenin from the liver (Patino and Sullivan, 2002).

Cytochrome P450 aromatase (P450) is the key enzyme for conversion of testosterone to estadiol-17β in the granulosa cells (Simpson *et al.* 1994). It is the product of the cyp 19 gene. In contrast to the human *cyp19* gene, which is believed to occur as a single copy in the haploid genome and has multiple tissue-specific promoters and first exons, the goldfish has at least two separate and distinct *cyp19* loci. *cyp19b*/P450aromB is constitutively expressed at high levels in the brain and is further up-regulated by estrogen, which is the basis for 6- to 8-fold seasonal variations in enzyme protein and messenger RNA (mRNA) (Gelinas *et al.*1998). *cyp19a*/P450aromA is expressed in ovary where enzyme levels and mRNA are relatively low. The cDNAs encoding the zebrafish orthologs of P450aromB and P450aromA, also determine the patterns of expression by tissue-type in adult fish and at defined developmental stages in embryos and larvae (Kishida *et al*.2001). P450arom cDNAs have been isolated and characterised in several teleost species, including rainbow trout (Tanaka *et al.* 1992), Catfish (Trant *et al*. 1994), medaka (Fukada *et al*. 1996), tilapia (Chang *et al*. 1997) and red sea bream (Gen *et al*. 2001). Early vitellogenesis is characterised by further increase in plasma FSH and E2, and increased expression of ovarian FSH receptor (Kwok *et al.* 2005), In salmonids, steroidogenic thecal cells supply androgen substrate to ovarian granulosa cells that express p450 sromatase and produce E2 (Lubzens *et al.* 2010)

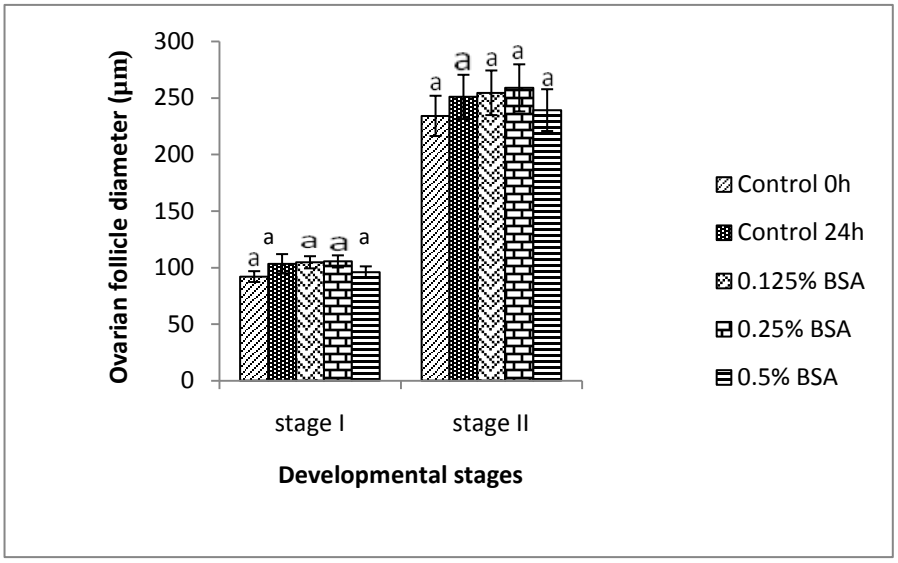
In order to further assess the growth of the ovarian follicles after 24h culture, vtg1 gene and p450aromA gene were studied to evaluate their pattern of expression in stage I, II and III ovarian follicle developmental stage and a quantitative RT-PCR approach to investigate the level of gene expression. **4.2 Results**

4.2.1 Development of *in-vitro* culture method for zebrafish ovarian tissue fragments

4.2.1.1 Effect of BSA on early stage ovarian follicle growth competence within the tissue fragment

The growth of stage I and stage II ovarian follicles within the ovarian tissue fragment after culturing in various concentrations (0.125, 0.25 and 0.5%) of BSA in 90% L-15 for 24 h culture at 28°C are shown in Figure 4.1. The results showed that there has not been an increase in diameter in stage I and stage II follicles cultures with BSA following 24 h incubation compared to the control follicles (No BSA) Figure 4.1a at 0h and 24 h. Results obtained by TB staining showed that BSA exposure (0.25% and 0.5%) induced a decrease in membrane integrity (Figure 4.1b). There were no significant differences when 0.125% BSA was used.

(a)



(b)

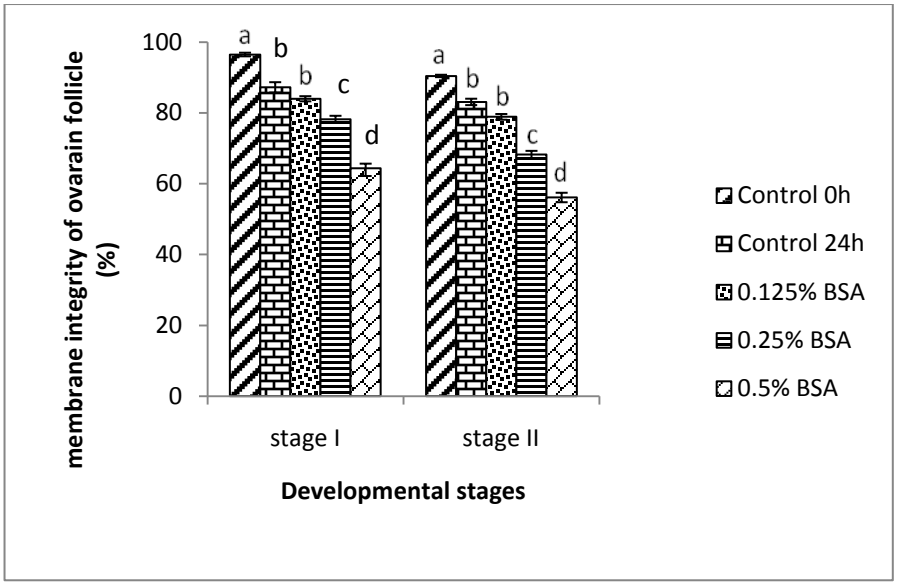
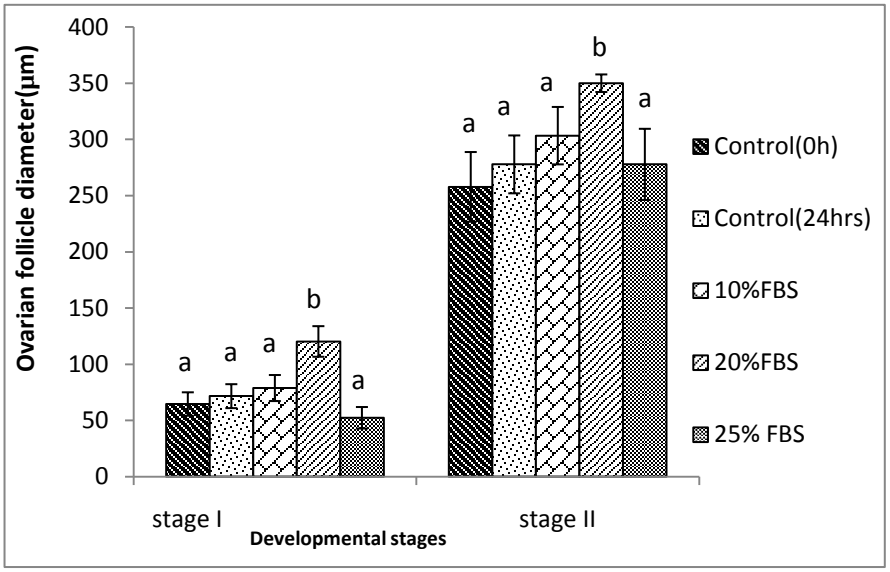


Figure 4.1: The diameter of stage I and II ovarian follicles (a) and membrane integrity of stage I and stage II ovarian follicles (b) within the ovarian tissue fragments in 0.125, 0.25 and 0.5% bovine serum albumin made up in 90% L-15 medium after 24 h culture at 28°C. Follicles before culture were used as controls (0h). Error bars represent Standard Errors of the Mean. Groups with no common superscript differ significantly ($P<0.05$).

4.2.1.2 Effect of FBS on early stage ovarian follicle growth competence within the tissue fragment

The growth of stage I and stage II ovarian follicles within the ovarian tissue fragment after culturing in various concentrations (10, 20 and 25%) of FBS in 90% L-15 for 24 h culture at 28°C are shown in Figure 4.2a. The results showed that the diameter of stage I and stage II ovarian follicle increased with samples cultured with 20% FBS. There was no significant difference in diameter between control and follicles cultured with 10% or 25% FBS ($P>0.05$). And the membrane permeability results are shown in Figure 4.2b. The results indicated that 10 and 20% FBS treatment on the tissue fragments did not show any difference from the control group but the treatment with 25% FBS decreased the membrane integrity of the fragments significantly. Hence 20% FBS was compared with other growth factors in the subsequent experiment.

(a)



(b)

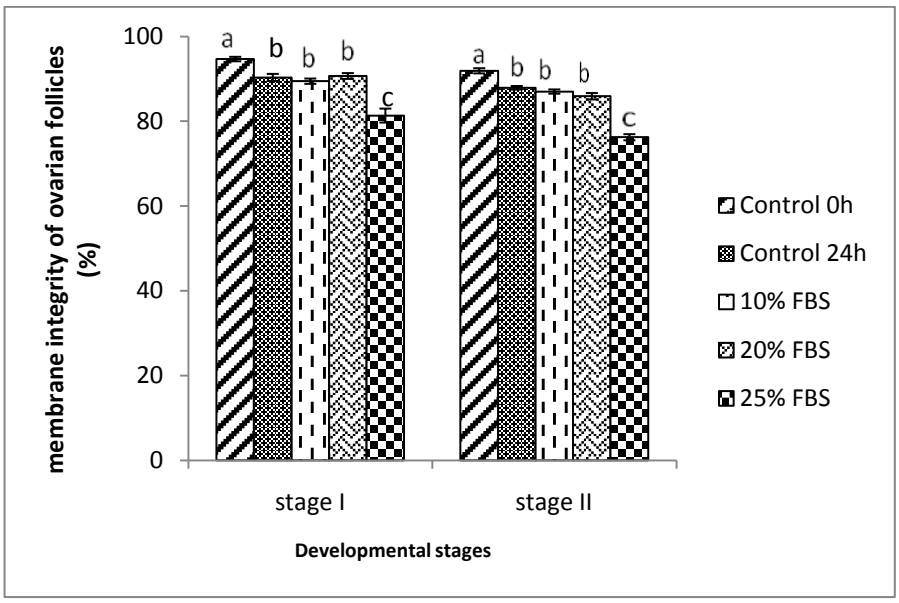


Figure 4.2: The diameter of stage I and II ovarian follicles (a) and membrane integrity of stage I and stage II ovarian follicles (b) within the ovarian tissue fragments in 10, 20 and 25% fetal bovine serum made up in 90% L-15 medium after 24 h culture at 28°C. Follicles before culture were used as controls (0h). Error bars represent Standard Errors of the Mean. Groups with no common superscript differ significantly ($P<0.05$).

4.2.1.3 Effect of hCG on early stage ovarian follicle growth competence within the tissue fragment

The growth of stage I and stage II ovarian follicles in ovarian tissue fragments after culturing in 10IUhCG (Tsai *et al* 2010) along with 0.125% BSA and 20% FBS in 90%L-15 for 24 h culture at 28°C are shown in Figure 4.3a. There were no significant differences in follicle diameter between controls (0h) and the treated groups for stage I and stage II follicles within the tissue fragments. There were no significant differences in membrane integrity assessed by TB staining between follicles incubated in hCG with 20% FBS and those for the control groups at 24h but there is significant difference when treated in hCG with 0.125% BSA (Figure 4.3b).

(a)

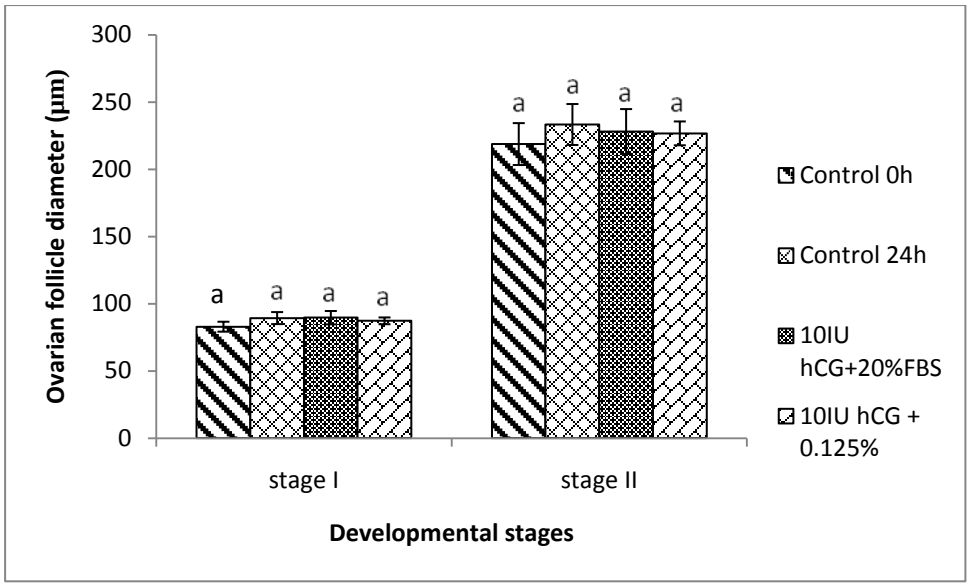


Figure 4.3a: The effect of 10IU/ml human chorionic gonadotropin with 0.125% bovine serum albumin and 20% fetal bovine serum on stage I and II ovarian follicles within the ovarian tissue fragments in 90% L-15 medium at pH 9.0 after 24 h culture at 28°C. Follicles before culture were used as controls (0h). Error bars represent Standard Errors of the Mean. Groups with no common superscript differ significantly ($P<0.05$).

(b)

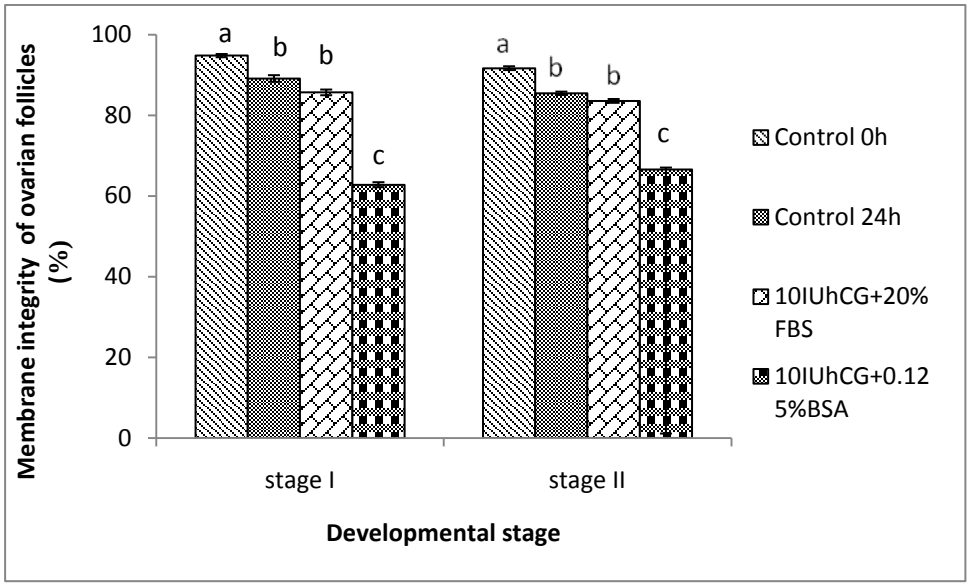


Figure 4.3b : The membrane integrity of stage I and II ovarian follicles within the ovarian tissue fragments in 90% L-15 medium at pH 9.0 after 24 h culture at 28°C containing 100mIU/ml follicle stimulating hormone with 0.125% bovine serum albumin and 20% fetal bovine serum. Follicles before culture were used as controls (0h). Error bars represent Standard Errors of the Mean. Groups with no common superscript differ significantly ($P<0.05$).

4.2.1.4 Effect of FSH on early stage ovarian follicle growth competence within the tissue fragment

The growth of stage I and stage II ovarian follicles within the ovarian tissue fragment after culturing in various concentrations (40, 60, 80, 100 and 120 mIU/ml) of FSH in 90% L-15 for 24 h culture at 28°C are shown in Figure 4.4a and membrane integrity of stage I and stage II ovarian follicles (Figure 4.4b). The results showed that the diameter of stage I and stage II ovarian follicle increased with samples cultured with 100mIU/ml FSH. Hence 100 mIU/ml FSH is considered in the subsequent experiment.

(a)

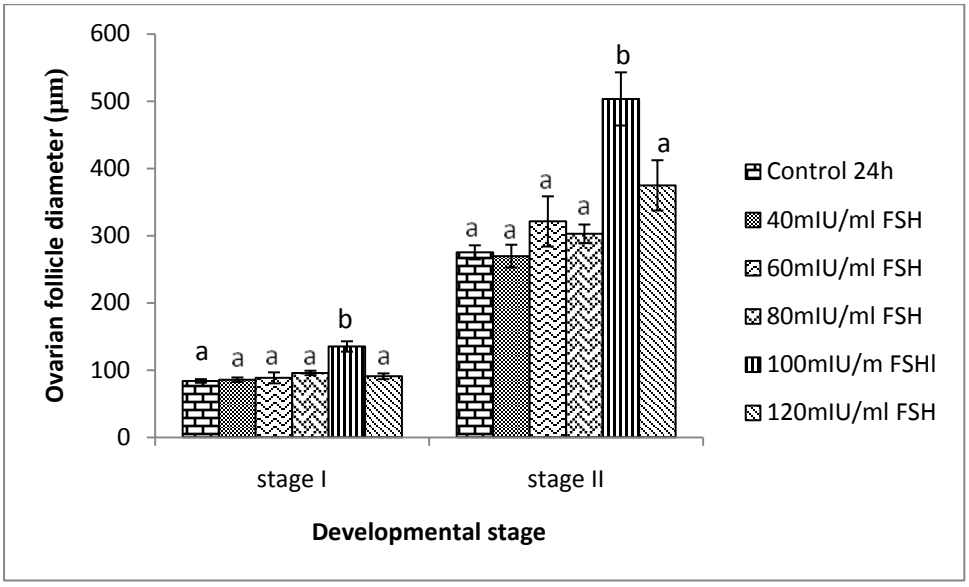


Figure 4.4a : The effect of Follicle stimulating hormone (40, 60, 80, 100 and 120 mIU/ml) on stage I and II ovarian follicles within the ovarian tissue fragments in 90% L-15 medium at pH 9.0 after 24 h culture at 28°C. Follicles cultured in 90% L-15 medium were used as controls (24h). Error bars represent Standard Errors of the Mean. Groups with no common superscript differ significantly ($P<0.05$).

(b)

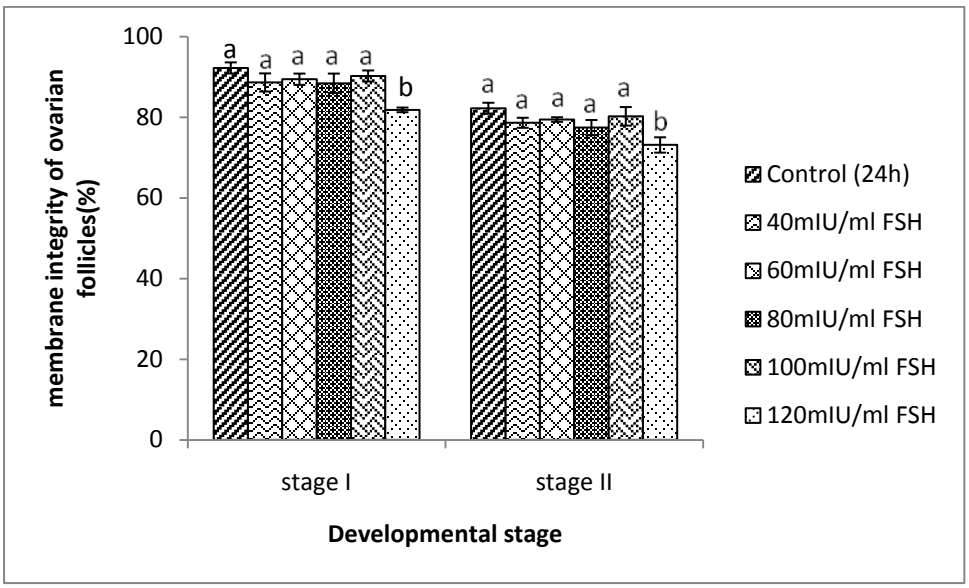


Figure 4.4b: The viability of stage I and II ovarian follicles within the ovarian tissue fragments in 90% L-15 medium at pH 9.0 after 24 h culture at 28°C containing different concentration of Follicles stimulating hormones. Follicles cultured in 90% L-15 medium were used as controls (24h). Error bars represent Standard Errors of the Mean. Groups with no common superscript differ significantly ($P<0.05$).

The growth of stage I and stage II ovarian follicles in ovarian tissue fragments after culturing in 100mIU/ml FSH along with 0.125% BSA in comparison to 100mIU/ml FSH with 20% BSA in 90%L-15 for 24 h culture at 28°C are shown in Figure 4.4c. The results showed that treatment with FSH and FBS showed increased in diameter in both stage I and stage II ovarian follicles within the fragments. There were no significant differences in membrane integrity assessed by TB staining between follicles incubated in FSH with 20% FBS and those for the control groups at 24h Figure 4.4d.

(c)

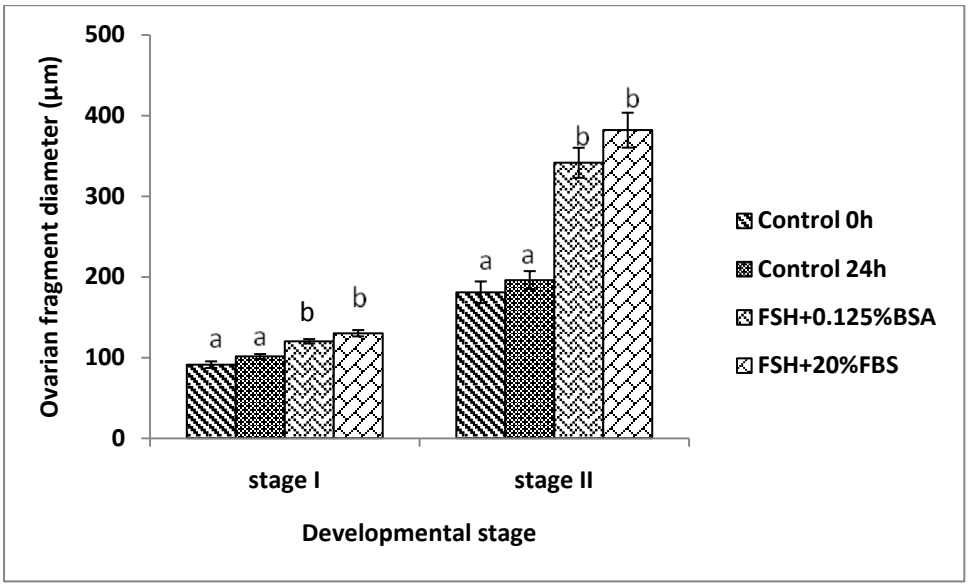


Figure 4.4c: The effect of 100mIU/ml follicle stimulating hormone with 0.125% bovine serum albumin and 20% fetal bovine serum on stage I and II ovarian follicles within the ovarian tissue fragments in 90% L-15 medium at pH 9.0 after 24 h culture at 28°C. Follicles before culture were used as controls (0h). Error bars represent Standard Errors of the Mean. Groups with no common superscript differ significantly ($P<0.05$).

(d)

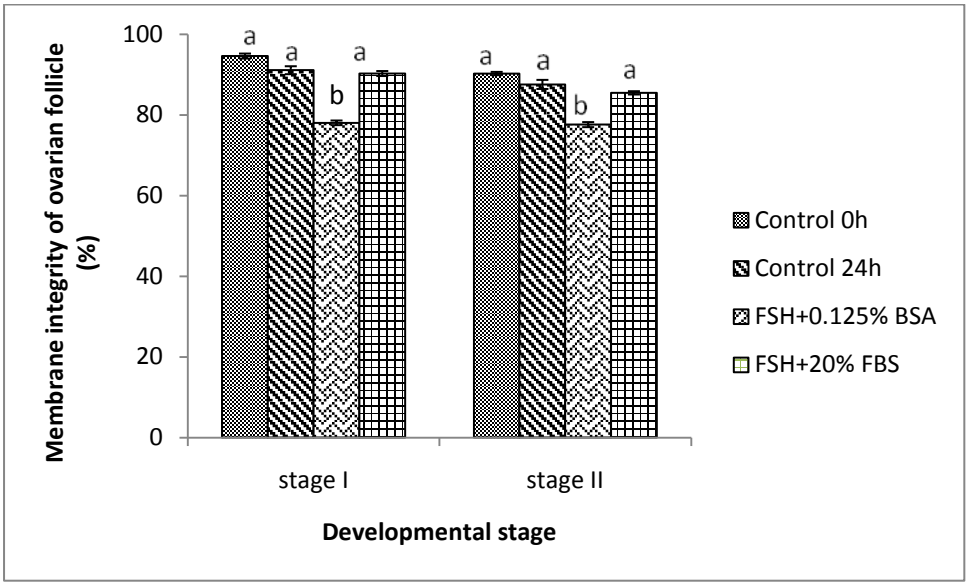


Figure 4.4d: The membrane integrity of stage I and II ovarian follicles within the ovarian tissue fragments in 90% L-15 medium at pH 9.0 after 24 h culture at 28°C containing 100mIU/ml follicle stimulating hormone with 0.125% bovine serum albumin and 20%FBS. Follicles before culture were used as controls (0h). Error bars represent Standard Errors of the Mean. Groups with no common superscript differ significantly ($P<0.05$).

4.2.2 Experimental design

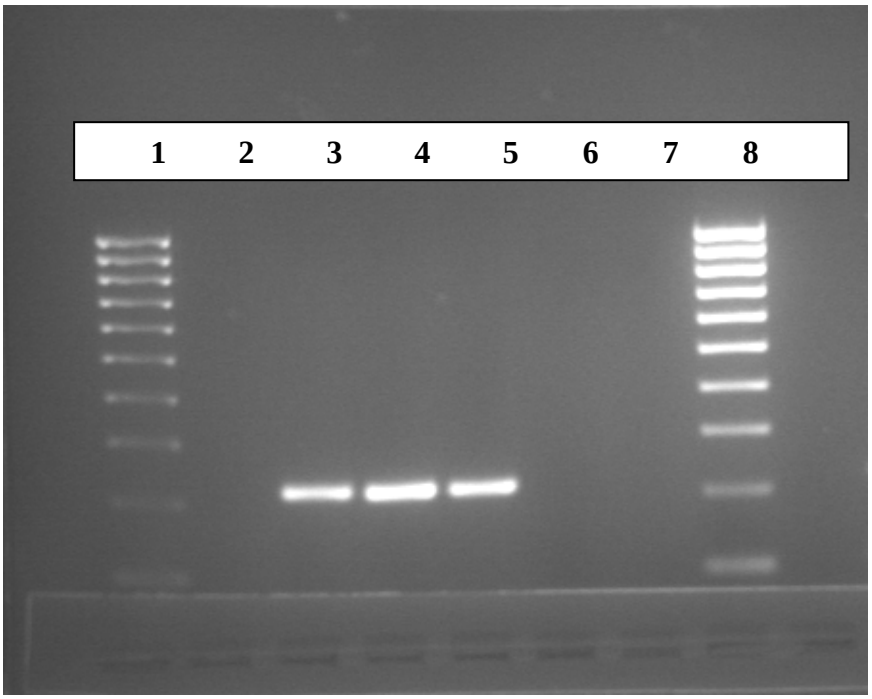
The two step reverse transcriptase PCR (RT-PCR) was used to determine patterns of gene expression in different developmental stages of zebrafish ovarian follicles. Gene expression studies were performed for P450arom A and Vtg1 genes. Stage I, II and III ovarian follicles were collected and subjected to RNA extraction, cDNA synthesis and PCR. PCR product was analysed using agarose gel electrophoresis. Housekeeping gene EF1-α was used as internal control. EF1-α was used in the study as this gene was shown to have the highest stability during zebrafish studies (Lin *et al.* 2009; McCurley and Calland, 2008). Housekeeping genes are responsible for basic cellular function and expressed at relatively similar levels across many types of cells.

4.2.2.1 Determination of P450arom A gene expression in zebrafish ovarian follicles at different ovarian follicle developmental stages

Gene expression was evaluated in different developmental stages-stage I, stage II, and stage III ovarian follicles. PCR product was analysed on 2% agarose gel stained with 0.5 µg ethidium bromide staining and visualised under Genosmart UV gel documentation system (VWR, UK). EF1-α housekeeping gene was used for each ovarian follicle stage RNA, PCR was also performed to check any variation in RNA. No template control was used to check reagent purity.

Expression of P450arom A was observed from stage I, stage II and stage III ovarian follicles (Figure 4.5). The band intensity was observed to be higher in stage II ovarian follicle. PCR product size was determined using Hyperladder^{TM} V which was 221 bp for P450arom A gene.

(a)



(b)

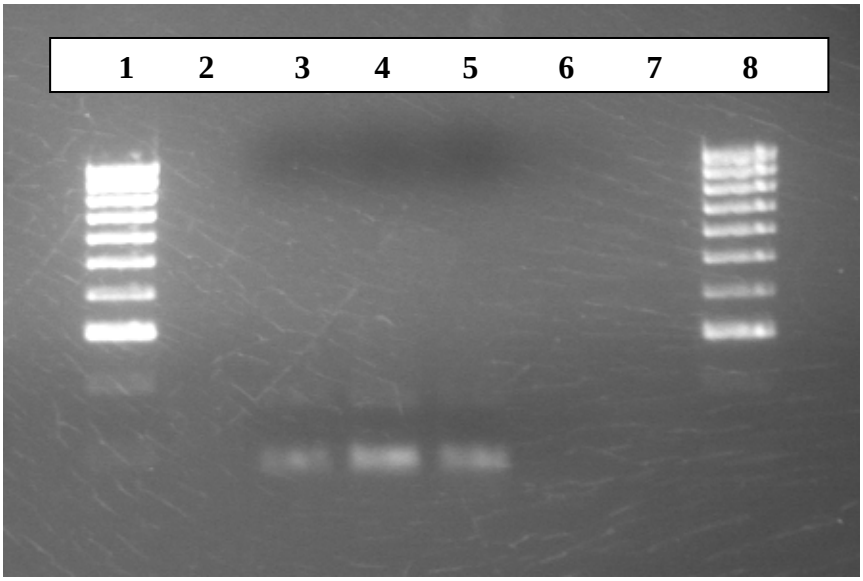


Figure 4.5 Agarose gel electrophoresis (2% agarose) of P450aromA PCR product. EF1-α was used as an internal control. Lanes 3-5(a) show P450aromA PCR product of stage I, stage II and stage III ovarian follicles respectively. Lanes 3-5(b) show EF1-α PCR product of stage I, stage II and stage III ovarian follicles respectively. No template controls for P450arom A(a) (lane 6) were also used in this experiment. No template controls for EF1-α (b) (lane 6) were also used in this experiment.Hyperladded^{TM}V (25 bp ladder) (lane 1 and 8) was used to determine PCR product size 221 bp for P450arom A gene and 87bp for EF-1α.

4.2.2.2 Determination of Vtg1 gene expression in zebrafish ovarian follicles at different ovarian follicle developmental stages

Expression of Vtg1 gene was observed from stage I, stage II and stage III ovarian follicles (Figure 4.6). PCR product size was determined using Hyperladder^{TM} V which was 100 bp for Vtg1 gene.

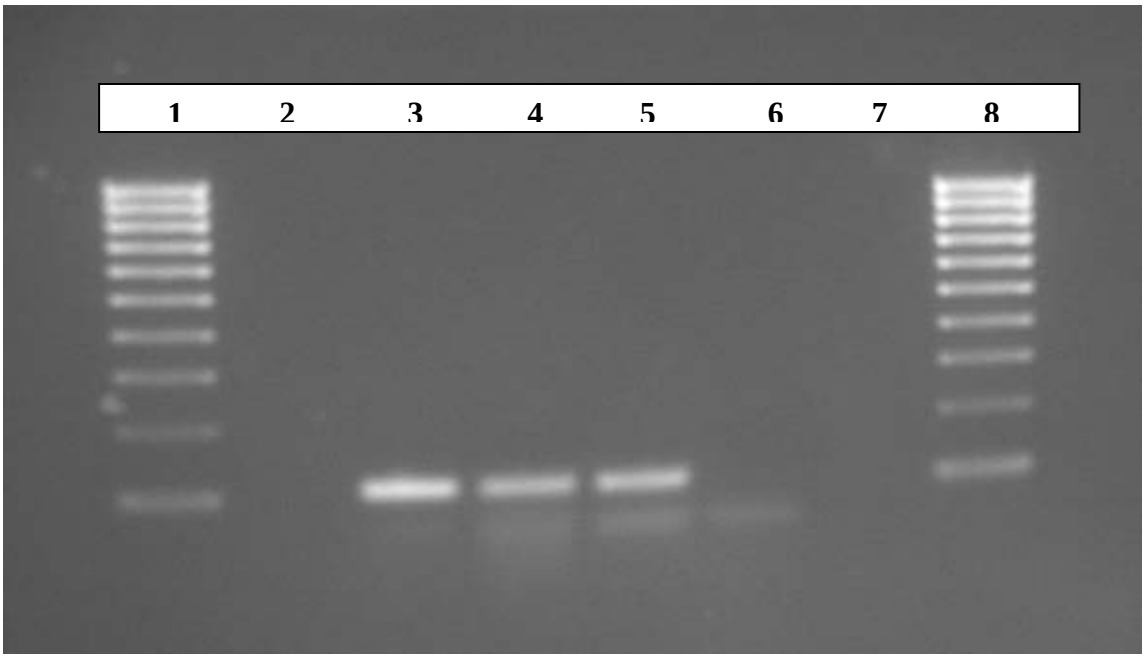


Figure 4.6 Agarose gel electrophoresis (2% agarose) of Vtg1 PCR product. Lanes 3-5 show Vtg1 PCR product of stage I, stage II and stage III ovarian follicles respectively. No template controls for P450arom A (lane 6) are also used in this experiment. Hyperladded^{TM}V (25 bp ladder) (lane 1 and 8) was used to determine PCR product size 100 bp for Vtg1 gene.

4.2.3 Experimental design

Based on the gene expression profile obtained from previous study (section 4.2.2) the gene expression levels were quantified using quantitative RT-PCR. This experiment investigates the level of expression at different ovarian follicle development stages. In order to assess level at stage I, II and III ovarian follicles quantitative analysis has been done. Quantification measures the relative change in mRNA expression level.

4.2.3.1 Studies on the expression of P450arom A gene on different ovarian follicle developmental stages

The results showed that the expression of p450aromA gene was higher in stage II compared to stage I and stage III ovarian follicles.

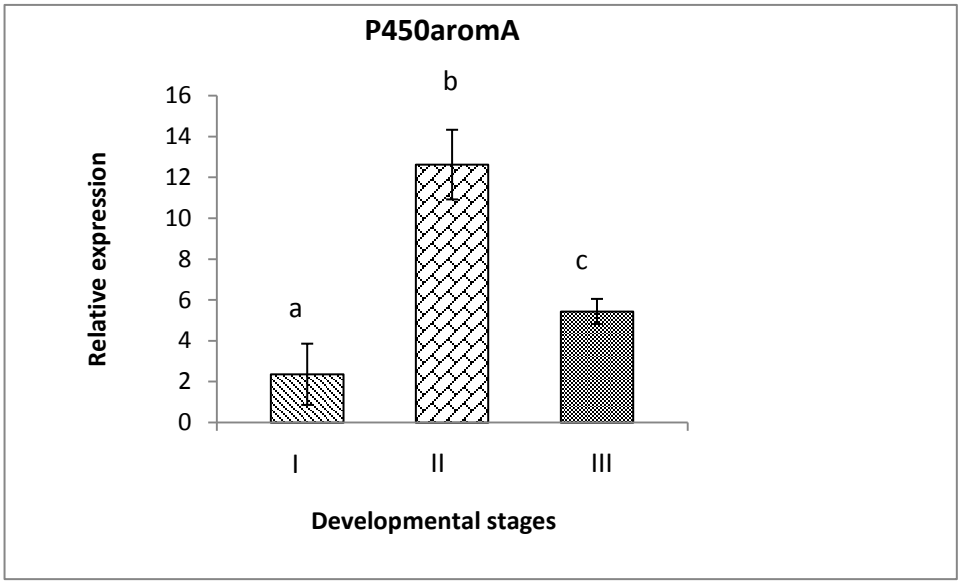


Figure 4.7 Relative mRNA expression of P450aromA in zebrafish stage I, stage II and stage III ovarian follicles. The values are normalised to EF1-α. Values represent the mean SEM of the relative expression as determined by RT-PCR. Different letters indicate significant differences.

4.2.3.2 Studies on the expression of Vtg1 gene on different ovarian follicle developmental stages

The results showed that the expression of Vtg1gene was higher in stage III when compared to stage I and stage II ovarian follicles.

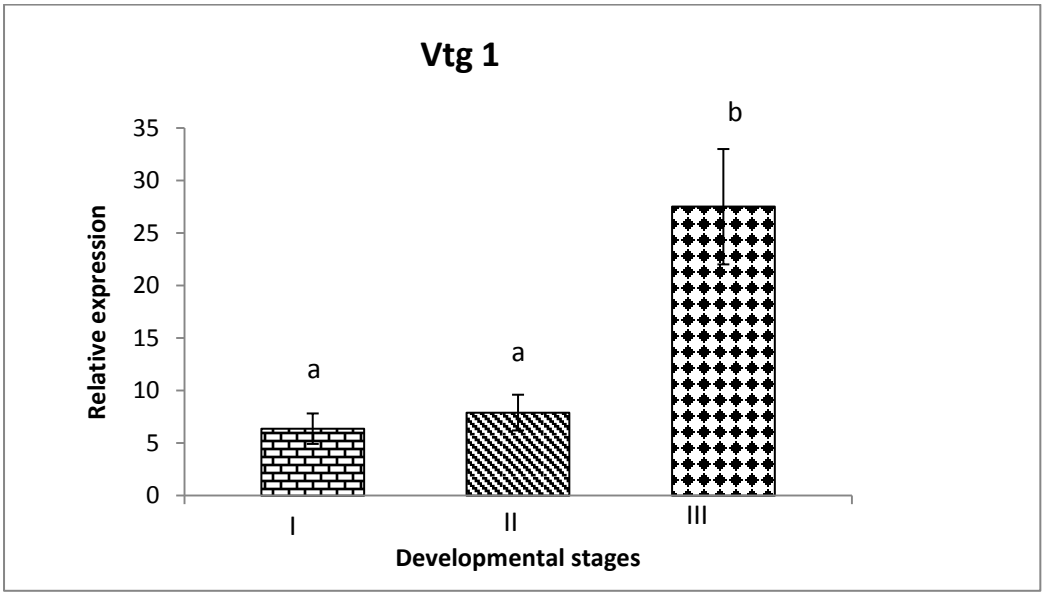
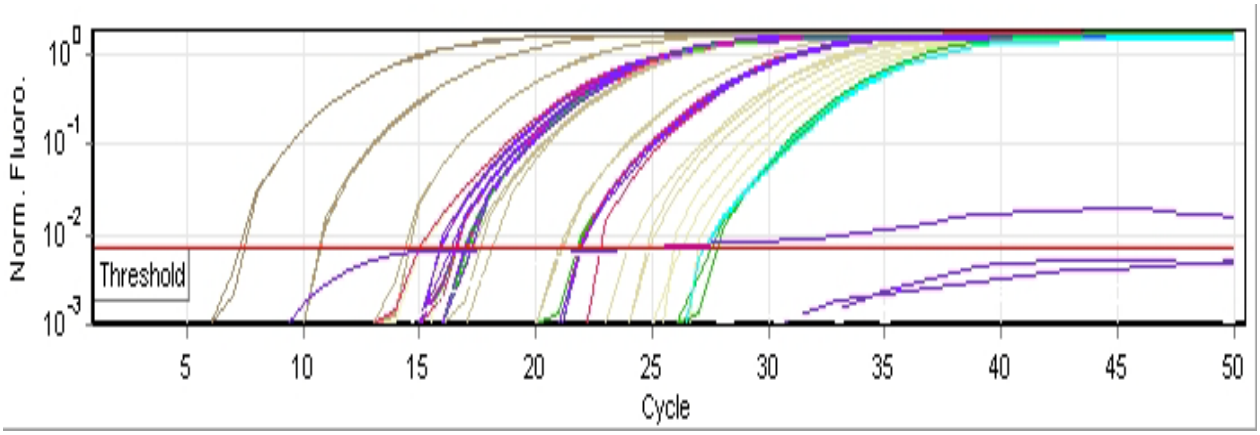


Figure 4.8 Relative mRNA expression of Vtg1 in zebrafish stage I, stage II and stage III ovarian follicles. The values are normalised to EF1-α. Values represent the mean SEM of the relative expression as determined by RT-PCR. Different letters indicate significant differences.

4.2.3.3 Fluorescence measurements melt curves and standard curve evaluation of P450arom A and Vtg1 genes

Fluorescence levels within each tube were measured at the end of each cycle (Figure 4.9). Amplification efficiency and a correlation coefficient from standard curve of cDNA at different 10-fold dilution were also determined for each gene using accompanied real time PCR software (Figure 4.10). All the target genes have acceptable efficiencies and correlation coefficients from 0.7 to 1.1 (Pfaffl 2003a). Melting curve was performed to ensure that the primers amplified a single product. The melting cure (Figure 4.11) indicates no contamination in samples.

P450aromA



Vtg1

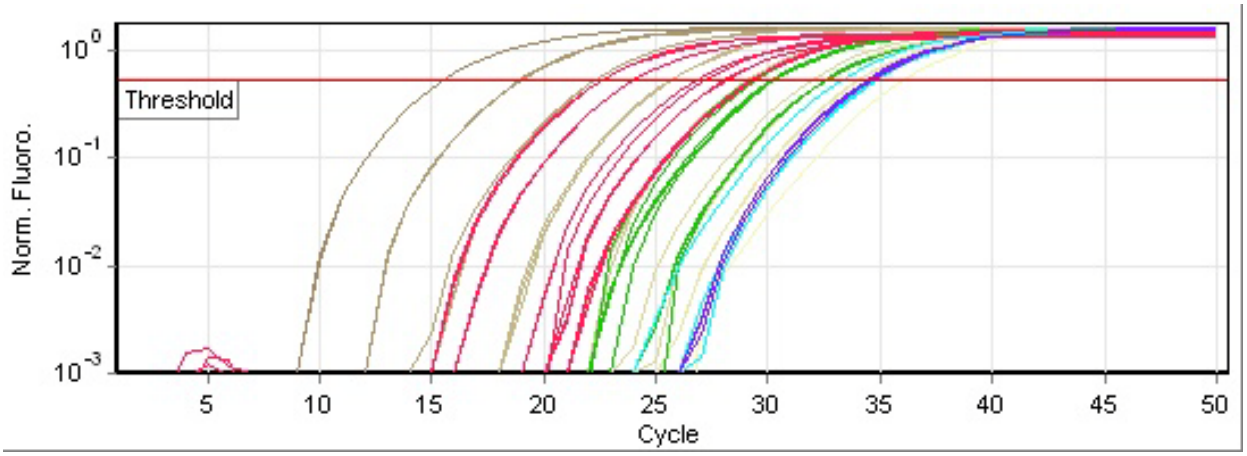
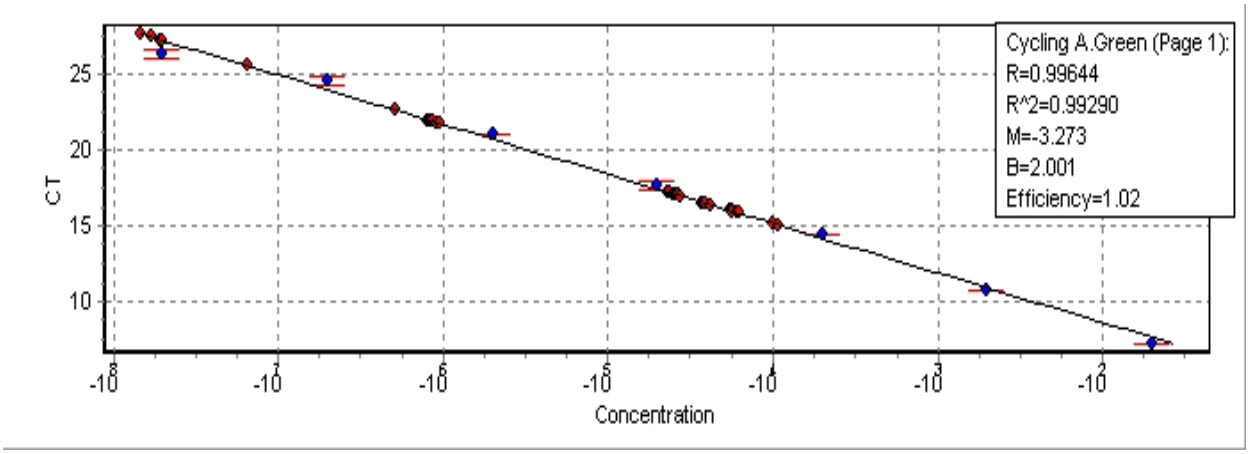


Figure 4.9 Example of fluorescence measurement of P450arom A and Vtg1 genes. Fluorescence graphs shows fluorescence measurement obtained for standard (brown), negative control (light blue) and the other colours related to ovarian follicle samples.

P450aromA



Vtg1

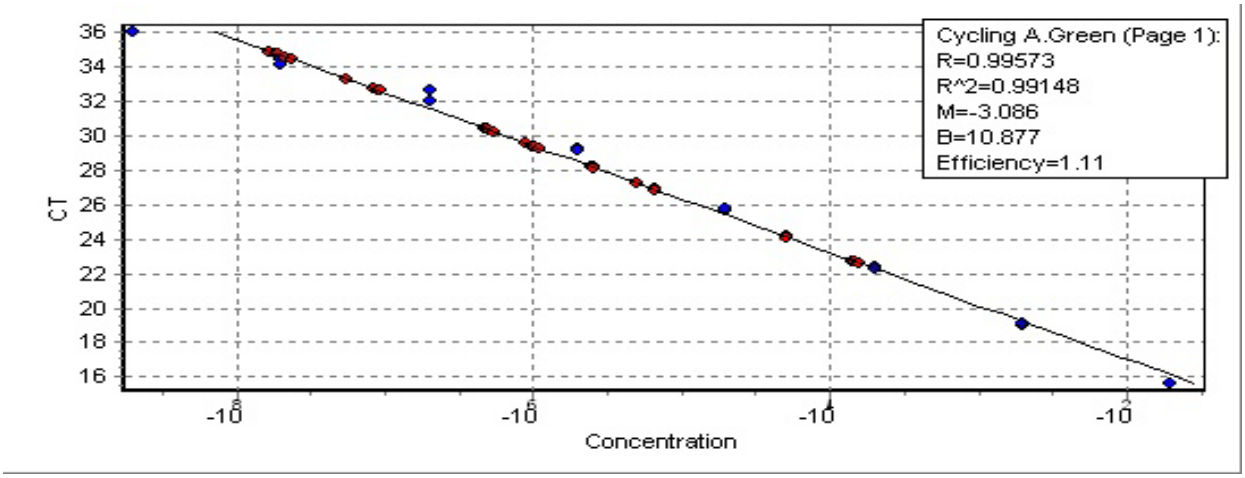
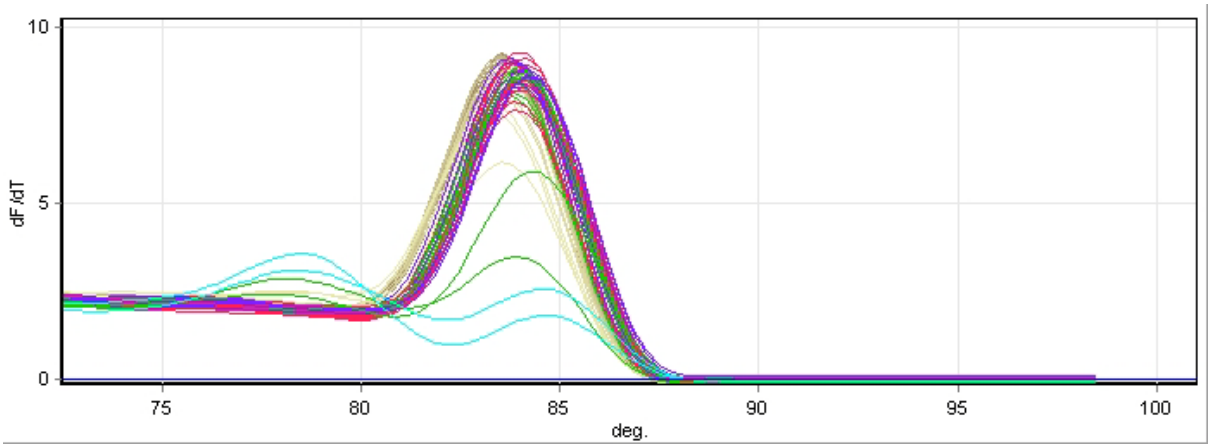


Figure 4.10 Example of standard curves for P450arom A and Vtg1 genes. Mean ± SEM Ct values are plotted against the log concentrations of the standards for 10-fold dilutions. Each standard curve has an R^2 value > 0.99, due to an equal number of cycles separating standards of 10-fold dilution concentration difference. Each curve has an efficiency value within acceptable range of between 0.7 and 1.1 (Pfaffl 2003a).

P450aromA



Vtg1

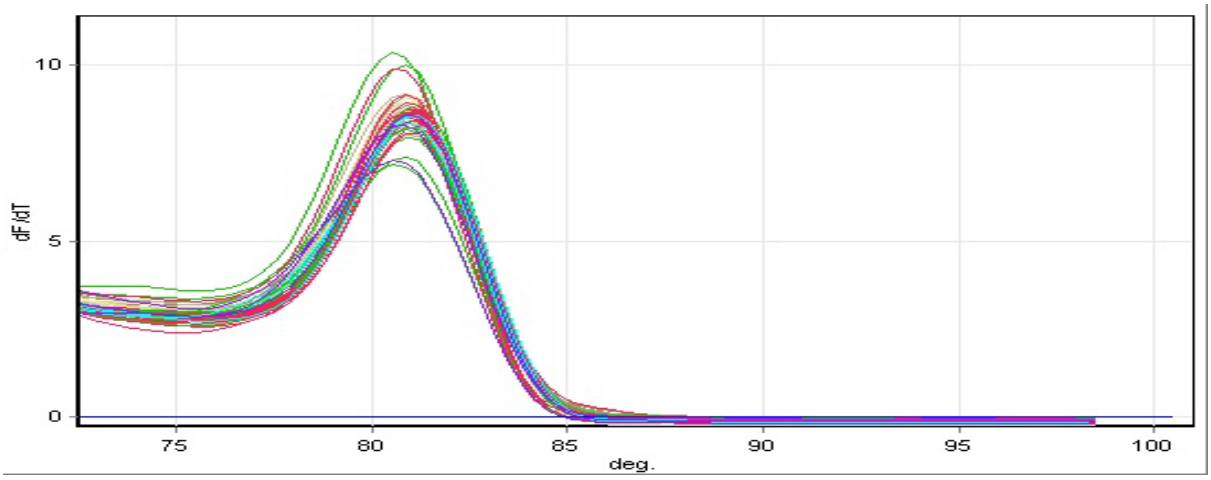
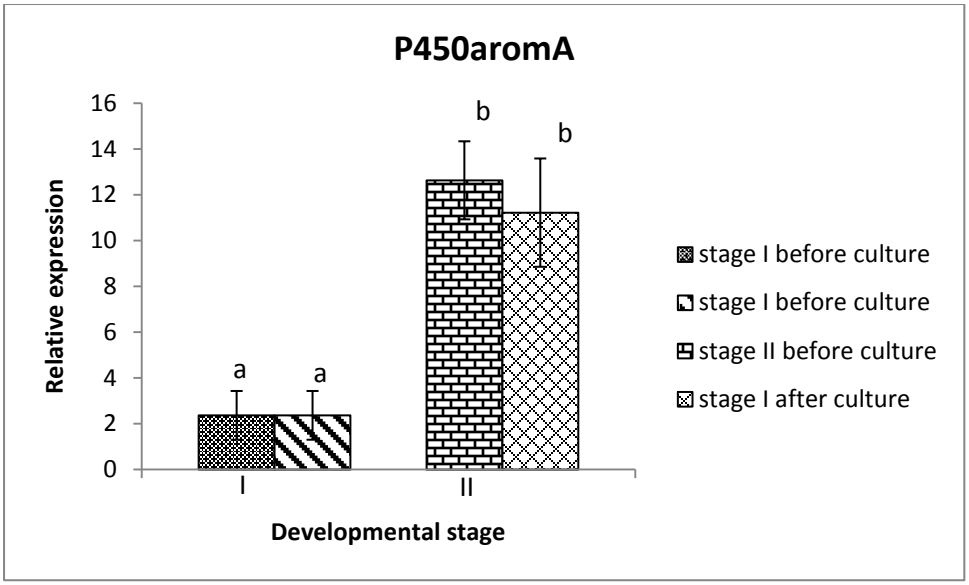


Figure 4.11 Example of melting curves for P450arom A and Vtg1 genes. Standard (brown), negative control (light blue) and the other colours related to ovarian follicle samples. All samples produced same peak, therefore sample have no contamination, mispriming and primer dimmers.

4.2.3 Studies on the expression of p450aromA gene and Vtg1 gene on different ovarian follicle developmental stages after *in vitro* culture treatment

Results from these experiments show that after 24 h *in-vitro* culture, the level of expression of P450aromA gene on stage I showed the level of expression of stage II obtained prior to culture. This indicates that stage I have developed to stage II follicles. Similarly the level of expression of Vtg1 gene on stage II showed the level of expression of stage III obtained prior to culture, indicating stage II have developed to stage III follicles. As a result P450aromA and vtg 1 gene can be used as markers to assess the growth of stage II and stage III ovarian follicles *in vitro*.

(a)



(b)

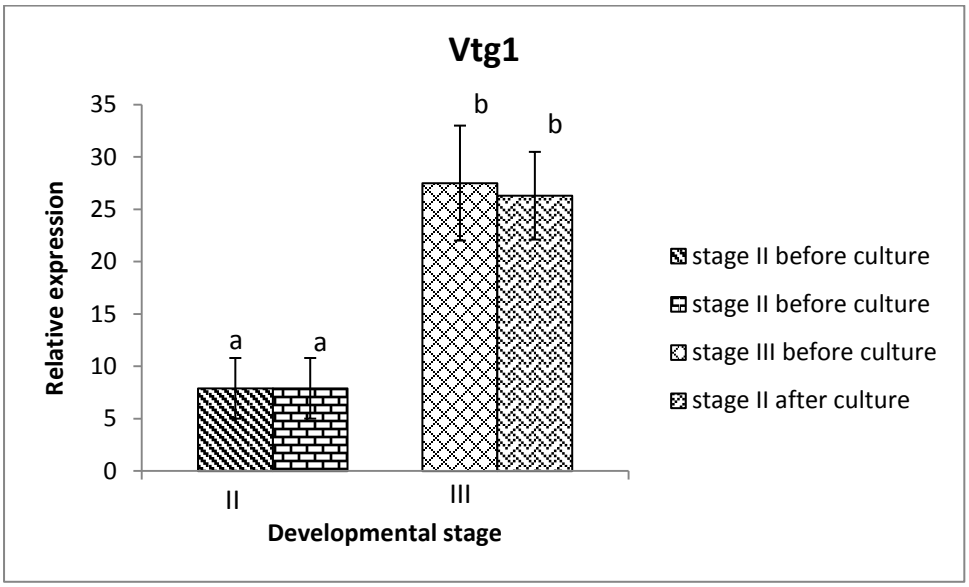


Figure 4.12: Relative mRNA expression of P450aromA gene (a) and Vtg1gene (b) after 24h *in-vitro* culture showing the growth from stage I to stage II follicles and from stage II to stage III follicles respectively. The values are normalised to EF1-α. Values represent the mean SEM of the relative expression as determined by RT-PCR. Different letters indicate significant differences.

4.3 Discussions

4.3.1 Effect of media supplements on the ovarian tissue fragment

Results obtained in the present study showed that after 24h of *in-vitro* culture, increase in follicle diameters were observed when ovarian fragments were incubated in 20% FBS when compared to the other concentrations. FBS has been shown as an effective growth factor in most fish culture and has been reported to increase the cellular growth rate (Goswami *et al*. 2009, Kumar *et al*. 2001) but it has not been used in zebrafish cultures until now. The FBS is also proven to be the essential supplement for the survival and optimal growth of fish cells (Lakra *et al.* 2010). FBS at concentration of 20% has been proven to favourable for cell growth and attachment for fin and heart tissues of common carp, after subculture the replication rate of the cell lines increased as the FBS concentration increased from 5- 20% (Lakra *et al*. 2010). The result obtained in the present study is also in line with the studies undertaken by Otala *et.al* (2002) on human ovarian tissues, Chen *et al*. (2004) and Kang *et al.* (2003) on marine fish. They reported in their study that culture medium supplemented with FBS preserved the integrity of the oocyte granulosa-stroma interaction, which is important for the development of early follicles.

Incubation of follicles with 0.5% BSA did not increase follicle diameter when compared to the controls whilst incubation with 20% FBS induced a significant increase in follicle diameter. Furthermore, incubation of fragments in 0.5% BSA in some samples induced damage to early stage follicles within the tissue fragments. Although lower concentrations of BSA were investigated, it showed lower membrane integrity when compared to FBS. In medaka, it has been reported that BSA is effective for the maturation of oocytes after LH surge (Iwamatsu, 1973). Seki *et al.* (2008) reported that BSA was proven effective for the cytoplasmic maturation of later stage zebrafish oocytes. But the *in-vitro* studies conducted on human ovarian tissue showed that incubation with albumin induced cell death (Otala *et al*. 2002). In the present study, FBS was shown to have a significant effect in stimulating the

ovarian follicle growth competence. This is in line with the study undertaken by Newton *et al*. (1999) who reported that FBS increased oocytes development in murine when compared to those cultured in BSA. In zebrafish oocytes, the mechanism of the effect of FBS on maturation is unknown, but FBS get endocytosed into the fish oocytes. Further studies are necessary to understand the mechanism of FBS on zebrafish oocytes. In the present study, 20% FBS was shown to have a significant effect in stimulating the ovarian follicle growth competence.

4.3.2 *Effect of gonadotropins on the ovarian tissue fragments*

In teleosts, as in other oviparous vertebrates, estrogens play important roles in reproduction. Biochemical analyses and molecular cloning studies on teleosts have demonstrated that there are two distinct gonadotropins (GTHs) namely, GTH-1, which is homologous to FSH; and GTH-II, which is homologous to LH (Melamed *et al.*1998). FSH and LH are expressed differently during the reproductive cycle. The level of FSH is high in oocytes growth stage and LH is high in the maturation stage (Clellan and Peng, 2008, Weil *et al*. 1995, Kwok *et al*. 2005). Since fish gonadotropins are not easily available, hormones from mammalian sources have been commonly used as the alternatives in various studies in fish (Tsai *et al*. 2010). In contrast to the high specificity of mammalian gonadotropins, the specificity of gonadotropins to their receptors has been shown to be less apparent in teleosts according to the limited studies in salmonids and catfish (Oba *et al.* 1999; Vischer *et al.* 2003). The only report on receptor specificity in cyprinids was conducted in an Indian carp using purified GTH receptors. However the study used heterologous salmon FSH and LH and its results interpreted with caution because of the high plasticity of gonadotropin-receptor interaction in teleosts (So *et al*. 2005).

The results obtained after the exposure to hCG did not aid follicle growth when compared with controls. This result is in line with the study undertaken by Wu *et al*. (2000) on zebrafish ovary that the treatment with hCG does not respond on stage I and stage II follicles, and also on stage III oocytes unless they are larger than 0.52mm. However the promotion of oocyte maturation competence by hCG has been

well documented in teleosts (Pang and Ge, 2002) they have reported that hCG significantly promoted the maturation of zebrafish stage III oocytes. Also studies with hCG treatment has shown to stimulate the growth in only later stage ovarian follicles in human (Filicori *et al*. 2002).

The results obtained from the present study showed that the treatment with FSH induced follicle size increase. It is known that FSH treatment increases the number of preantral and small antral follicles in mouse, it is also reported that the follicular growth up to antrum formation is controlled by FSH (Wang *et al*. 1993). FSH is involved in early folliculogenesis (Javed *et al*. 2008). In zebrafish, FSH act in early folliculogenesis and is essential to an adequate development up to the vitellogenesis (Kwok *et al.* 2005). The presence of FSH receptor in granulose cells suggests that FSH promote follicular development and growth (Magalhaes, 2009). However in Salmonoids, it has been proposed that FSH is likely to be important for promoting follicle growth in the ovary (Tyler *et al*. 1991). In trouts, FSH is elevated during pre-vitellogenesis, whereas LH appears during final oocyte maturation and ovulation in females (Gomez *et al.*1999). Furthermore, Meduri *et.al* (2008) has reported that FSH receptors appear during early stage ovarian follicle development. Treatment with FSH significantly increased the follicular diameter in most of mammalian *in-vitro* cultures (Rosetto *et al*. 2009, Rajarajan *et al.* 2006). FSH is essential for the differentiation of granulose cells and it regulates the transzonal connection between the oocytes and surrounding granulose cells (Chaves *et al.* 2012).

4.3.3 Optimum *in-vitro* culture protocol for zebrafish ovarian tissue fragments

An *in-vitro* culture protocol for zebrafish ovarian tissue fragments is identified here for the first time. The results obtained from the present study showed that FSH was most effective on the tissue fragments containing stage I and stage II ovarian follicles. The tissue fragments were cultured in 90% L-15 medium (pH 9) containing 100mIU/ml FSH with 20% FBS. The treatment increased the follicular diameter from stage I to stage II and from stage II and stage III. A different protocol will be required in order to grow stage III oocytes to more advanced developmental

stages as this requires vitellogenin. Hence further studies on oocyte maturation are required. However, the *in-vitro* culture method developed here will provide a reliable method for the practical use of ovarian tissue fragments containing stage I and stage II follicles.

4.3.4 The levels of expression of P450aromA gene and Vtg 1 genes at different stages of ovarian follicular development

The objective of this study was to investigate the levels of expression of P450aromA gene and Vtg 1 genes at different stages of ovarian follicular development in zebrafish and to employ the genes as biomarkers to assess the growth of ovarian follicles according to their developmental stages. The different developmental stage used for RNA extraction includes the granulosa and theca layers of the grown ovarian follicles. The gene expressions monitored were located in the fully-grown follicles. Little is known about the expression of the genes involved throughout follicle development in the zebrafish.

The present study (Section 4.2.3.1) demonstrated that the expression of P450aromA gene was higher in stage II ovarian follicles when compared to stage III ovarian follicles. This is in agreement with the studies undertaken by Ings *et al.* (2006) in zebrafish, where they reported that the expression of P450aromA peaked in previtellogenic follicles and dropped off to almost non-detectable levels in maturing follicle. Kumar *et al.* (2000) also showed that P450aromA expression decreased as follicles matured in the channel catfish. Other studies have found that the expression of P450aromA peaks during mid-vitellogenesis with a drop in expression during maturation in medaka (Fukada *et al.* 1996) and tilapia (Chang *et al*. 1997), although expression was not measured in earlier developmental stages. P450arom mRNA levels increase in association with increase of enzyme activity during vitellogenesis in medaka (Fukada *et al*. 1996). In salmonids, P450 arom enzyme activity and its mRNA levels increse during the vitellogenic oocyte growth (Kagawa *et al.*2003). Studies have shown that the highest levels of expression of P450aromA expression during maturation in artificially matured Japanese eels (Matsubara *et al.* 2003) and artificially matured red seabream (Gen *et al.* 2001), although this could be due to

hormonal stimulation, so therefore may not reflect the natural expression patterns of these species (Ings *et al.* 2006).

The present study (Section 4.2.3.2) demonstrated that the expression of Vtg1 gene was higher in stage III follicles when compared to stage I and stage II ovarian follicles. Vtgs are the most abundant proteins in the mature teleost oocytes. In late stage zebrafish oocytes, very large amounts of Vtgs in their large forms were observed (Ziv *et al.* 2008). The variability of protein and mRNA levels defines the specific maturation stage. Until recently, liver was assumed to be the main site for Vtg synthesis in teleosts. Levi *et al.* (2011) had reported that Vtgs are well expressed in ovary. The higher expression levels of Vtg 1 gene, may suggest the differential regulation by E_2 from differences in the estrogen in the promoter region (Levi *et al.* 2009). The mRNA expression reflects the *in vivo* gene expression in zebrafish, in which maturational competence was acquired through *in vitro* gonadotropin stimulation (Bobe *et al.* 2004).

In (Section 4.2.4), results from these experiments show that after 24 h *in-vitro* culture, the level of expression of P450aromA gene on stage I showed the level of expression of stage II obtained prior to culture and the level of expression of Vtg1 gene on stage II showed the level of expression of stage III obtained prior to culture. This shows that stage I has grown to stage II follicles and stage II has grown to stage III follicles respectively. Hence P450aromA and vtg 1 gene can be used as markers to assess the growth of stage II and stage III ovarian follicles *in vitro*.

The present gene expression study is focussed on mRNA levels of the target genes, and neither the corresponding proteins levels nor the functionality were assessed. P450aromA and Vtg1 genes have proved to be simple and sensitive markers for assessing the growth competence of the developing zebrafish ovarian follicles. In zebrafish, it is possible to use these genes to distinguish the growth pattern and confirm the structural difference in terms of the ovarian follicle development.

4.4 Summary

The results from these experiments demonstrated that the zebrafish ovarian tissue fragments containing stage I and stage II follicles can be cultured *in-vitro* for 24h, treated in 90% L-15 medium (pH 9) containing 100mIU/ml FSH with 20% FBS. It showed growth competence from stage I to stage II and from stage II to stage III respectively. Growth assessment can be confirmed by determining the expression of P450arom A and Vtg1 gene. The result from this study demonstrates that the *in-vitro* culture method and marker developed can be a positive indication for understanding the mechanism of maturation in zebrafish oocytes *in-vitro*.

CHAPTER 5 DEVELOPMENT OF CRYOPRESERVATION PROTOCOL FOR ZEBRAFISH OVARIAN FRAGMENTS USING CONTROLLED SLOW COOLING

5.1 INTRODUCTION

Cryopreservation of gametes is an important method of conservation of germplasm and has wide range of applications in aquaculture and fisheries management. Cryopreservation is the most efficient method for long term storage of genetic materials. Though fish sperm cryopreservation has been a success, developing successful protocols for eggs and embryo cryopreservation still remains elusive. Several studies have been undertaken on cryopreservation of isolated fish ovarian follicles at different stages (Guan *et al.* 2008; Tsai *et al.* 2009; Zampolla *et al.* 2008), yet the protocols used lead to compromised viability. It has been reported that early stages of zebrafish ovarian follicles are less sensitive to chilling than late stages (Tsai *et al.* 2009) and that the membrane permeability of zebrafish immature oocytes to water and cryoprotectants is higher than in mature oocytes (Seki *et al.* 2007a; Seki *et al.* 2007b). Cryopreservation of follicles within ovarian tissue has attracted considerable attention in recent years. It has become a valid alternative to cryopreservation of oocytes in human (Hovatta *et al*. 1996; Newton *et al.* 1996; Abir *et al*. 2001; Gook *et al*. 2001; Oktay *et al.*2001), sheep (Gosden *et al.* 2002; Cecconi *et al.* 2004), cattle (Celestino *et al*. 2008), goat (Rodrigues *et al.* 2004) and pig (Borges *et al.* 2009). Along with the cryoprotectants, varying concentrations of serum and serum substitutes have been used for cryopreservation of ovarian tissue. In sheep, supplementation with 10% bovine calf serum was used (Gosden *et al*. 1994), in human 10% foetal bovin serum was used (Oktay *et al*. 1997). No comparative studies regarding serum supplementations in cryopreservation of zebrafish ovarian tissue have been carried out, they are therefore investigated here. The present study was undertaken to determine the effect of cryopreservation on zebrafish ovarian tissue fragments containing the stage I and stage II follicles. The effect of cryopreservation medium, the effect of cryoprotectant, cooling rate and ovarian follicle viability assessment after controlled slow cooling were investigated.

Ovarian follicle membrane integrity was assessed by trypan blue (TB) and fluorescein acetate-propidium iodide (FDA-PI) staining. ATP levels were measured to assess the metabolic status following cryopreservation.

5.2 Results

The penetrating cryoprotectants, methanol and ethanol were chosen for this part of study. Each of the cryoprotectants was used at the highest No Observed Effect Concentration (NOEC) according to the viability test (for stage I and stage II ovarian follicles), which was determined in toxicity studies (Chapter 3).

5.2.1 Effect of medium

KCl buffer and 90% L-15 medium were used as freezing medium in this study. Methanol (2M) was used as a cryoprotectant in this experiment. The results of TB staining assessed the membrane integrity of stage I and stage II ovarian follicles with freezing rates of 2°C/min from seeding temperature (-12.5°C) to -40°C and then frozen to -196°C are shown in Figure 5.1. The result showed that KCl buffer and 90% L-15 medium were not significantly different from each other. Stage I ovarian follicles survival within the tissue fragments in 90% L-15 medium+2M methanol and KCl buffer + 2M methanol after cryopreservation at a cooling rate of 2°C/min was 41.3±1.0% and 39.6±1.9%, and survival of stage II ovarian follicles within the ovarian fragments was 63.3±1.5% and 60.8±1.8% respectively. Also the post-culture results obtained from (Chapter 4) showed that 90% L-15 medium was the optimum culture medium for zebrafish ovarian tissue culture. Hence 90% L-15 medium was therefore used as the base medium for the subsequent freezing experiment.

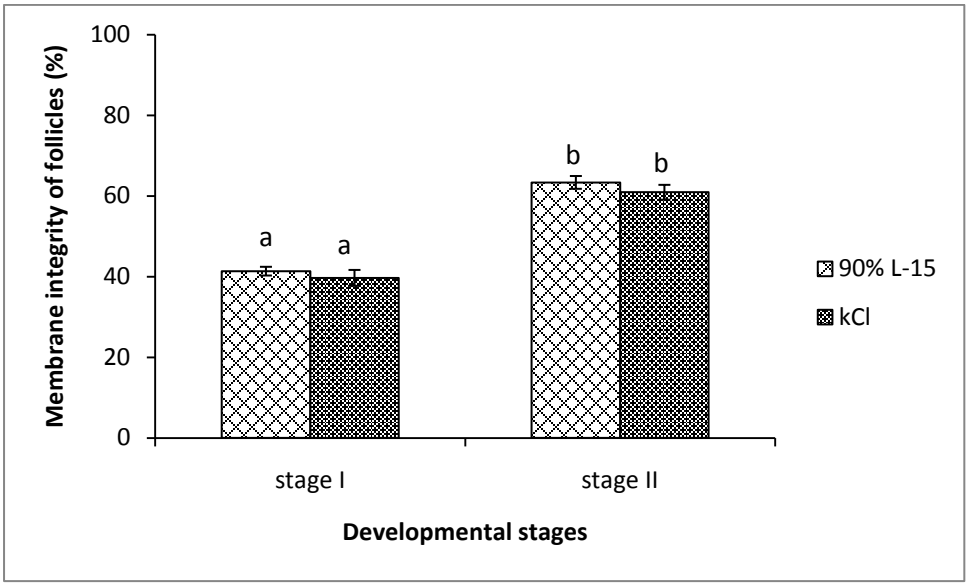
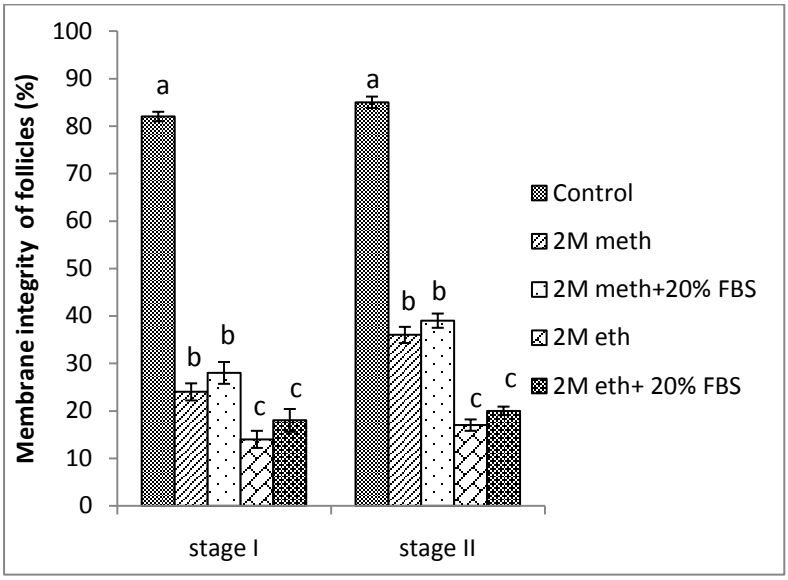


Figure 5.1 Effect of two freezing media on stage I and stage ovarian follicles survival with the tissue fragment. Ovarian follicles were frozen to -196°C in either 90% L-15 medium or KCl buffer in 2M methanol at cooling rate of 1°C/min. Stage I and stage II zebrafish ovarian follicles were incubated in 90% L-15 and KCl buffer in 2M methanol at room temperature for 10 min after thawing, and cryoprotectant was removed in one-step. Ovarian follicle membrane integrity was assessed with TB staining. Error bars represent standard errors of the mean. Different letters indicate significant differences between the stages ($p<0.05$).

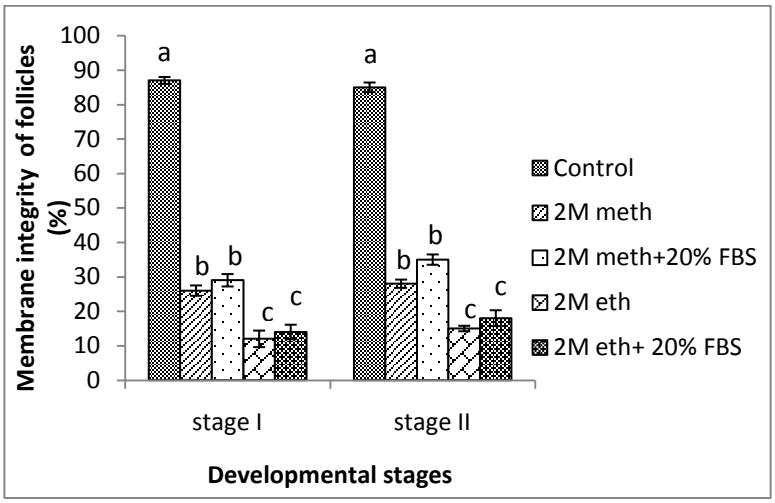
5.2.2 Effect of different cryoprotectants

The comparison of ovarian follicle survivals within the tissue fragments after cryopreservation in 2M methanol, 2M methanol+20% FBS, 2M ethanol and 2M ethanol + 20% FBS under different cooling conditions are shown in Figure in 5.2 (a-f). The cryoprotectants were made up in the 90% L-15 medium. Cryopreservation of zebrafish ovarian tissue fragments in 90% L-15 as the base medium along with different cryoprotectant combination was studied here for the first time. The results indicated that methanol diluted in 90% L-15 medium in combination with 20% FBS is a better cryoprotectant than ethanol for both stages showing the least damaging effect on membrane integrity. There were significant difference in stage I and II ovarian follicles within the tissue fragments when methanol or ethanol was used. The membrane integrity of the ovarian follicles within the tissue fragments was assessed with TB staining. The highest survival after cryopreservation was obtained with 2M methanol + 20% FBS. The addition of serum to the cryoprotectant combination increased the membrane integrity of the follicles. Hence this medium was used in subsequent experiments.

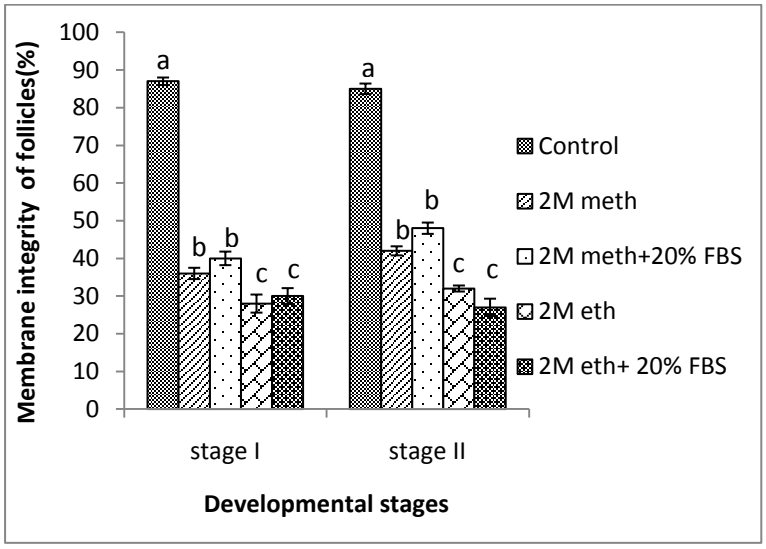
(a)



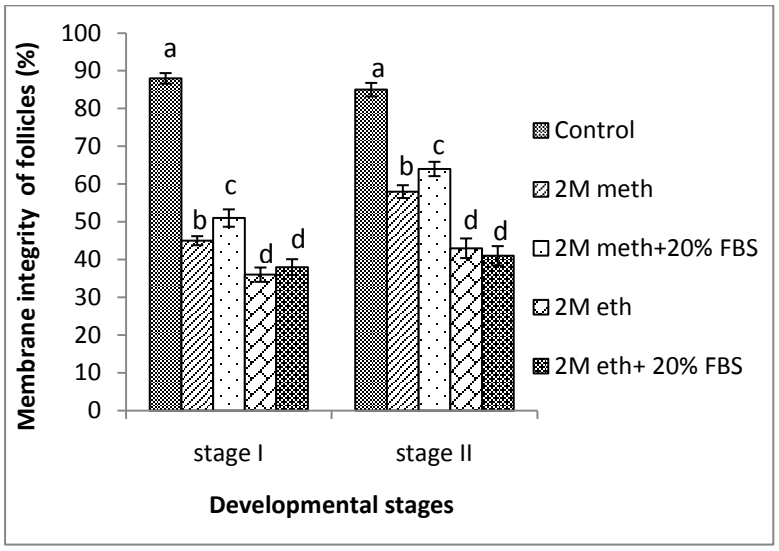
(b)


Membrane integrity of follicles (%)
100
90
80
70
60
50
40
30
20
10
0
a
b
b
c
c
a
b
b
c
c
Control
2M meth
2M meth+20% FBS
2M eth
2M eth+ 20% FBS
stage I
stage II
Developmental stages

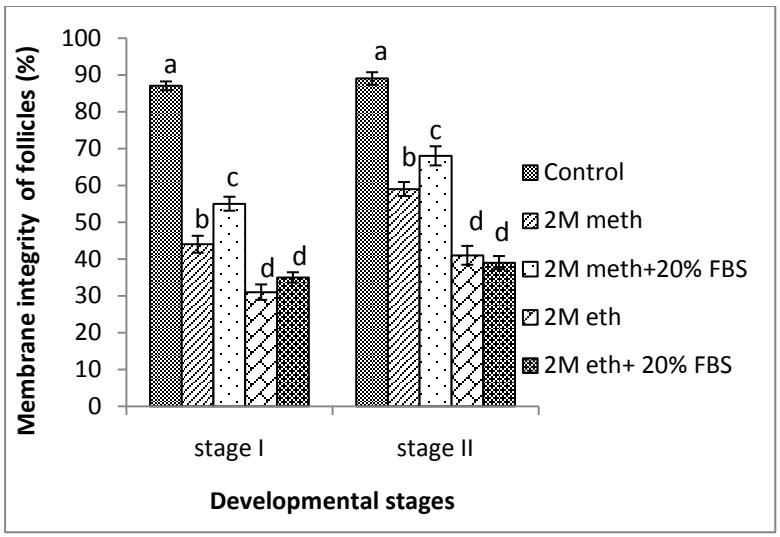
(c)


Membrane integrity of follicles(%)
100
90
80
70
60
50
40
30
20
10
0
a
b
b
c
c
a
b
b
c
c
Control
2M meth
2M meth+20% FBS
2M eth
2M eth+ 20% FBS
stage I
stage II
Developmental stages

(d)


Membrane integrity of follicles (%)
100
90
80
70
60
50
40
30
20
10
0
a
b
c
d
d
a
b
c
d
d
stage I
stage II
Developmental stages
Control
2M meth
2M meth+20% FBS
2M eth
2M eth+ 20% FBS

(e)


Membrane integrity of follicles (%)
100
90
80
70
60
50
40
30
20
10
0
a
b
c
d
d
a
b
c
d
d
stage I
stage II
Developmental stages
Control
2M meth
2M meth+20% FBS
2M eth
2M eth+ 20% FBS

(f)

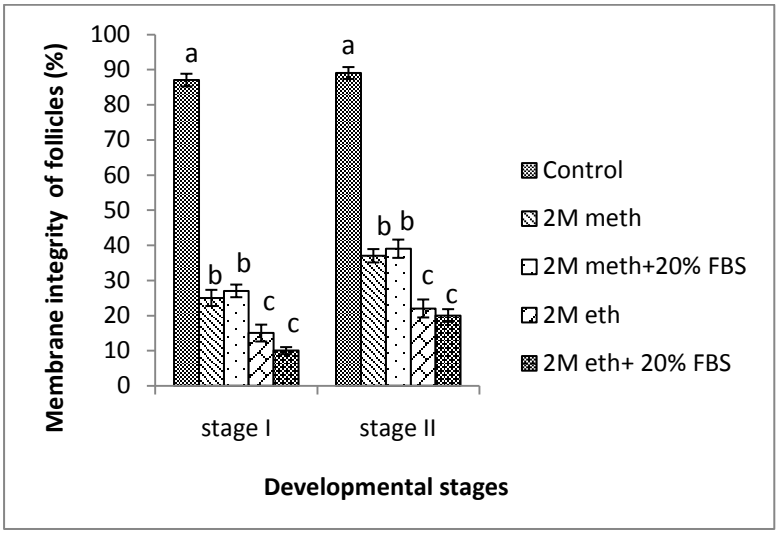


Figure 5.2: Comparison of different cryoprotectants (2M methanol, 2M methanol+20% FBS, 2M ethanol and 2M ethanol + 20% FBS made up in 90% L-15 medium) on ovarian fragments survival after cryopreservation and post-thaw incubation for 10min at room temperature. Ovarian fragments were incubated in the cryoprotective medium for 30 min at room temperature and then frozen to -196ºC, post-seeding cooling rates of 0.3ºC/min (a), 0.5ºC/min (b), 1ºC/min (c), 2ºC/min (d), 4ºC/min (e) and 7ºC/min (f). The cryoprotectant was removed in four steps. The membrane integrity was assessed with trypan blue staining. Error bars represent standard errors of the mean. Different letters indicate significant differences between the treatment groups ($p<0.05$).

5.2.3 Effect of post-seeding cooling rate

A number of post-seeding cooling rates, ranging from 0.3 -7°C/min were tested. Moderate cooling rates appear to be more advantageous when compared with faster or slower cooling rates (Figure 5.2). Ovarian follicles survivals obtained with 4°C/min post-seeding cooling rates were significantly higher when compared to other cooling rates, whilst survivals obtained with slower and faster rates were significantly lower. 4°C/min was therefore used in the subsequent experiments.

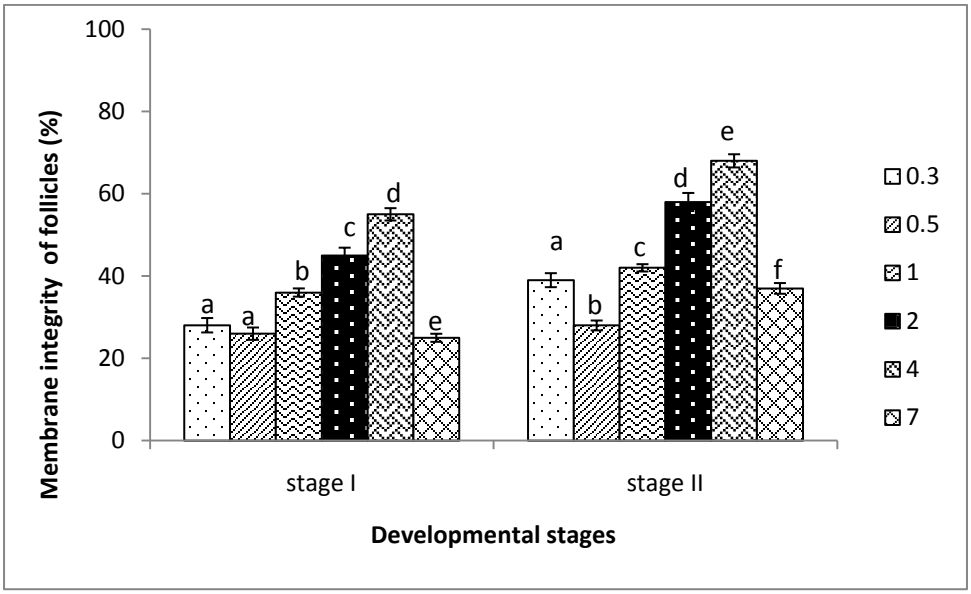


Figure 5.3 Effect of post-seeding cooling rate on stage I and stage II zebrafish ovarian follicles within the tissue fragments after cryopreservation and incubation at the room temperature. Ovarian follicles were frozen to 196°C in 2M methanol in 90% L-15 medium+20% fetal bovine serum at different cooling rates. The ovarian tissue fragments were incubated in 90% L-15 medium at room temperature for 10 min after thawing and four step cryoprotectant removal. Ovarian follicle membrane integrity was assessed using TB staining. Error bars represent standard errors of the mean. Different letters indicate significant differences between the treatment groups ($p<0.05$).

5.2.4 Effect of viability assessment method

The comparisons of the three viability assessment methods used in this study are shown in Fig 5.4 and 5.5. In experiment Fig 5.4, the comparisons of the two staining methods (TB and FDA-PI staining) were performed. TB staining assesses the membrane integrity of the follicles and FDA+PI staining assesses the membrane integrity and metabolic activity of the cells. Results showed that FDA+PI staining are more sensitive than TB staining and the sensitivity appeared to be increased with the increase in post-thaw incubation time. The percentage of undamaged follicles after cryopreservation assessed by FDA+PI was always lower than the follicles evaluated by TB. The FDA+PI proved to be more sensitive than TB staining of ovarian tissue fragments as microscopic observation showed a decrease of green fluorescence in the follicles with intact membrane after cryopreservation.

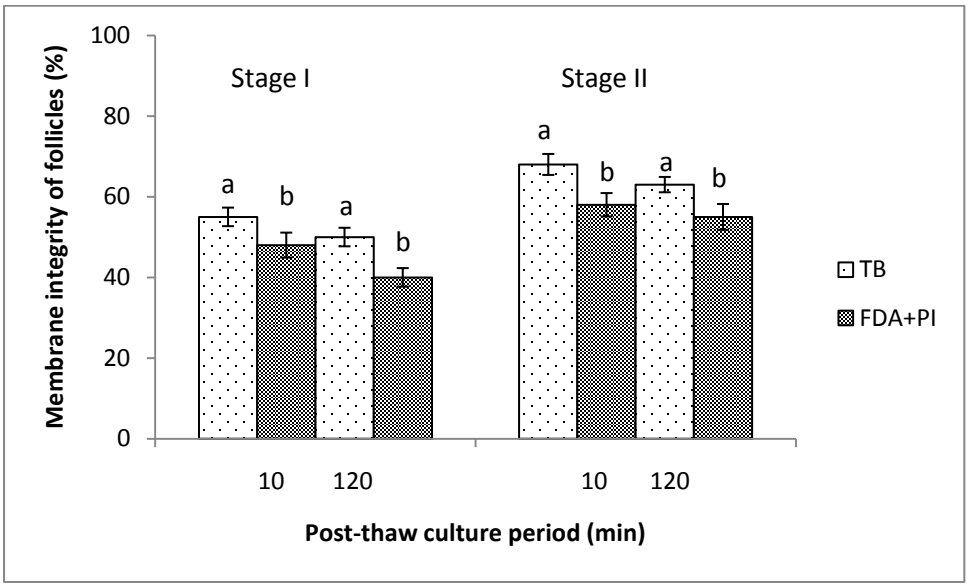


Figure 5.4: Membrane integrity of ovarian follicles within the fragments assessed using trypan blue staining (TB) and fluorescein diacetate + propidium iodide (FDA+PI) staining after cryopreservation and post-thaw incubation for 10min and 120 min (90% L-15 medium) at room temperature. The tissue fragments were incubated in 2M methanol in 90% L-15 medium+20% FBS for 30min at room temperature and then frozen to -196ºC at post-seeding cooling rate 4ºC/min. Cryoprotectants was removed in four-steps. Error bars represent standard errors of the mean. Different letters indicate significant differences between the staining methods ($p<0.05$).

The ATP levels in ovarian fragments were measured following cryopreservation. The results showed that the ATP levels were affected by cryopreservation (Figure 5.5). The ATP levels of the ovarian tissue fragments significantly decreased with 2M methanol+ 20% FBS in 90% L-15 medium.

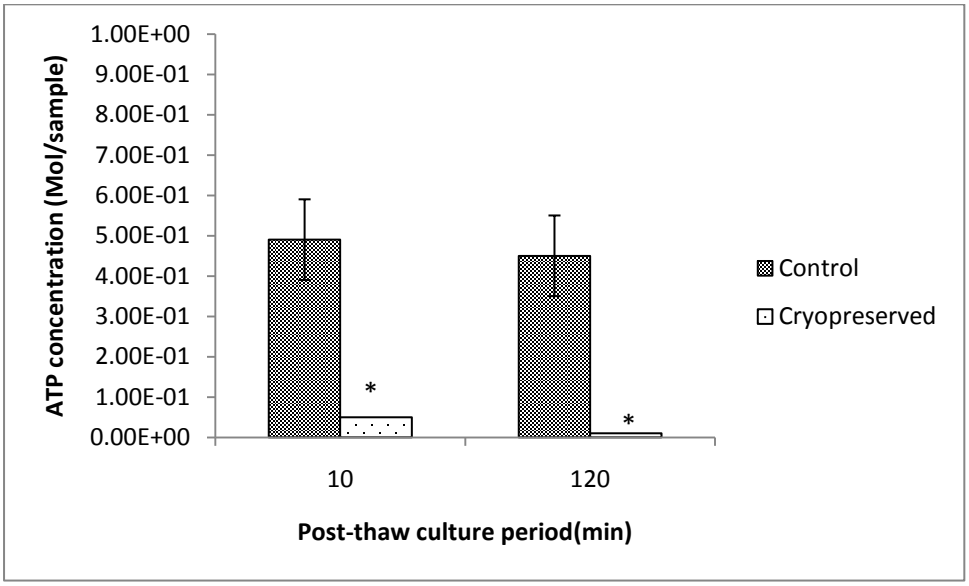


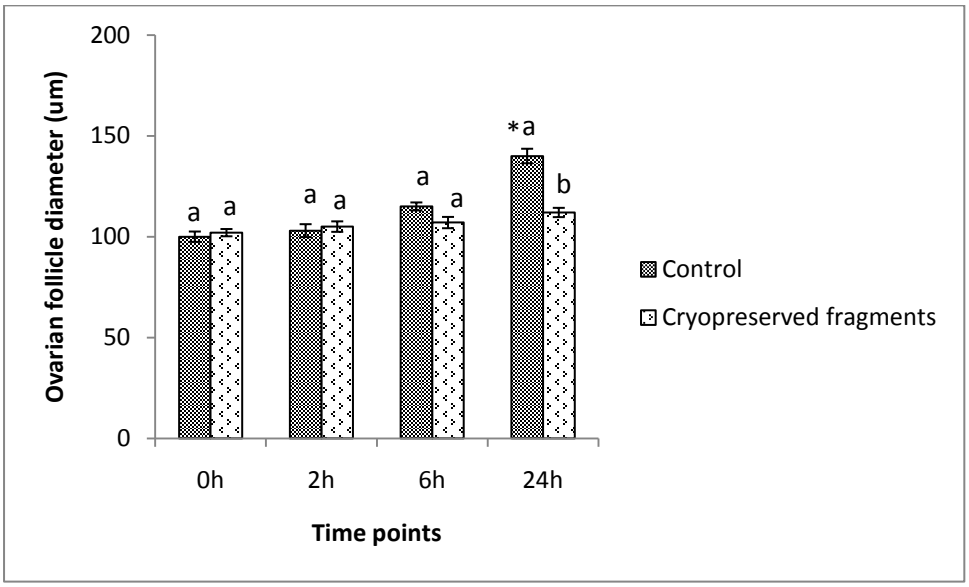
Figure 5.5: The Adenosine 5'-triphosphate (ATP) levels measured after cryopreservation and post-thaw incubation for 10min and 120 min (90% L-15 medium) at room temperature. The tissue fragments were incubated in 2M methanol in 90% L-15 medium+20% FBS for 30min at room temperature and then frozen to -196ºC at post-seeding cooling rate 4ºC/min. Cryoprotectants was removed in four-steps. Error bars represent standard errors of the mean. * Significantly different from corresponding control value, $p<0.05$

5.2.5 *In-vitro* culture of cryopreserved ovarian tissue fragments

The optimum *in-vitro* culture protocol developed in the previous experiment (Chapter 4) was used to assess the ovarian follicle growth competence after cryopreservation. The ovarian tissue fragments containing stage I and stage II follicles were cryopreserved using the freezing protocol identified in the previous experiments using controlled slow cooling. The tissue fragments were incubated in 2M methanol in 90% L-15 medium+20% FBS for 30min at room temperature and then frozen to -196ºC at post seeding cooling rate of 4ºC/min. Ovarian fragments were then incubated in 90% L-15 at room temperature for 10 min after thawing and cryoprotectants were removed in four steps. The ovarian fragments were then placed in the *in-vitro* medium containing 100mIU/ml FSH with 20% FBS and incubated for a 24h period. After 24h post-culture the follicle growth was measured using confocal microscopy and their membrane integrity was assessed using trypan blue staining.

The results showed that stage I and stage II follicles from the freeze-thawed group did not show any increase in diameter when measured at different time points (0h, 2h, 6h and 24h) (Figure 5.6). The results obtained from TB staining assessing the membrane integrity of the cryopreserved stage I and stage II follicles indicated a significant decrease when compared to the control (unfrozen groups).

(a)



(b)

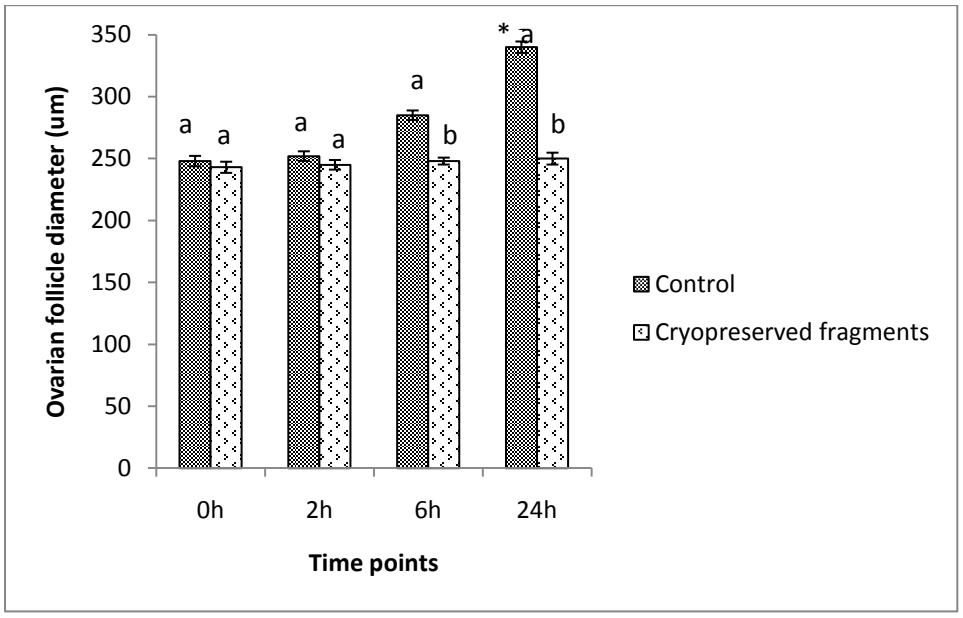


Figure 5.6: The growth of stage I (a) and stage II (b) ovarian follicles within the ovarian fragments after freeze-thawing and cultured in 90% L-15 medium (pH 9) containing 100mIU/ml follicle stimulating hormone(FSH) with 20% fetal bovine serum(FBS) for 24 hr at 28ºC. The tissue fragments were incubated in 2M methanol in 90% L-15 medium+20% FBS for 30min at room temperature and then frozen to -196ºC at post-seeding cooling rate 4ºC/min. Cryoprotectants was removed in four-steps. The diameters of ovarian follicles were measured with an ocular micrometer under microscope. Error bars represent standard errors of the mean. Different letters indicate significant differences between the control and cryopreserved groups ($p<0.05$) and * represent the significant difference between the time points.

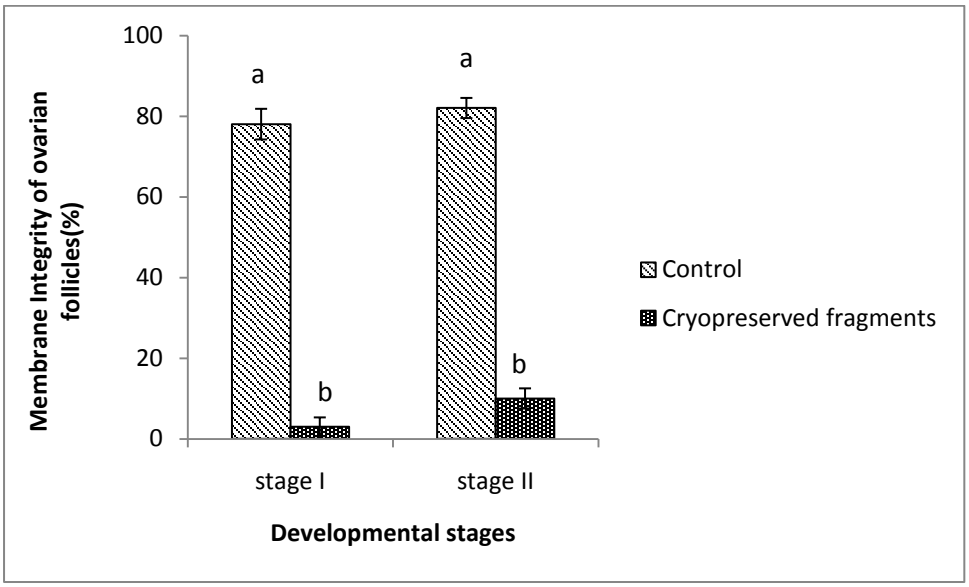


Figure 5.7: Viability of stage I and stage II follicles within the ovarian fragments after freeze-thawing and cultured in 90% L-15 medium (pH 9) containing 100mIU/ml follicle stimulating hormone (FSH) with 20% fetal bovine serum (FBS) for 24 hr at 28ºC. The tissue fragments were incubated in 2M methanol in 90% L-15 medium+20% FBS for 30min at room temperature and then frozen to -196ºC at post-seeding cooling rate 4ºC/min. Cryoprotectants was removed in four-steps. The viability was assessed by TB staining. Error bars represent standard errors of the mean. Different letters indicate significant differences between the control and cryopreserved groups ($p<0.05$).

5.2.6 The effect of using non-permeating cryoprotectants on the zebrafish ovarian tissue fragments

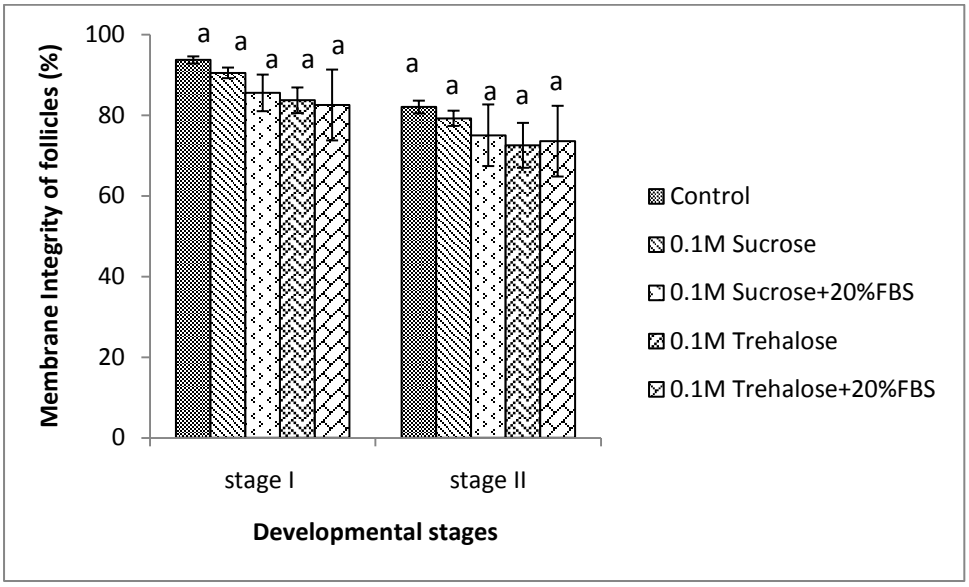
Saccharides are frequently used as extracellular cryoprotectants, often in combination with permeating cryoprotectants to maximize the cryoprotection. Sugars are useful in the stabilization of lipid membrane and proteins when cells are dehydrated (Crowe *et al.* 1998). As cells are dehydrated during cryopreservation, a balance must be maintained between the removal of free water that could form ice crystals without excessive removal of bound water, resulting in loss of structural support to the proteins and lipids. Therefore, sugars may be served as a replacement for bound water on the membranes to decrease the injury from dehydration stresses (Wright *et al.* 2004). One of the major advantages of sugars is their high glass transition temperature compared with permeating cryoprotectants (Chen *et al.* 2002). The high glass transition temperatures allow long-term storage of cells at high subzero and even supra zero temperatures. In fact, many organisms including arctic frogs, salamanders, some nematodes, insects, brine shrimps can survive at extremely low temperatures for years by undergoing a glassy state due to the accumulation of large amounts of intracellular sugars; therefore sugars offer a unique prospect for high-sub zero temperature storage of mammalian cells (Crowe *et al.* 1992).

In order to identify the disaccharides in a suitable concentration for zebrafish ovarian tissue cryopreservation, studies on the toxicities with sucrose and trehalose were carried out. As methanol was found to be the most effective cryoprotectant for cryopreservation in the previous experiments, 2M methanol was used in these experiments. Studies on toxicities were carried out under the following conditions: 2M methanol along with different concentrations of sucrose and trehalose, 2M methanol +20% FBS along with different concentrations of sucrose and trehalose were incubated for 30 min at room temperature and the membrane integrity was assessed using TB staining.

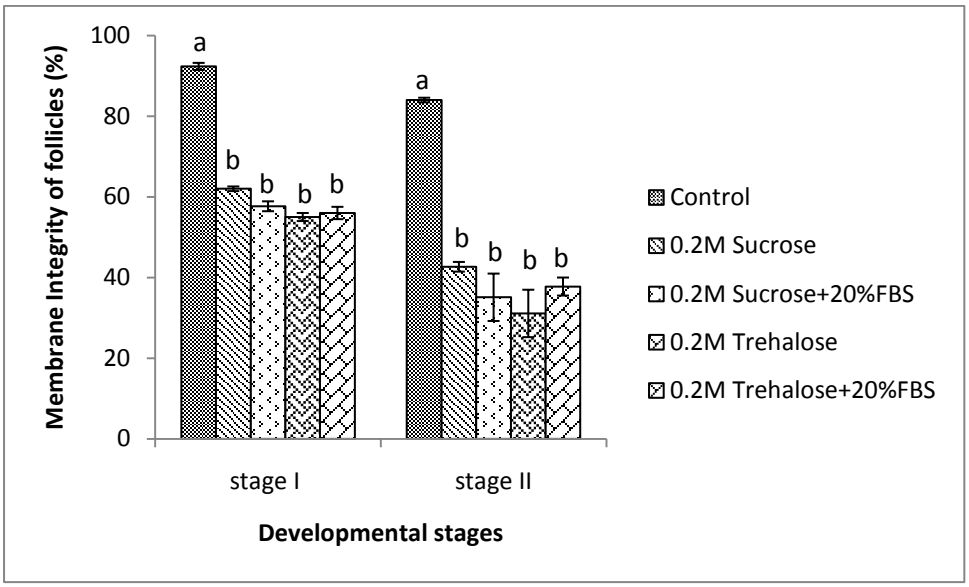
The effect of cryoprotectants at different concentrations on stage I and stage II follicles within the ovarian fragments are shown in Figure 5.8 (a-c). The results indicated that 0.1Msucrose+2M methanol in 90%L-15 medium were the least toxic

cryoprotectants when compared with other combinations. Hence this cryoprotectant combination was used for the subsequent freezing experiment.

(a)



(b)



(c)

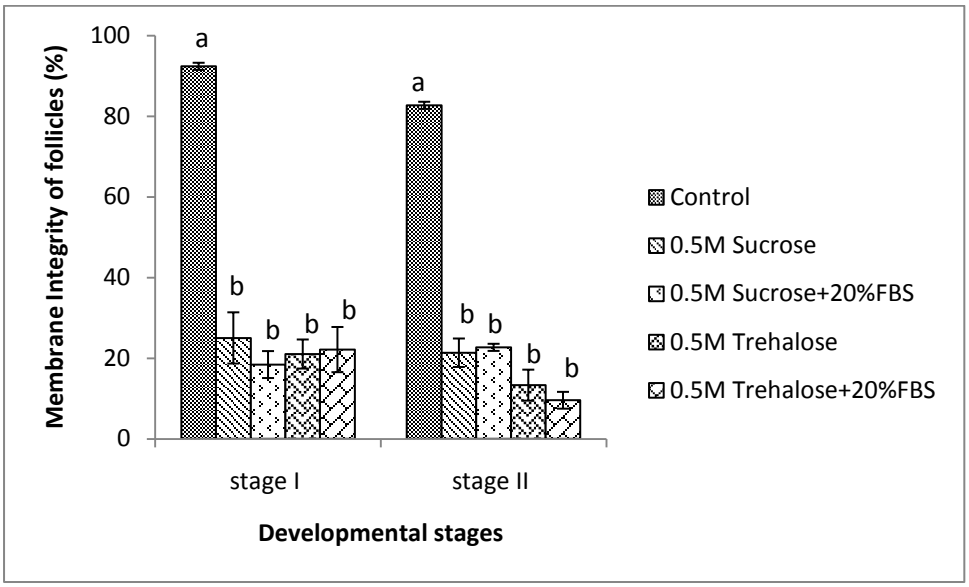


Figure 5.8: Membrane integrity of stage I and stage II zebrafish ovarian follicles in ovarian tissue fragments assessed with TB staining. The tissue fragments were exposed to different concentration of sucrose and trehalose 0.1M (a), 0.2M (b) and 0.5M (c) in 2M methanol for 30min at room temperature. Controls were incubated in 90% L-15(pH 9) for 30 min at room temperature. Different letters indicated significant differences between control and the cryoprotectant treated groups ($P<0.05$).

5.2.7 Effect of non-permeating cryoprotectants on ovarian tissue fragments

The comparison of ovarian follicles survivals within the tissue fragments after cryopreservation in 2M methanol+0.1M sucrose, 2M methanol+20% FBS+0.1M sucrose, 2M methanol+0.1M trehalose and 2M methanol+20% FBS+0.1M trehalose made in L-15 medium are shown in Figure 5.9. The results indicated that 2M methanol+20% FBS+0.1M Sucrose in L-15 medium is better combination cryoprotectant for both stages. There were significant difference in stage I and stage II follicles within the fragments when sucrose and trehalose was used with 2M cryoprotectant. The viability of the ovarian follicles within the tissue fragments was assessed using TB staining. Although the highest survival after cryopreservation was obtained with 2M methanol+20% FBS+0.1M sucrose the viability of the follicles lowered significantly to the control.

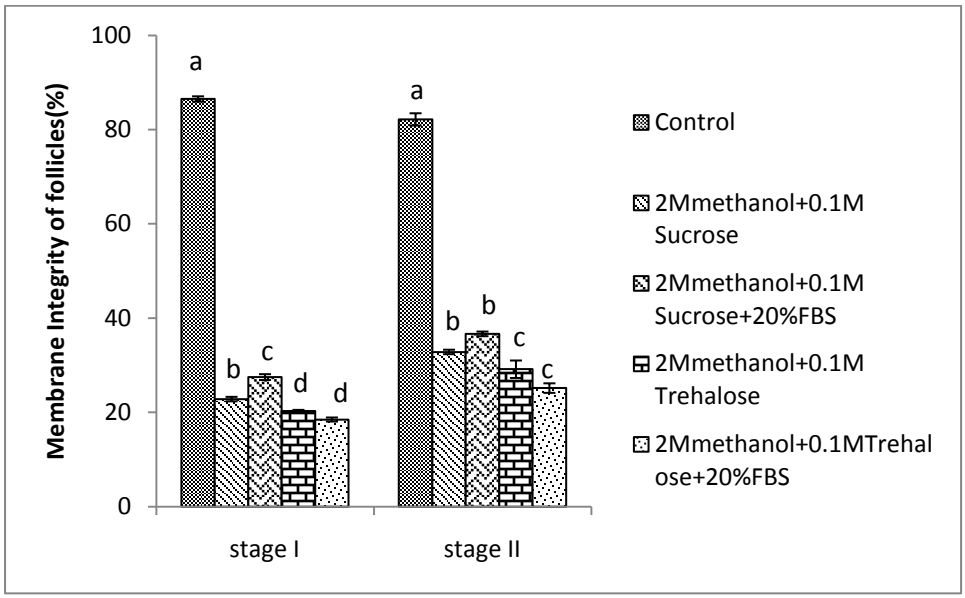


Figure 5.9 Comparison of different cryoprotectants 2M methanol+0.1M Sucrose, 2M methanol+0.1M Sucrose+20% FBS, 2M methanol+0.1M Trehalose and 2M methanol+0.1M Trehalose+20% FBS on ovarian fragments after cryopreservation and post-thaw incubation for 10min in 90% L-15 medium at room temperature. Ovarian fragments were incubated in the cryoprotective medium for 30 min at room temperature and then frozen to -196ºC, post-seeding cooling rates 4ºC. The cryoprotectant was removed in four steps. The membrane integrity was assessed with TB staining. Error bars represent standard errors of the mean. Different letters indicate significant differences between the control and cryopreserved groups ($p<0.05$).

5.2.8 Effect of cryopreservation with non-permeating cryoprotectant on the membrane integrity

The ovarian tissue fragments were incubated in 2M methanol+20% FBS+ 0.1M sucrose for 30min at room temperature and were frozen to -196°C at post seeding cooling rate 4°C/min. After cryopreservation the cryoprotectant was removed in four steps and the survival was assessed using TB and FDA+PI staining. The results showed that cryopreservation using the non-permeating cryoprotectant appeared to have significant effect on the membrane integrity of the cryopreserved group assessed by trypan blue and FDA-PI tests.

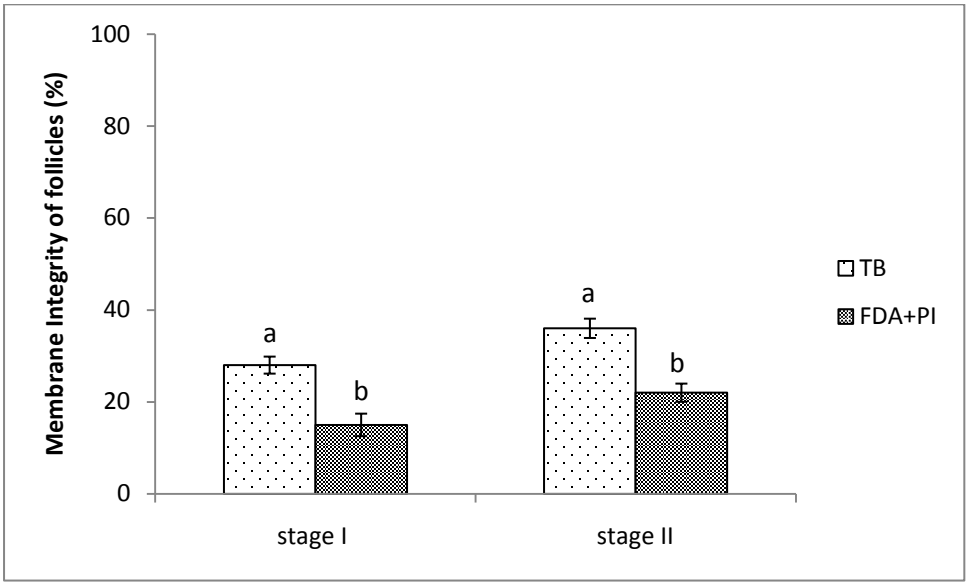


Figure 5.10: The ovarian follicles within the fragments viability assessed using trypan blue (TB) and fluorescein diacetate + propidium iodide (FDA-PI) staining after cryopreservation and post-thaw incubation for 10min at room temperature. The tissue fragments were incubated in 2M methanol+20% FBS+0.1M Sucrose in 90% L-15 medium for 30min at room temperature and then frozen to -196ºC at post-seeding cooling rate 4ºC/min. Cryoprotectants was removed in four-steps. Error bars represent standard errors of the mean. Different letters indicate significant differences between the treatment groups ($p<0.05$).

5.2.9 Effect of cryopreservation with non-permeating cryoprotectant on ATP levels

The ATP levels in the ovarian tissue fragments were measured following cryopreservation. The ovarian tissue fragments were incubated in 2M methanol+20% FBS+ 0.1M sucrose for 30min at room temperature and were frozen to -196°C at post seeding cooling rate 4°C/min. After cryopreservation the cryoprotectant was removed in four steps and the survival was assessed using ATP assay. The results showed that ATP levels of the ovarian tissue fragments significantly decreased after cryopreservation with sugars.

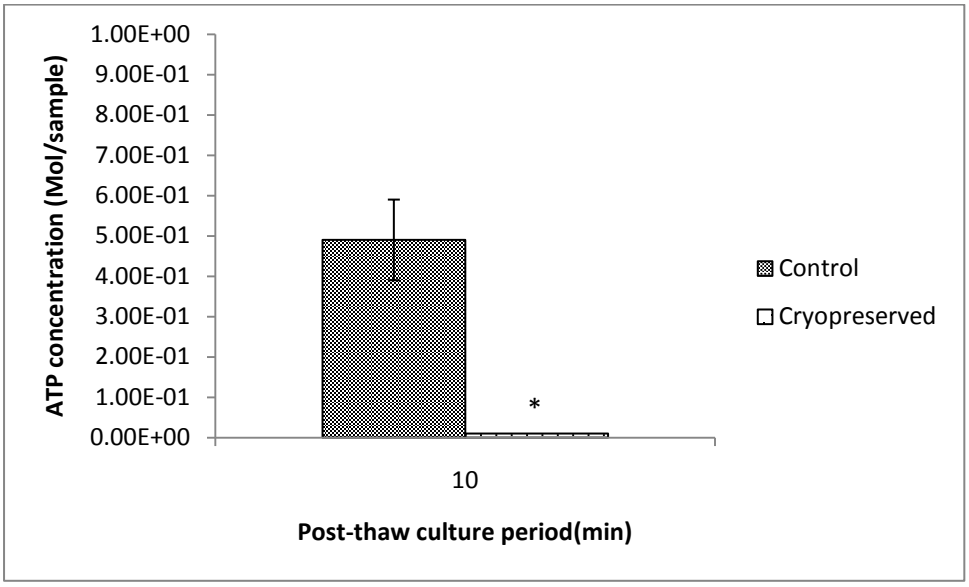


Figure 5.11: The Adenonosine 5'-triphosphate (ATP) levels measured after cryopreservation and post-thaw incubation for 10min at room temperature. Control: ATP levels were assessed in ovarian fragments held in 90% L-15 medium at room temperature; Treated: ATP levels were assessed after cryopreservation of ovarian fragments in 2M methanol+20% FBS+ 0.1M sucrose Error bars represent standard errors of the mean. * Significantly different from corresponding control value, $p<0.05$.

5.3 Discussion

Cryopreservation of fish ovarian tissue has proven to be a major challenge. Low permeability and surface volume ratio and high internal water are the fundamental reason for the failure of fish eggs and embryos cryopreservation (Saragusty and Arav, 2011). Several factors that assist survival of ovarian tissue cryopreservation include cryoprotectants, cryopreservation medium, cooling rates, removal of cryoprotectants.

5.3.1 Effect of medium

L-15 medium has been widely used as base medium in various ovarian tissue cryopreservations in human (Schmidt *et al.* 2003). L-15 medium is also used in various fish ovarian follicle culture and *in-vitro* maturation. It has been reported by Onions (2008) that L-15 medium has no negative effects on freezing of whole ovine ovary. Under physiological conditions, sodium ions diffuse freely into the cell, but the excess is removed by sodium pumps to balance the considerable proportion of the cell's energy outflow (Wolfe, 1993). During freezing, the rise in the extracellular solute concentration due to the ice formation helps the flow of water out of the cell and also increases diffusion of sodium into the cell. In this process, the sodium pump may be expected to stop due to decrease in temperature. It is therefore likely that the intracellular sodium will have increased by the time the cell is transferred to LN2. This situation will still exist immediately after thawing and could lead to post-thaw damage. Hence sodium containing freezing medium would help overcome this problem (Stachecki *et al.* 2000). The present study also showed that L-15 medium is advantageous in using as a base medium in freezing experiments.

5.3.2 Effect of different cryoprotectants

Cryoprotectants are essential component to maintain the cell viability and functions for storage at very low temperature. The results obtained in the present study showed that methanol is a better cryoprotectant than ethanol for freezing the zebrafish ovarian tissue fragments. These results are in agreement with the results obtained with zebrafish embryos, oocytes and isolated follicles (Zhang *et al.* 1993; Liu *et al.* 2001; Guan *et al.* 2008; Tsai *et al.* 2009; Zampolla *et al.* 2009). The relative effectiveness of methanol as a cryoprotectant is thought to be due to its rapid penetration and low toxicity (Zhang and Rawson, 1995). The results in the present study also showed that the viability of the cells increased when methanol was used in the combination with FBS. These results are in agreement with the study undertaken by YunHa *et al.* (2005) on human embryonic stem (ES) cells, which reported that FBS in the combination of cryoprotectant increased the survival rate after cryopreservation, showing that serum is needed for the cryopreservation of human ES cells, freezing cells without FBS is associated with a poor survival rate (YunHa *et al.* 2005). This study is also in agreement with studies in fish cells (Choresca *et al.* 2011; Han *et al.* 2011) and European eel sperm (Macro-Jimenez *et al.* 2006) that addition of FBS in the freezing medium increased the cell viability. Also in the study of cryopreservation of rat hepatocytes, the best survival rate was found with cryoprotectant and 30% FBS (Son *et al.* 2006). The cell survival was found to be 80% higher when compared to the cryoprotectant without serum. FBS is a mixture that contains various low level antibodies and growth factors that are beneficial to cells. It has been reported that cryopreservation of oocytes may lead to hardening of the zona pellucida and the addition of FBS can protect the mouse zona from hardening as long as it is present throughout the thawing procedure (Schroeder *et al.* 1990, George *et al.* 1993, Carroll *et al.* 1993). Hence FBS is beneficial combination along with cryoprotectants in a freezing protocol.

5.3.3 Effect of post-seeding cooling rate

In the present study, the optimal post-seeding cooling rate for zebrafish ovarian tissue fragments was found to be 4ºC/min. This rate is comparable to the study with isolated early ovarian follicles identified as 2- 4ºC/min (Tsai *et al.* 2009) but much higher than 0.3ºC/min identified for stage III isolated ovarian follicles (Guan *et al.* 2008). As stage III ovarian follicles larger with limited membrane permeability, slower post-cooling rates would be required for their cryopreservation (Zhang *et al.* 2005). During the process of ovarian tissue freezing, if the cooling rate is fast the intracellular water will have no time to flow out and the cells within the tissue fragments will be unable to maintain the equilibrium, and ice crystals will form intracellularly. The cooling rate must also be slow enough to overcome the difficulty of heat transfer and equilibrium due to the space between the inner cells and the ovarian tissue fragment surface. However, slowly cooled cells suffer damage due to long exposure to high electrolyte concentrations, excessive cell dehydration, and mechanical effects of the external ice. Thus the cooling rates should be fast enough to minimize the long exposure of ovarian tissues to the adverse freezing conditions, but slow enough to avoid the damaging effects of intracellular ice formation. Therefore it requires higher post-seeding cooling rate for ovarian tissue fragments.

5.3.4 Effect of viability assessment method

In the present study after cryopreservation of the zebrafish ovarian tissue fragment their viability was assessed using TB staining, FDA+PI staining and ATP assay. The results showed that the cryopreservation appeared to have effect on the membrane integrity and metabolic activity as assessed by TB and FDA+PI staining (Figure 5.4). TB staining is one of most common method to assess the cell viability by assessing the cells membrane integrity (Narayana *et al.* 2005). In TB dye, the chromophore is negatively charged and does not penetrate the cell unless the membrane is damaged. The present study showed that FDA+PI staining is more

sensitive than TB staining because after cryopreservation of the ovarian tissue fragment the follicles stained by FDA+PI showed a lower level of green fluorescence when compared to the TB stained groups. The FDAs reaction is characterised by the appearance of bright –green fluorescence inside the cell. In viable cells the fluorescein is unable to pass through intact membrane accumulating in the cytoplasm of the cells, while damaged cells show a distinct loss of fluorescein through the cell membrane. And PI is a DNA-binding probe which can be performed as single or in combination with other fluorochromes (Cai *et al.* 2005). Live cells with intact membranes are differentiated by their ability to exclude the dye that easily penetrates dead or damaged cells, intercalating with DNA and RNA to form bright red fluorescence. Since the dye is excluded by intact cell membranes, PI is an effective stain to identify non-viable cells (Zampolla, 2009). The live follicles produced fluorocein, producing bright green stain remain unstained by PI. These results are in agreement with the previous studies on isolated follicles (Zampolla *et al.* 2009, Tsai *et al.* 2009).

In the present study the ATP levels in ovarian tissue fragments were measured following cryopreservation (Fig 5.5). Cryopreservation induced a significant decrease in viability with 2M methanol + 20% FBS when compared to the control. The ATP content in the cell is a reliable parameter for evaluation of viability of different types of cells, including fish sperm cells (Perchec *et al.* 1995), fish oocytes (Wendling *et al.* 2004). ATP plays an essential role in gamete maturation and embryo development, and high quality oocytes contain significantly higher levels of ATP (Bradbury *et al.* 2000). The result from the present study showed that ATP assay is the most sensitive method for assessing the quality of follicles in tissue fragments after cryopreservation. Guan *et al*. (2008) also reported a decrease of ATP levels following cryopreservation of isolated stage III follicles and same results were observed in the study conducted by Tsai *et al*. (2009), where ADP/ATP ratio was evaluated.

5.3.5 *In-vitro* culture of cryopreserved ovarian tissue fragments

When the cryopreserved ovarian tissue fragment were cultured using the optimum protocol developed (Chapter 4), the results showed that the ovarian follicles within the fragment did not show any growth after the *in-vitro* treatment (Figure 5.6). The results also showed that the ovarian follicles viability was significantly lower than those of the controls after 24h culture (Figure 5.7). These results are in agreement with the previous information on cryopreserved ovarian tissue fragments which showed damage in the membrane integrity and metabolic activity of the cryopreserved tissues (Sec 5.2.4). The primary causes of cellular damage during cryopreservation is (a) intracellular ice formation occurs from rapid super-cooling inside the cell (b) an increase in solute concentration as the sample cools down and extracellular ice forms, solutes become concentrated in the remaining liquid. The cryopreserved ovarian follicles within the fragments did not show any growth, possibly this may due to the damage of theca and granulosa cells by intracellular ice formation. The freezing/thawing process severely damages preantral follicles (Cecconi *et al.* 2002). This is because the early follicles are sensitive to cryodamage. The physiological differentiation of mammalian ovarian follicles is strictly dependent on the existence of a bidirectional function between the germinal and somatic cell compartments. This function is applied either through the production of paracrine/autocrine factors or through the presence of functional gap junctions (Simon *et al.*1997). Further studies are required on the effect of cryopreservation procedures and the possible intracellular ice formation.

5.3.6 The effect of using non-permeating cryoprotectants on the zebrafish ovarian tissue fragments

In this study, different non-permeating cryoprotectants were tested. Methanol, the intracellular compound that impair the crystal ice formation inside the cells and two extracellular compounds (sucrose and trehalose) which improve freezing osmotic imbalance was used.

The result in the present study showed that 0.1M sucrose+2M methanol in 90% L-15 medium were the least toxic cryoprotectants with higher follicle survival when compared with the other combination. But after cryopreservation of the ovarian tissue fragments the membrane integrity and ATP level of the ovarian follicles decreased significantly to the control. Osmotic shock is one of the main problems occurring during the freezing process. And this can be minimized by using non-permeating cryoprotectants, such as sucrose which minimize the osmotic stress of the cell. When inside the cell, sugars may serve to stabilize membranes, proteins and supra-molecular structures during dehydration (Crowe *et al.* 1998). However, sugars are unable to cross the cell membrane naturally, limiting their beneficial effects to enhancing cellular dehydration by osmotic pressure. Sugars such as sucrose and trehalose have been widely used as natural cryoprotectants for freezing. The precise mechanism by which disaccharides act to preserve biological systems during freezing and drying is not well understood. The fact that they do not enter in cells is the main advantage, facilitating their removal after thawing (Rodrigues *et al*. 2008). But the use of sucrose or trehalose has led to low rate of zebrafish ovarian follicles viability. These results are in line with the study on aminotic fluid stem cells cryopreservation, which showed significant decrease in viability on using sucrose or trehalose (Janz *et al.* 2012). This may be because these sugars as extracellular agents do not prevent the formation of ice crystal within the cells. Some studies have demonstrated the beneficial nature of having intracellular sugars offering additional protection for the cell during freezing. Hence the use of sugars as a extracellular agent is not beneficial in the improvement of freezing protocol for zebrafish ovarian tissue fragments. Instead develop the technique to insert sugars into the oocyte which has demonstrated success in mouse models and in human oocytes (Wright *et al.* 2004).

5.4 Summary

The aim of the present study was to develop a cryopreservation protocol by using a controlled slow cooling method for zebrafish ovarian tissue fragments. Cryopreservation of ovarian tissue fragments containing stage I and stage II fragments were carried here for the first time. The results from the present study indicated that the tissue fragments can be incubated in 2M methanol+ 20% FBS in L-15 medium for 30 min at room temperature and then frozen to -196º C at post-seeding cooling rate 4ºC/min and the removal of the cryoprotectant in 4 steps. After cryopreservation the survival rate of the follicles was assessed using TB staining, FDA+PI staining and ATP assay. The survivals after cryopreservation procedure were 55.4±2.3% for stage I and 68.2±1.9% for stage II using TB staining and 48.2±2.9% for stage I and 54.2±2.6% for stage II follicles using FDA+PI staining. The results obtained from ATP assay showed compromised survival of the ovarian follicles. The results obtained from this study provides an improved cryopreservation protocol since it enhanced survival of the follicles with the use of 2M methanol + 20% FBS in 90% L-15 medium when compared to the previous study (Guan *et al.* 2008; Tsai *et al.* 2009) with 4M methanol in KCl buffer which are highly toxic to the cells. The present study also indicated that the use of non-permeating cryoprotectants is not beneficial for the zebrafish ovarian tissue cryopreservation since it decreased the survival rate of the follicles when compared to the use of permeating cryoprotectants. The results also showed that 90% L-15 medium in the freezing medium did not show any significant difference from KCl buffer on the survival of follicles after cryopreservation. The results obtained after the *in-vitro* culture of the cryopreserved fragments showed that there was no growth of follicles obtained after *in-vitro* culture treatment and the survival rate of the cryopreserved follicles were significantly lower to those of the non-cryopreserved follicles. Although a successful cryopreservation protocol for ovarian tissue fragments has not been achieved, the present study provides useful information for future cryopreservation protocol development.

CHAPTER 6 CONCLUSION

6.1 Reiteration of Aims

Cryopreservation of fish ovarian tissue fragments can be a viable alternative to cryopreservation of oocytes and embryos. Cryopreservation of ovarian tissues are advantageous over the embryos and oocytes as they can be cultured and cryopreserved in small pieces which are rich in primary follicles. There are several advantages in cryopreservation of ovarian tissue fragments; including the storage of large number of ovarian follicles, and the ovarian follicle remains in their natural three-dimensional structure which can tolerate cryopreservation.

Studies on isolated zebrafish ovarian follicle cryopreservation have been carried out in our laboratory (Isayeva *et al.* 2004; Plachinta *et al.* 2004; Guan *et al.* 2008; Tsai *et al.* 2009), the results from these studies showed that early stage oocytes are less sensitive to chilling when compared to the late stage oocytes (Tsai *et al.* 2009) and the sensitivity to cryoprotectant also appeared to increase with development stage. Cryopreservation of early stage follicles showed compromised viability using TB staining but the result after ATP assay indicated that it failed to preserve cells energy systems after cryopreservation. Previous study on the *in-vitro* culture of stage I and stage II follicles also showed that the cryopreserved ovarian follicles did not grow after 24h culture. The protocol developed in the previous study did not support the growth of follicles possibly be due to the exposure of follicles to hCG which has been reported that it does not respond to zebrafish stage I and stage II follicles (Wu *et al.* 2000). Hence developing an *in-vitro* culture method for zebrafish ovarian tissue fragments is a significant step since if cryopreservation of the tissues can be achieved, *in-vitro* procedures for the ovarian follicle development after cryopreservation would be needed. Developing a biomarker is also important after *in-vitro* culture study; because although the growth in size and development from one stage to another can be assessed by measuring the diameter the use of biomarkers would provide more accurate information on the follicle development.

The growth in size of the follicle can be mistaken due to the increase in cell size due to the uptake of solutions, but biomarkers measure the level of expression at each development stage and confirm the specific stage.

Hence the main aim of the present study was to develop an *in-vitro* culture protocol and cryopreservation protocol for the zebrafish ovarian tissue fragments.

In order to achieve the aim the following investigation were carried out: (i) Studies on cryoprotectant toxicity to ovarian tissue fragments containing stage I and stage II follicles. Although toxicity studies have been carried out on isolated follicles, studies on cryoprotectant toxicity to zebrafish ovarian tissue fragments were studied here for the first time. (ii) Development of *in-vitro* culture protocol for zebrafish ovarian tissue fragments and growth assessment of stage I and stage II ovarian follicles using biomarkers. *In-vitro* cultures of tissue fragments containing early follicles provide an alternative for generating mature oocytes. This system will contribute significantly to the utilization of cells and tissues after thawing. The development of biomarkers has been studied here for the first time on zebrafish ovarian follicles. This study is important to assess the specific development stage after *in-vitro* culture. (iii) Development of cryopreservation protocols using controlled slow cooling for zebrafish ovarian tissue fragments. Cryopreservation of isolated ovarian follicles carried out in previous studies were not successful, hence cryopreservation of zebrafish ovarian tissue fragments using controlled slow cooling were studied here for the first time since the tissue fragments may tolerate cryopreservation better.

(i) Studies on cryoprotectant toxicity to ovarian tissue fragments containing stage I and stage II follicles: In the present study, as the first step in designing cryopreservation protocol the impact of cryoprotectants on zebrafish ovarian tissue fragments was studied. Although cryoprotectant protects cells against freezing damage, they can be toxic and cause damage to the cells especially used at higher concentrations (Fahy *et al.*1990). In order to identify the CPA in a suitable concentration for zebrafish ovarian tissue cryopreservation, studies on toxicities of five permeating cryoprotectants methanol, ethanol,

dimethyl sulfoxide (DMSO), ethylene glycol (EG) and propylene glycol (PG) were carried out. The cryoprotectant toxicity tests were carried out on tissue fragments containing stage I and stage II follicles and the follicle survival rate was assessed using TB staining, FDA+PI staining and ATP assay. The staining method assessed the membrane integrity and metabolic activity of the cell and ATP assay assessed the ATP level of the fragments assessing the quality of the follicles within the fragments. The result obtained in this study provided useful information for ovarian tissue fragment cryopreservation protocol design.

(ii) Development of *in-vitro* culture protocol for zebrafish ovarian tissue fragments and growth assessment of stage I and stage II ovarian follicles using biomarkers: The second part of the study (Chapter 4) focussed on developing an *in-vitro* culture protocol for zebrafish ovarian tissue fragments containing stage I and stage II follicles. Since it has been reported that stage I and stage II ovarian follicles are less sensitive to chilling damage when compared to later stage indicating stage II is the most suitable stage for cryopreservation(Tsai *etal.* 2009), tissue fragments containing early stage follicles has been used in this study. Developing an *in-vitro culture* protocol for ovarian tissue fragments is very important since cryopreserved ovarian tissue fragments would need to be matured *in vitro*. In this study, the effect of growth supplements- foetal bovine serum (FBS) and bovine serum albumin (BSA) and growth factors- human chorionic gonadotropin (hCG) and follicle stimulating albumin (FSH) in culture medium were evaluated. The survival of follicles within the fragments was assessed using TB staining and their follicle growth was measured using confocal microscopy. The results obtained in this study would provide reliable method to grow stage I and stage II follicles *in-vitro*. Chapter 4 is focussed on developing a biomarker to assess the growth of ovarian follicles. Although follicle growth can be assessed by measuring the diameter of the follicles, biomarkers would provide important information on the follicular development stage. Selection of genes is important since the selected gene should be associated with the

developmental process through ovarian follicle development. Vtg1 gene and p450aromA gene were used in this study. Both these genes are well expressed in the ovary. The Vtgs are the yolk precursor proeins, and the expression of Vtgs in under hormone regulation and is sex and stage specific (Wang *et al.* 2005). P450aromA gene is also used to study the level of expression in follicles. P450aromA genes are found to be expressed during estradiol production prior vitellogenesis (Nagahama, 1994; Jalabert, 2005). In this study, vtg1 gene and p450aromA gene were studied to evaluate their pattern of expression in stage I, II and III ovarian follicle developmental stage and a quantitative RT-PCR approach to investigate the level of gene expression. The results from this study provide important information to distinguish the growth pattern and confirm the structural difference in terms of the ovarian follicle development. The method developed here can be used for assessing the growth pattern of the ovarian follicles *in-vitro* and after cryopreservation.

(iii) Development of cryopreservation protocols using controlled slow cooling for zebrafish ovarian tissue fragments: Several studies have been undertaken on cryopreservation of isolated follicles at different stages, although the protocol used lead to a compromised viability. The present study is focussed on developing cryopreservation protocol of ovarian tissue fragments. Based on the toxicity results obtained from previous study (Chapter 3), the effect of cryopreservation on zebrafish ovarian tissue fragments were investigated using controlled slow cooling. The present study differs from the previous studies undertaken and indicates the effect of lower concentration of cryoprotectants during cryopreservation. In order to optimize the cryopreservation protocol for the tissue fragments, studies using non-permeated cryoprotectant were also investigated. The effects of cryopreservation medium, cryoprotectants, cooling rate and ovarian follicle viability assessment after controlled slow cooling method were investigated. The survival rate was assessed using TB staining, FDA+PI staining and ATP assay. The effect of cryopreserved cells after *in-vitro* culture was also

investigated. Results from these studies would provide optimized cryopreservation protocol for zebrafish ovarian tissue fragments.

6.2 Review of the main findings

6.2.1 Studies on cryoprotectant toxicity to ovarian tissue fragments containing stage I and stage II follicles

The objective of this study was to identify the least toxic cryoprotectant to ovarian tissue fragments. One of the important factors that lead to successful cryopreservation is the addition of cryoprotectants during the course of freezing. In this study, toxicity of five penetrating cryoprotectants: methanol, ethanol, dimethyl sulfoxide (DMSO), ethylene glycol (EG) and propylene glycol (PG) at a range of concentration (1M -4M) were carried out. Methanol has been shown to be the most effective cryoprotectant to isolated zebrafish ovarian follicles study in our laboratory (Zhang *et al*. 1996, Plachinta *et al*. 2004, Tsai *et.al* 2008, Zampolla *et al*. 2008). Ethanol has also been found to be an effective cryoprotectant for catfish (Muchlisin *et al*. 2009) sperm cryopreservation but it has not been used for zebrafish embryos or ovarian follicles cryopreservation before. Ethanol has been used in this study for the first time. The survival of the follicles were assessed using TB staining, FDA+PI staining and ATP assay. The results showed that methanol and ethanol were the least toxic cryoprotectant compared to the other tested cryoprotectants. Cryoprotectant toxicity increased in the order of methanol/ ethanol, DMSO, PG and EG. The No Observed Effect Concentrations (NOECs) of the five tested cryoprotectants for stage I ovarian follicles are 2M for both methanol and ethanol and 1M for DMSO, PG and EG. The NOECs for stage II ovarian follicles are 1M for all five cryoprotectants. The study also indicated that the sensitivity of stage II ovarian follicles in the tissue fragments were more sensitive than stage I follicles when exposed to different cryoprotectants. It was also observed that ATP assay was the most sensitive method

for assessing the quality of the follicles in the tissue fragments after cryoprotectant exposure. Based on the results obtained from this study, 2M methanol and 2M ethanol will be used as the optimal cryoprotectant for the development of the zebrafish ovarian tissue cryopreservation protocol. In the present study the optimal cryoprotectant which are least toxic to the early stage ovarian follicles within the tissue fragments has been identified.

6.2.2 Development of *in-vitro* culture protocol for zebrafish ovarian tissue fragments and growth assessment of stage I and stage II ovarian follicles using biomarkers

The aim of this study was to develop an *in-vitro* culture protocol for zebrafish ovarian tissue fragments containing stage I and II follicles. Studies on the effect of using growth supplements - foetal bovine serum (FBS) and bovine serum albumin (BSA) and growth factors - human chorionic gonadotropins (hCG) and follicle stimulating albumin (FSH) were carried out. The culture media plays an important role in the development of *in-vitro* culture systems, the addition of growth supplements and growth factors in the culture system can increase the success of the oocyte maturation and fertilization. Viability was assessed using TB staining; follicle growth was measured using confocal microscopy and the follicle developmental stage was confirmed using biomarkers. The results obtained from this study showed that after 24h culture, increase in follicle diameter was observed when the ovarian fragments were incubated in 20% FBS when compared to the other concentrations and other growth supplements. The results also showed that 100mIU/ml FSH was most effective in stimulating follicle growth when compared with other concentrations of FSH and hCG. The results indicated that the tissue fragments cultured in 90% L-15 medium (pH 9) containing 100mIU/ml FSH with 20% FBS showed increase in follicle diameter from stage I to stage II and stage II to stage III ovarian follicles.

Although follicle growth can be assessed by measuring the diameter of the follicles, the follicular developmental stage can be confirmed using a biomarker. In order to develop a biomarker to identify stage II and stage III ovarian follicles after *in vitro* culture, studies on vtg1 gene and p450aromA gene were examined to evaluate their pattern of expression in stage I, II and III ovarian follicle developmental stage and a quantitative RT-PCR approach to investigate the level of gene expression. The results demonstrated that the expression of P450aromA gene was higher in stage II ovarian follicles when compared to stage III ovarian follicles and the expression of Vtg1 gene was higher in stage III follicles when compared to stage I and stage II ovarian follicles. After 24 h *in-vitro* culture, the level of expression of P450aromA gene on stage I showed the level of expression of stage II obtained prior to culture. This indicates that stage I have developed to stage II follicles. Similarly the level of expression of Vtg1 gene on stage II showed the level of expression of stage III obtained prior to culture, indicating stage II have developed to stage III follicles. As a result P450aromA and vtg 1 gene can be used as markers to assess the growth of stage II and stage III ovarian follicles *in vitro*.

The successful *in-vitro* culture method and biomarker developed here will provide a reliable method for the practical use of ovarian tissue fragments and assessing ovarian follicles development competence *in-vitro*.

6.2.3 Development of cryopreservation protocols using controlled slow cooling for zebrafish ovarian tissue fragments

The aim of this study was to develop a cryopreservation protocol for zebrafish ovarian tissue fragments using controlled slow cooling. In this study effect of permeating (Chapter 3) and non-permeating cryoprotectant (Chapter 5) with and without the addition of serum in the ovarian tissue fragments cryopreservation was investigated. The effect on freezing medium and the optimal cooling rate for ovarian tissue cryopreservation was investigated. To design the optimal controlled slow cooling procedure, several factors need to be considered: type and concentration of

cryoprotectants, freezing medium, cooling rate, ice-seeding temperature, thawing rate and removal of cryoprotectants. The survival rate of the follicles after cryopreservation was assessed using TB and FDA+PI staining. TB staining method assesses the membrane integrity and FDA+PI staining method assesses the membrane integrity and metabolic activity of the cryopreserved cells. The ATP level was also measured after cryopreservation using the ATP assay. The growth of the cryopreserved follicles within the fragments was assessed using *in-vitro* culture method (Chapter 4).

The quality of the follicles was studied by evaluating the membrane integrity and metabolic activity of the follicles.

The results from these studies showed that, a) L-15 medium or KCl buffer in the freezing medium did not show any significant difference in their survival rate for both the stages. Hence L-15 medium was used as a base medium in subsequent experiments. L-15 medium was used in this study since it has been widely used in ovarian tissue cryopreservation studies and found to be the optimal freezing medium (Schmidt *et al.* 2003) and KCl buffer was used in this study since it has been used in previous studies on isolated ovarian follicles (Guan *et al.* 2008; Tsai *et al.* 2009). b) In these experiments, combination of 2M methanol and 2M ethanol, 20% FBS in methanol and ethanol as the cryoprotectant mixture, different combination of sucrose and trehalose in 2M methanol as the cryoprotectant mixture were tested. It has been reported that on using serum in the combination with cryoprotectants increased the survival rate after cryopreservation (YunHa *et al.* 2005). The results showed that 2M methanol along with 20% FBS was a better cryoprotectant than other tested cryoprotectant for zebrafish ovarian tissue fragments. c) The result also indicated that the optimal cooling rate for the ovarian tissue fragments cyropreservation was found to be 4ºC/min. d) The result also indicated that ATP assay informed about the quality of follicles of the cryopreserved fragments and was therefore more accurate assessment method for the follicles survival after cryopreservation. e) To further understand the growth of the cryopreserved fragment, *in-vitro* culture of the cryopreserved fragments was evaluated and the results indicated that there was no growth in follicles after 24h *in-vitro* culture compared to the non-cryopreserved follicles within the tissue fragments. In the present study, optimal cryopreservation

protocol for zebrafish ovarian tissue fragments containing stage I and stage II follicles has been identified.

6.3 Conclusions

In the present study the aim to achieve an *in-vitro* culture protocol was successful and this can be used to grow the early stage follicles *in-vitro*. The biomarkers developed here in this study are also a successful approach to evaluate the growth of follicles *in-vitro*. Although the optimal cryoprotectant which are least toxic to the early stage ovarian follicles within the tissue fragments has been identified in this study, a successful cryopreservation protocol for the tissue fragments has not been achieved due to the morphological interactions and loss of metabolic activity of the follicles, but the outcome of this study have provided information for future ovarian tissue cryopreservation protocol development.

In the present study original contributions to knowledge has been made in the following areas:

(i) Identifying a potential cryoprotectant for zebrafish ovarian tissue fragments. Although toxicity studies have been carried out on isolated follicles, studies on cryoprotectant toxicity to zebrafish ovarian tissue fragments were studied here for the first time. The effect of ethanol was studied here for the first time on zebrafish follicles. It was found that methanol and ethanol was the least toxic cryoprotectant to the tissue fragments at 2M concentration for stage I follicles and 1M concentration for stage II follicles. Stage II follicles was found to be more sensitive to higher concentrations of cryoprotectants than stage I follicles. Methanol has been shown to be the least toxic cryoprotectant for isolated follicles in our laboratory (Tsai *et al.* 2008;

Zampolla *et al.* 2008), the results from the present study on methanol toxicity are in agreement with the previous findings. The present study also indicated that the sensitivity of stage II follicles within the tissue fragments were more sensitive than stage I follicles. These results were also in agreement with the previous findings on isolated follicles (Tsai *et al.* 2008).The information on the cryoprotectant toxicity to the tissue fragments obtained in this study provided important and essential information prior to any further development of optimal cryopreservation protocols for zebrafish ovarian tissue fragments.

(ii) An *in-vitro* culture protocol was developed for the zebrafish ovarian tissue fragment containing stage I and II follicles. The culture conditions developed here for the ovarian tissue fragments have been studied for the first time. An optimal protocol was identified; the ovarian tissue fragments were cultured in 90% L-15 medium (pH 9) containing 100mIU/ml FSH with 20% FBS. This culture method showed growth of stage I and II follicles to stage II and stage III respectively. The protocol developed here is a major step forward, since these tissue fragments enable the storage of large number of ovarian follicles, which offer a source of immature oocytes that could be matured *in-vitro*. The present study showed that the stage I and stage II follicles within the fragments reached the size of stage II and stage with FSH and FBS treatment after 24h culture when compared to the previous study (Tsai *et al.* 2010). In the present study, the use of hCG (Tsai *et al.* 2010) on the tissue fragments did not aid follicle growth when compared to FSH. This result is in agreement with Wu *et al.* (2000) on zebrafish follicles that hCG does not respond on oocytes unless they reach the size of 0.52mm (stage III) but they promote maturation of stage III oocytes.

Identifying the expression of genes for assessing the follicle growth in zebrafish has been studied here for the first time. Increase in size of the cells after *in-vitro* culture could be due to osmolality, but the use of biomarkers confirms the ovarian follicle development stage. Identifying P450aromA

gene and Vtg1 gene as biomarkers for assessing the growth competence of the early stage zebrafish ovarian follicles is another important part of this study. P450aromA gene can be used to assess the growth of stage II follicles and Vtg1 gene can be used for stage III follicles. This study provided new information to assess the growth of follicles *in-vitro*.

(iii) Ovarian tissues cryopreservation are advantageous over the embryos and oocytes as they can cryopreserved in small pieces and can store large number of follicles. In addition, ovarian follicle can remain in the natural three-dimensional structure which may tolerate cryopreservation (Matrinez-Madrid *et al.* 2004). Identifying optimum protocol using controlled slow cooling for zebrafish ovarian tissue fragments. Several important facts were discovered in the controlled slow cooling studies: 2M methanol along with 20% FBS was found to be the optimal cryoprotectant for the cryopreservation of the tissue fragments containing stage I and stage II follicles. 4°C/min cooling rates was indicated as the optimal cooling rate. The study also showed that 90% L-15 medium in the freezing medium did not show any significant difference from KCl buffer on the survival of the follicles after cryopreservation. The cooling rates of the present study are in agreement with the previous studies on isolated follicles (Tsai *et al.* 2009) that reported 2-4°C/min cooling rates was indicated as the optimal cooling rate for isolated early stage follicles. The present study also showed that 2M methanol was more effective for cryopreservation of the ovarian fragments although subsequent cryopreservation induced decreases in ATP levels.

The optimal protocol for cryopreservation of zebrafish ovarian tissue fragments is identified as: incubation of the ovarian tissue fragments in 2M methanol+ 20% FBS in 90%L-15 medium for 30 min at room temperature, load tissue fragments into 0.5ml plastic straws and place the straws in a programmable cooler (Planer KRYO 550); controlled slow cooling at 2ºC/min from -12.5ºC to seeding temperature (-7.5 ºC for 2M methanol), manual seeding and hold for 5min, freezing from seeding temperature to -

40ºC at 4ºC/min; from -40ºC to -80ºC at 10ºC/min and hold for 10 min. Samples were then plunged in LN(-196 ºC) and held in LN for atleast 10 min. Samples were thawed using water bath at 28ºC. Removal of cryoprotectant should be conducted in 4 steps. This study provides improved cryopreservation protocol since it enhanced survival of the follicles with the use of 2M methanol +20% FBS in 90% L-15 medium when compared to the previous study (Guan *et al.* 2008; Tsai *et al.* 2009) with 4M methanol in KCl buffer which are highly toxic to the cells. The present study showed the highest survival obtained for stage II follicles within the tissue fragment was 68.2±1.9% and 55.4±2.3% for stage I follicles within the fragment using TB staining, but showed significant decrease in ATP levels. When the cryopreserved ovarian tissue fragments were cultured, the results showed that the ovarian follicles did not show any growth in follicles after *in-vitro* treatment, and the survival rate of the cryopreserved follicles were significantly lower to those of the non-cryopreserved follicles. This could be due to the cellular damage of the theca and granulosa cells during cryopreservation. Although the development of successful cryopreservation protocol for zebrafish ovarian tissue fragments has not been achieved in the present study, the outcomes of this study have provided useful information for future cryopreservation protocol development.

6.4 Future work

Following the findings obtained from the present study, further studies need to be carried out in the following areas:

6.4.1 Vitrification Studies

In the present study on the cryopreservation of zebrafish ovarian tissue fragments using controlled slow cooling procedure the results indicated a compromised survival rate after staining method, but when the cryopreserved fragments were cultured the follicles were not able to grow *in-vitro* compared to the non-cryopreserved follicles. This may be due to the damage of thecal and granulosa

cells affected by intracellular ice formation (Cecconi *et al*.2004).This could be avoided by using vitrification process which eliminates the structural damage and cell injury related to intracellular ice formation.

Vitrification has been stated as a promising option, and has been performed for various fish embryos (Liu *et al.* 1998; Robles *et al.* 2003; Cabrita *et al.* 2003; Chen and Tian, 2005). However there is no commonly accepted method for vitrification of fish embryos or oocytes. Despite the successful use of ovarian tissue cryopreservation in humans and some domestic mammals, vitrification of fish oocytes has been almost unexplored to date (Guan *et al.* 2010). Therefore more research is needed regarding vitrification of fish ovarian tissues.

6.4.2 Microinjection of cryoprotectants

Although in the present study using sucrose or trehalose in the extracellular environment did not show any improvement in the cryopreservation procedure it could be possible to improve cryopreservation if they are induced internally. The formation of intracellular ice is one of the major causes to cell death during freezing and thawing (Mazur, 1984). Reduction of the ice nucleation temperature is normally achieved by introducing cryoprotectants that change the process of crystallisation and enhance the outflow of intracellular water. Depression of intracellular ice temperature below that of extracellular ice is believed to be essential to successful cryopreservation using slow cooling protocol (Hagerdon *et al.* 2004). The impact of microinjections on the physical process of crystallisation with zebrafish embryos has been studied earlier (Kopeika *et al.* 2006), but has not been successful. And this has not been studied in zebrafish oocytes so far. But this novel approach of injecting sugar into the oocytes has demonstrated success in the mouse oocytes (Eroglu *et al.* 2008) and also in human oocytes (Eroglu *et al.* 2001). Hence study needs to be carried forward on the approach of injecting sugars into the ovarian follicles within the tissue fragments.

6.4.3 *In-vitro* maturation studies

In the present study, an optimal protocol for *in-vitro* culture of ovarian tissue fragments containing stage I and stage II follicles was developed. This offers a reliable way to store large number of ovarian follicles, which offers a source of immature oocytes. However, the present study maintains the follicles growth from stage I to stage II and stage II to stage III within the fragments respectively and did not grow the follicles to reach the maturation stage. The primary growth phase is usually characterised by pre-vitellogenic oocytes, while the secondary growth phase is characterised by vitellogenic oocytes and maturation phase by advanced vitellogenic oocytes undergoing germinal vesicle migration (Adebiyi *et al.* 2011). To grow follicles from early stages to maturity *in-vitro* has proven to be difficult, only few works has been reported on the reproductive biology of fish (Khan *et al.* 1990; Christianus *et al.* 1999; Picton *et al.* 2003; Muchlisin and Azizah, 2009). Hence further study on the artificial induction of ovulation therefore requiring certain conditions that allow the oocyte to undergo accurate biological reaction leading to ovulation needs to be carried out.

Oocyte maturation in lower vertebrates is activated by maturation-inducing hormone (MIH), which acts as unidentified receptors on the oocyte surface and induces the activation of maturation-promoting factor (MPF) in the oocyte cytoplasm (Tukomoto *et al.* 2005). During the course of maturation, oocytes undergo morphological changes which lead to meiotic cell cycle and germinal vesicle break down (GVBD). In zebrafish two types of MPR (membrane progesterone receptor) which is a MIH receptor, α and β were identified (Zhu *et al.* 2003). 17α, 20β-DHP has been shown to induce oocyte maturation by stimulating the *de-novo* synthesis of cyclin B, a regulatory subunit of MPF.

6.5 Closing Words

The research was focused on two major areas (i) Development of *in-vitro* culture protocol for early stage ovarian follicles within the tissue fragments (ii) Development of cryopreservation protocol for ovarian tissue fragments. The work in

this research has led to the successful development of an *in-vitro* culture protocol. This research has also demonstrated various optimizations for controlled slow cooling cryopreservation protocol. Since the attempts to develop a controlled slow cooling cryopreservation protocol have not been successful, it is intended that vitrification technique would be studied to develop a cryopreservation protocol for ovarian tissue fragments. This work has advanced number of areas for further investigation, which would redefine the current situation in cryopreservation of fish maternal genome.

References

Abir, R., Fisch, B., Nitke, S., Okon, E., Raz, A., Ben Rafael, Z. (2001) 'Morphological study of fully and partially isolated early human follicles.' *Fertil. Steril.,* 75, 141-146.

Agca Y. (2000) 'Cryopreservation of Oocyte and Ovarian tissue', *ILAR*, 41, 4.

Agca,Y., Liu,J., Rutledge, J.J., Critser, E.S., Critser, J.K. (2000) 'Effect of osmotic stress on the developmental competence of germinal vesicle and metaphase II stage bovine cumulus oocytes complexes and its relevance to cryopreservation.' *Mol Reprod Dev.* 55: 212-219.

Agca, S., Mullen, J., Liu, J., Johnson-Ward, K., Gould, A., Chan, J., Critser,J.K. (2005) 'Osmotic tolerance and membrane permeability characteristics of rhesus monkey (*Macaca mulatta*) spermatozoa'. Cryobiology, 51, pp. 1–14.

Arakawa, T., Carpenter, J. F., Kita, Y. A. and Crowe, J. H. (1990) 'The basis for toxicity of certain cryoprotectants: A hypothesis', *Cryobiology*, 27(4), 401-415.

Arlotto, T., Schwartz, J.L., First, N.L., Leibfried, M.L (1996) 'Aspects of follicle and oocyte stage that affect in vitro maturation and development of bovine oocytes.' *Theriogenology*, 45: 943-956.

Ashwood-Smith, M.J., Morris, G.W., Flowler, R., Appleton, T.C., Ashorn, R. (1988) 'Physical factors are involved in the destruction of embryos and oocyte during freezing and thawing procedure.' *Human Reprod*; 3: 795-802.

Axelrod, H.R., Schultz, L.P. (1955). 'A handbook of tropical aquarium fishes.' *McGraw-Hill Book Co,* New York.

Babiak, I., Glogowsk,Y., Brzuska, J.E., Szumiec, J., Adamek, J. (1995) 'Cryopreservation of sperm of common carp *Cyprinus carpio'*. *Aquaculture Res.* 28: 567-571.

Babin, P.J, Cerda, J, Lubzens, E. (2007). The fish Oocyte : From Basic studies to biotechnological Applications. 1-37.

Bank, H.L., Brockbank, K.G. (1987) 'Principles of Cryobiology', *J Card Surg*, 2(1):137-43.

Baust,J.G., Baust,J.M. (2007) ' Advances in Biopreservation', in: Taylor, M.J. *Biology of cell survival in the cold: The Basic for biopreservation of tissues and organs*. CRC press. 15-62

Ball, B.A., Vo, A. (2001) 'Osmotic tolerance of equine spermatozoa and the effects of soluble cryoprotectants on equine sperm motility, viability and mitochondrial membrane potential'. *J.Androl.* 22, pp 1061-1069.

Bass, L.D., Denniston, D.J., Maclellan L.J., McCue, P.M. Squires, E.L. (2004) 'Methanol as cryoprotectant for equine embryos'. *Reprod Fertil Dev,* 16(2): 163-164.

Bavister, B.D. (1995) 'Culture of pre-implantation embryos: facts and anti facts'.*Hum Reprod Update*; 1:91-148.

Blanco, M.R., Simonetti, L., Palermo, P. (2000) 'Morphological survival and in vitro maturation of immature bovine oocytes exposed to EGTA with or without cryopreservation.' *In vitro Cell Dev. Biol*., 36(3): Part. II, 2042

Blanco, M. R., Demyda, S., Moreno, M., Genero, E. (2011) '[Developmental competence of in vivo and in vitro matured oocytes: A review' Biotechnology *and Molecular Biology* Review Vol. 6(7), pp. 155-165.

Bobe, J., Nguyen, T., Jalabert, B. (2004) 'Targeted gene expression profiling in the rainbow trout (Oncorhynchus mykiss) ovary during maturational competence acquisition and oocyte maturation.' *Biol. Reprod.* 71, 73–82.

Bogliolo,L., Ariu, F., Fois, S., Rosati, I., Zedda, M.T., Leoni, G., Succu, S., Pau,S., Ledda, S. (2007) ' Morphological and biochemical analysis of immature ovine oocytes vitrified with or without cumulus cells' *Theriogenology*, 68(8), 1138-1149.

Bokor, Z., Müller, T., Bercsényi, M., Horváth, L., Urbányi, B., Horváth, A. (2007) 'Cryopreservation of sperm of two European percid species, the pikeperch (*Sander*

lucioperca) and the Volga pikeperch (*S. volgensis*).' *Acta. Biol. Hung.* 58(2): 199-20.

Borges, E.N., Silva, R.C., Futino, D.O., Rocha-Junior, C.M., Amorim, C.A., Bao, S.N., Lucci, C.M., (2009). Cryopreservation of swine ovarian tissue: effect of different cryoprotectants on the structural preservation of preantral follicle oocytes. Cryobiology 59, 195–200.

Borini, A., Sciajno, R., Bianchi, V., Sereni, E., Flamigni, C., Coticchio, G. (2006), 'Clinical outcome of oocyte cryopreservation after slow cooling with a protocol utilizing a high sucrose concentration', *Human Reproduction,* 21 (2), 512–517.

Brockbank, K.G., Song, Y,C., Khirabadi, B.S., Lightfoot, F.G., Boggs, J.M., Taylor, M.J. (2000) ' Storage of tissues by vitrification'. *Transplant Proc,* 32 (1), 3-4.
Bunge, R.G., Keetee, W.C., Sherman, J.K. (1954) 'Clinical use of frozen semen' *Fertility and sterility*, 5:520-529.

Bradbury, D.A, Simmons, T.D, Slater K.J, Crouch S.P.M. (2000). Measurement of the ADP:ATP ratio in human leukaemic cell lines can beused as an indicator of cell viability, necrosis and apoptosis. *J.Immunol Methods*, 240:79–92.

Buratowski, S. (1994). 'The basics of basal transcription by RNA polymerase II.' *Cell* 77:1-3.

Bustin, S. (2000) 'Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays', *Journal of Molecular Endocrinology,* 25(2), 169-193.

Bustin, S.A. (2002) 'Quantification of mRNA using real-time reverse transcription PCR (RT-PCR): trends and problems' *J Mol Endocrinol* 25, 169-193.

Cai, K., Yang, J., Guan, M., Ji, E., Li, Y., Rens, W. (2005) 'Single UV excitation of Hoechst 33342 and propidium iodide for viability assessment of rhesus monkey spermatozoa using flow cytometry' *Arch Androl* 51(5): 371-383.

Carroll, J., Wood, M.J, Whittingham, D.G. (1993) ‘Normal fertilization and development of frozen-thawed mouse oocytes: protective action of certain macromolecules.’ *Biol Reprod*; 48:606–612.

Cao, E., Chen, Y., Cui, Z. (1993) ‘Effect of freezing and thawing rates on denaturation of proteins in aqueous solutions’ *Biotechnology and Bioengineering*, 82(6): 684-690.

Cabrita, E., Sarasquete, C., Martinez-paramo, S., Robles, V., Beirao, J., Perez-Cerezales, S., Herraez, M.P. (2010) ‘Cryopreservation of fish sperm: applications and perspectives’ *Journal of Applied Ichthyology*, 26(5): 623-635.

Chao, N.-H., Chiang, C.-P., Hsu, H.-W., Tsai, C.-T. and Lin, T.-T. (1994) 'Toxicity tolerance of oyster embryos to selected cryoprotectants', *Aquatic Living Resources*, 7(02), 99-104.

Chang, X.T., Kobayashi, T., Kajiura, H., Nakamura, M., Nagahama, Y. (1997) ‘Isolation and characterization of the cDNA encoding the tilapia (Oreochromis niloticus) cytochrome P450 aromatase (P450arom): changes in P450arom mRNA, protein and enzyme activity in ovarian follicles during oogenesis.’ *J. Mol. Endocrinol*. 18:57–66.

Chang, E.F., Yan,Y.L., Guiguen, Y., Postlethwait, J., Chung, B.C. (2001) ‘Two Cyp19 (P450 Aromatase) Genes on Duplicated Zebrafish Chromosomes Are Expressed in Ovary or Brain’ *Mol. Biol. Evol.* 18(4):542–550. 2001.

Chen,C.P., Denlinger, D.L. (1992) ‘ Reduction of cold injury inflies using an intermittent pulse of high temperature’. *Cryobiology*, 29:138-143.

Chen, C. (1986) Pregnancy after human oocyte cryoprevervation. *Lancet*, i, 884-886.

Chen, S.U., Lien, Y.R., Tsai, Y.Y *et al*. (2002) ‘ Successful pregnancy occurred from slowly freezing human oocytes using the regime of 1.5 mol/l 1,2-propanediol with 0.3 mol/l sucrose’ *Human Reproduction* 17, 1412.

Chen, S.L., Ren, G.C., Sha, Z.X., Shi, C.Y. (2004) ‘Establishment of a continuous embryonic cell line from Japanese flounder Paralichthys olivaceus for virus isolation’ *Diseases of aquatic organism* 60: 241-246.

Chernov, A. A. (1984). Modern Crystallography III-CrystalGrowth. Springer-Verlag, Berlin.

Choresca CH Jr, Gomez DK, Kim JH, Han JE, Shin SP, Lee BC, Park SC. (2011) ‘Cryopreservation of goldfish caudal fin explants using glycerol as a cryoprotecant.’ *Cryo Letters.*;32(1):57-61.

Cheung, W.W.L., Watson, R., Morato, T., Pitcher, T.J., Pauly, D. (2007) ‘Intrinsic vulnerability in the global fish catch’ *Mar Ecol Prog Ser* 333: 1-12.

Clegg. J.S., Seitz. P., Seitz. W and Hazlewood, C.F. (1982) ‘Cellular response to extreme water loss: The water-replacement hypothesis.’ *Cryobiology* 19:306-316.

Cecconi, S. (2002) ‘Growth and differentiation of small ovarian follicles’ *J.Reprod. Dev*. 48, 431–445.

Cecconi, S., Barboni, B., Coccia, M., Mattioli, M. (1999) ‘In vitro development of sheep preantral follicles.’ *Biol. Reprod.* 60, 594–601.

Cecconi,S., Capacchietti,G., Russo,V., Berardinelli, P., Mattioli, M.,Barboni, B. (2004) ‘In Vitro Growth of Preantral Follicles Isolated from Cryopreserved Ovine Ovarian Tissue’ *Biol. Reprod.* 70: 12-17.

Celestino, J.J.H., Santos, R.R., Lopes, C,A,P.,Martins, F.S., Matos, M, H, T., Melo, M.A.P., *et al.*(2008) ‘ Preservation of bovine preantral follicle viability and ultra-structure after cooling and freezing of ovarian tissue’ *Animal Reprod Sci.* 108(3-4): 309-318.

Clelland, E.S., Tan, Q., Balofsky, A., Lacivita, R., Peng, C (2007) ‘Inhibition of premature oocyte maturation: a role for bone morphogenetic protein 15 in zebrafish ovarian follicles.’ *Endocrinol* 148: 5451–5458.

Cossins, A. R. (1983) 'The adaptations of structure and function to temperature ' in Cossin, A. R., Sheterline, P., ed. *Cellular Acclimatisation to Environmental Change* Cambridge: Cambridge University Press, 1-32.

Crowe, J.H., Hoekstra, F.A., Crowe, L.M. (1992) Anhydrobiosis. *Annual Reviews of Physiology* 54, 579-599.

Crowe, J.H., Crowe, L.M., Carpenter, J.F. (1993) ‘Preserving dry biomaterials: the water replacement hypothesis’ Part II. *BioPharm*; 28:40–44.

Crowe, J.H., Leslie, S.B., Crowe, L.M. (1994) ‘Is vitrification sufficient to preserve liposomes during freeze-drying?’ *Cryobiology*; 31:355–366.

Crowe, J.H., Carpenter, J.F., Crowe, L.M. (1998) ‘The role of vitrification in anhydrobiosis’ *Annual Reviews of Physiology* 54:579-599.

Curry, M.R. (2000) ‘Cryopreservation of semen from domestic livestock’ *Reviews of Reproduction*, 5: 46–52.

Day, J.G., McLellan, M.R. (1995) ‘Cryopreservation and freeze-drying protocols’ In Method in molecular biology, Ed Ludlow, J.W. Protein phosphate protocols, Vol 93, *Humana Press*.

Denlinger,D., Lee, R.E. (2010) ‘ Low temperature biology of insects’, in Lee, R.E.: *A primer on insect cold-tolerance,* Cambridge University Press,3-16.

Devireddy, R.V., Swanlund, D.J., Roberts, K.P., Pryor, J.L and Bischof, J.C. (2000) ‘The effect of extracellular ice and cryoprotective agents on the water permeability parameters of human sperm plasma membrane during freezing’ *Human Reproduction*15:1125–1135.

Dinnyes, A., Dai, Y., Jiang, S., Yang, X. (2000) 'High developmental rates of vitrifies bovine oocytes following parthogenetic activation, in vitro fertilization, and somatic cell nuclear transfer. *Biology of Reproduction* 62, 513-518.

Didion, B.A., Pomp, D., Martin, M.J., Homanics, G.E., Market, C.L. (1990) 'Observations on the cooling and cryopreservation of pig oocytes at the germinal vesicle stage' *J Anim Sci* 68: 2803-2810.

Dollo, V.H., Yi, S.X., Jr. Lee, R.E. (2010) ' High temperature pulses decreases chilling injury and elevate ATP levels in the fresh fly, Sarcophaga crassipaplis', *Cryobiology*, 60: 351-353.

Donnez, J., Dolmans, M.M., Demylle, D., Jadoul, P., Pirard, C., Squifflet, J., Martinez-Madrid,B., Langendonckt, A.V. (1994) 'Live birth after orthothopic transplantation of cryopreserved ovarian tissue' *The Lancet*, 364(9443), 1405-1410.

Drobins, E.Z., Crowe, L.M., Berge, T., Anchordoguy, T.J., Overstreet, J.W., Crowe, J.H. (1993) 'Cold shock damage is due to lipid phase transition in cell membranes: A demonstration using sperm as a model', *J Exp Zool* 265:432-437.

Ecker, R.E., Smith, L.D. (1971) 'Influence on exogenous ions on the event of maturation in Rana pipiens oocytes' *Journal of cell physiology* 77,61-70.

Edidin, M. (1970) 'A rapid quantitative fluorescence assay for cell damage by cytotoxic antibodies.' *J.Immunol* 104:1303.

Eroglu, A., Bailey,S., Toner,M., Toth, T.L. (2009) 'Successful Cryopreservation of Mouse Oocytes by Using Low Concentrations of Trehalose and Dimethylsulfoxide' *Biology of Reproduction* , 80: 70–78

Everaats, J.M., Shugart, L.R., Gustin, M.K., Hawkins, W.E., Walker, W.W. (1993) 'Biological markers in fish: DNA integrity, haematological parameters and liver somatic index' In Stegeman J.J., Moore, M.N., Hahn, M.E Edn. *Responses of marine organism to pollutants* 2: 101-107.

Fabbri, R., Porcu, E., Marsella, T., Rocchetta, G., Venturoli, S., Flamigni, C. (2001) ' Human oocyte cryopreservation: new perspectives regarding oocytes survival.' *Human Reprod*. 16: 411-416.

Fabbri. R., Venturoli, S., D'Errico, A., Iannascoli, C., Gabusi, E., Valeri, B., Seracchioli. R., Grigioni, W.F. (2003) 'Ovarian tissue banking and fertility preservation in cancer patients: histological and immune histochemical evaluation' *Gynecol Oncol*., 89(2), 259-66.

Farrant, J. (1980) 'General observations on cell preservation. In: Low temperature preservation in medicine and biology Eds. Ashwood-smith M.J. and Farrant,J. *Pitman medical,* 1-18.

Fahy, G. M., Lilley, T. H., Linsdell, H., Douglas, M. S. J. and Meryman, H. T. (1990) 'Cryoprotectant toxicity and cryoprotectant toxicity reduction: In search of molecular mechanisms', *Cryobiology,* 27(3), 247-268.

Fahy, G. M., MacFarlane, D. R., Angell, C. A. and Meryman, H. T. (1984) 'Vitrification as an approach to cryopreservation', *Cryobiology,* 21(4), 407-26.

Fahy, G. M. (1986) 'The relevance of cryoprotectant "toxicity" to cryobiology', *Cryobiology,* 23(1), 1-13.

Filicori, M ., Cognigni, GE, Samara, A., Melappioni, S., Perri, T., Cantelli, B., Parmegiani, L., Pelusi, G., DeAloysio, D.(2002): The use of LH activity to drive folliculogenesis: Exploring uncharted territories in ovulation induction. *Hum Reprod Update* ;8:543–57

Finn, R.N., Kristoffersen, B.A. (2007) 'Vertebrate Vitellogenin Gene Duplication in Relation to the "3R Hypothesis": Correlation to the Pelagic Egg and the Oceanic Radiation of Teleosts.' PLoS ONE 2(1): e169. doi:10.1371/journal.pone.0000169.

Fleming, A. (2007) 'Zebrafish as an alternative model organism for disease modelling and drug discovery: implications for the 3Rs' *NC3Rs #10* Zebrafish as an alternative model organism.

Franks,F. (1985) ' Biophysics and Biochemistry at low temperatures' *Cambridge university Press,* New York.

Franks, F., Mathias, S.F., Galfre, P., Webster, S.A.D., Brown, D. (1983) 'Ice nucleation and freezing in undercooled cells.' *Cryobiology,* 20: 298-309.

Frazer, C.A and Hall, M.R. (1999). Studies on primary cell cultures derived from ovarian tissue of Penaeus monodon. *Methods in cell science.* 21:213-218

Fuller, B.J., Paynter, S., Watson, P.F. (2004). 'Life in the frozen state' CRC Press, Boca Raton, FL.

Fukui, Y. (1990) 'Effect of follicle cells on the acrosome reaction fertilization and developmental competence of bovine oocytes matured in vitro.' *Mol. Reprod. Dev*., 26: 40-46.

Fukada,S., Tanaka, M., Matsuyama,M., Kobayashi, D., Nagahama, Y. (1996) 'Isolation, characterization, and expression of cDNAs encoding the medaka (Oryzias latipes) ovarian follicle cytochrome P450 aromatase.' *Mol. Reprod. Dev*. 45:285–290.

Forsyth,M., MacFarlane, D.R. (1990) 'A study of hydrogen bonding in concentrated diol/water solutions by proton NMR correlations with glass formation' *J. Phys. Chem.*, *94* (17), pp 6889–6893.

Fouladi, A.A.N., Waddington, D., Campbell, K.H. (1998) 'Maintenance of bovine oocytes in meiotic arrest and subsequent development in vitro: a comparative evaluation of antral follicle culture with other methods' *Biology of Reproduction*, 59, 255-262.

Gao, D.Y., Liu, J., McGann, L.E., Watson, P.F., Kleinhans, F.W., Mazur, P., Critser, E.S., Critser, J.K. (1995) 'Prevention of osmotic injury to human spermatozoa during addition and removal of glycerol' *Human Reprod.*, 10, 1109-1122.

Gao, D.Y., Mazur, P., Critser, J.K., 1997. 'Fundamental cryobiology of mammalian spermatozoa'. In: Karow, A.M., Critser, J.K. (Eds.), *Reproductive Tissue Banding*: Scientific Principles. Academic Press, San Diego, pp. 263–328.

Ge, W. (2005) 'Intrafollicular paracrine communication in the zebrafish ovary: The state of the art of an emerging model for the study of vertebrate folluculogenesis' *Molecular and Cellular Endrocrinology* 237(1-2): 1-10.

Gelinas, D., Pitoc, G.A., Callard, G.V (1998) ‘ Isolation of a goldfish brain cytochrome P450 aromatase cDNA: mRNA expression during the seasonal cycle and after steroid treatment’ *Mol. Cell. Endocrinol.*, 138 pp. 81–93.

Gentle, A., Anastasopoulos, F. and McBrien, N. A. (2001) 'High-resolution semi-quantitative real-time PCR without the use of a standard curve', *BioTechniques*, 31(3), 502, 504-6, 508.

George, M.A., Johnson, M.H.(1993) ‘ Use of fetal bovine serum substitutes for the protection of the mouse zona pellucida against hardening duringcryoprotectant addition.’ *Hum Reprod*; 8:1898–1900.

Ghafari, F., Gutierrez, C.G., Hartshorne ,G.M.(2007). ‘Apoptosis in mouse fetal and neonatal oocytes during meiotic prophase one’. *BMC Developmental Biology*, 7,87

Gilmore, J.A., Liu, J., Gao, D.Y., Critser, J.K (1997). ‘Determination of optimal cryoprotectants and procedures for their addition and removal from human spermatozoa’. *Human Reprod*, 12, 112-118.

Gliedt, D.W., Rosenkrans, C.F., Rorie, Jr. R.W., Munyon, A.L., Peirson, J.N., Miller, G.F., and Rakes, J.M. (1996) ‘Effect of media, serum, oviductal cells and hormones during maturation on bovine embryo development in vitro.’ *J Dairy Sci*, 79: 536-542.

Goetz, F.W., Theofan, G. (1976) ‘In-vitro stimulation of germinal vesicle breakdown and ovulation of yellow perch (Perca flavescens) oocytes. Effect of 17α-hydroxy-20β-dihydroprogesterone and prostaglandins. *Gen Comp Endrocrinol* 37: 273-285.

Goswami,M., Lakra, W.S., Rajaswaminathan, T., Rathore, G. (2010). Development of cell culture system from the giant freshwater prawn Macrobrachium rosenbergii(de Man). *Mol Bio Rep*,37(4): 2043-8.

Gomez, J.M., Weil, C., Ollitrault, M., Le Bail, P.Y., Breton, B., Le- Gac, F. (1999) ‘Growth hormone (GH) and gonadotropin subunit gene expression and pituitary and plasma changes during spermatogenesis and oogenesis in rainbow trout (Oncorhynchus mykiss).’ *General and Comparative Endocrinology* 113 413–428.

Gook, D.A., McCully, B.A., Edgar, D.H. and McBain, J.C. (2001)'Development of antral follicles in human cryopreserved ovarian tissue following xenografting.' *Hum. Reprod*., 16, 417-422.

Gosden, R.G., Baird, D.T., Wade, J.C., Webb, R. (1994). Restoration of fertility to oophorectomized sheep by ovarian autografts stored at −196 degrees C. *Hum. Reprod*. 9, 597–603.

Gosden, R.G., Mullan, J., Picton, H.M., Yin, H. and Tan, S.L. (2002) Current perspective on primordial follicle cryopreservation and culture for reproductive medicine. *Hum. Reprod. Update, 8*, 105-110.

Grout, B. W. W. and Morris, G. J. (1987) 'Freezing and cellular organization' in B, W., W, Grout; G, J, Morris, ed. *The Effects of Low Temperature on Biological Systems*, London: Edward Arnold, 72-119.

Gstraunthaler, G. (2003) 'Alternatives to the use of fetal bovine serum: serum-free culture'. *Altex* 20, 4.

Guan, M., Rawson, D. M. & Zhang, T. (2008), 'Cryopreservation of zebrafish (Danio rerio) oocytes using improved controlled slow cooling protocols', *Cryobiology*, 56**,** 204-208.

Guan, M., Rawson, D.M.,Zhang, T.(2010) 'Cryopreservation of Zebrafish (*Danio Rerio*) Oocytes by Vitrification' Cryoletters, 31(3), pp. 230-238.

Guthrie, H.D., Liu, J., Critser, J.K. (2002) 'Osmotic tolerance limits and effects of cryoprotectants on motility of bovine spermatozoa'. *Biol. Reprod*, 67, pp 1811-1816.

Han, J.E., Choresca, Jr. Ca. H., Koo, O.J. , Oh, H.J, Hong, S.G., Kim, J.H., Shin, S.P., Jun, J.W., Lee, B.C., Park, S.C. (2011)' Establishment of glass catfish (Kryptopterus bicirrhis) fin-derived cells.' *Cell Biol Int Rep* 18, 1–5.

Harries, J.E., Sheahan, D.A., Jobling, S., Matthiessen, P., Neall, P., Sumpter, J.P., Taylor, T.,

Zaman, N., (1997) ‘Estrogenic activity in five United Kingdom rivers detected by measurement of vitellogenesis in caged male trout.’ *Environ. Toxicol*. Chem. 16, 534-542

Harrison, R.A., Vickers, S.E. (1990) ‘Use of fluorescent probes to assess membrane integrity in mammalian spermatozoa’ J.Reprod Fertil, 88, 343-352.

Harvey, B., Ashwood-Smith, M.J. (1982) ‘Cryopretectant penetration and supercooling in the eggs of samonid fishes.’ *Cryobiology*. 19: 29-40.

Hays, L.M., Crowe, J.H., Wolkers, W., Rudenko, S. (2001) ‘Factors affecting leakage of trapped solutes from phospholipid vesicles during thermotropic phase transitions’, *Cryobiology*, 42 (2):88-102.

Hagedorn, M., Kleihans, F.W., Wildt, D.E. (1996) ‘Water permeability studies in dechorionated zebrafish embryos’, *Cryobiology,* 33,646 Abstract.

Hagedorn, M., Hsu, E. W., Pilatus, U., Wildt, D. E., Rall, W. R. and Blackband, S. J. (1996) 'Magnetic resonance microscopy and spectroscopy reveal kinetics of cryoprotectant permeation in a multicompartmental biological system', *Proceedings of the National Academy of Sciences,* 93(15), 7454-7459.

Heid, C. A., Stevens, J., Livak, K. J. and Williams, P. M. (1996) 'Real time quantitative PCR', *Genome Research,* 6(10), 986-994.

Heppell, S.A., Denslow, N.D., Folmar, L.C., Sullivan, C.V. (1995) ‘Universal assay of vitellogenin as a biomarker for environmental estrogens’ *Environ Health Perspect* 103(7):9-15.

Hess, E.A., Teague, H.S., Ludwick, T.M., Martig, R.C. (1957). ‘Swine can be bred with frozen semen’ *Ohio Fm Res*, 42: 100.

Hoetelmans, R. W., Prins, F. A., Cornelese-ten Velde, I., van der Meer, J., van de Velde, C. J. and van Dierendonck, J. H. (2001) 'Effects of acetone, methanol, or paraformaldehyde on cellular structure, visualized by reflection contrast microscopy and transmission and scanning electron microscopy', *Appl Immunohistochem Mol Morphol*, 9(4), 346-51.

Holt, W.V., Pickard, A.R. (1999) 'Role of reproductive technologies and genetic resource banks in animal conservation' *Rev Reprod*, 4: 143-150.

Hovatta, O., Silye, R., Krausz, T., Abir, R., Margara, R., Trew, G., Lass, A., Winston, R.M. (1996) Cryopreservation of human ovarian tissue using dimethylsulphoxide and propanediol±sucrose as cryoprotectants. *Hum.Reprod.,* 11, 1268-1272.

Hudson,L., Hay, F.C. (1980) 'Practical Immunology' 2ns Edn *Blackwell*, Oxford.

Hutchinson, T.H., Ankley, G.T., Segner, H., Tyler, C.R. (2006) 'Screening and Testing for endocrine disruption in fish-Biomarkers as signposts not traffic lights in risk assessment' *Environ health perspect* 114(S-1): 106-114.

Isayeva, A., Zhang, T., Rawson D.M. (2004) 'Studies on chilling sensitivity of zebrafish (Danio rerio) oocytes' *Cryobiology* 49(2), 114-122.

Iwamatsu, T., Takahashi S.Y., Sakai, N., Nagahama, Y., Onitake, K. (1987) 'Induction and inhibition of in-vitro oocyte maturation and production of steroids in fish follicles by forskolin' *J. Exp Zool* 241: 101-111.

Ings,J.S and VanderKraak, G, J. (2006). 'Characterization of the mRNA expression of Star and steriodogenic enzymes in zebrafish Ovarian follicles.' *Mol Reprod and Development* 73:943-954.

Jain, M.K. (1983) 'Non random lateral organization in bilayers and biomembranes. In: *Membranes Fluidity in Biology* Ed. Aloia R.C. Academic press, New York, 1-37.

Janz, F,L., Debes, A.A., Cavaglieri,R,C., Duarte, S.A., Ramao, C.M., Fernandes,A,m Zugaib, M., Bydlowski, S.P. (2012) 'Evaluation of Distinct FreezingMethods and Cryoprotectants forHuman Amniotic Fluid Stem Cells Cryopreservation' *Journal of Biomedicine and Biotechnology*. Volume 2012, Article ID 649353.

Jalabert, B. (1976) 'In-vitro oocyte maturation and ovulation in rainbow trout (Salmo gairdneri), northern pika (Essox lucius) and goldfish (Carassius auratus).' *J Fish Res Board Can.* 33:974-988.

Javed, A., Jamil, A., Zarchi, S.R., Kalantar, S.M., Anvari, M., Nazems, H. (2008) 'An *in vitro* Comparative Study of Follicle Stimulating Hormone(FSH) and Activin A Effects on the Maturation of PreantralFollicle-Enclosed Oocytes from Immature Syrian Mice' *Iranian Biomedical Journal* 12 (2): 85-92.

Jones,K.H., Senft, J. A. (1985) 'An improved method to determine cell viability by simultaneous staining with fluorescein diacetate-propidium iodide' *J Histochem Cytochem* 33: 77.

Kagawa, H., Gen, K., Okuzawa, K., Tanaka, H. (2003) 'Effects of leutinizing hormone and follicle stimulating hormone and insulin-like growth factor-I on aromatase activity and P450aromatase gene expression in the ovarian follicles of red seabream. *Biol Reprod* 68: 1562-1568.

Kang, M.S., Oh, M.J., Kim,Y.J., Kawai, K. & Jung S.J. (2003) 'Establishment and characterization of two new cell lines derived from flounder, Paralichtys olivaceus (Temminck & Schlegel).' *Journal of Fish Diseases* 26, 657-665.

Kempisty, B., Bukowska, D., Piotrowska, H., Zawierucha, P., Sniadek, P., *et al.* (2011) 'Selected molecular and microfluidic aspects of mammalian oocyte maturation-perspectives: a review' *Veterinarni Medicina*, 56, (8): 367-378

Kishida, M., Callard, G.V. (2001) 'Distinct cytochrome P450 aromatase isoforms in zebrafish (*Danio rerio*) brain and ovary are differentially programmed and estrogen regulated during early development' Endocrinology, 142 : 740–750.

Knoll-Gellida,A., Babin, P.J. (2007) 'Zebrafish ovarian follicle transcriptome.' In The *fish oocyte*: from basic studies to biotechnological applications (P.J.Babin, J.Cerada and E.Lubzens eds): Springer, 77-98.

Kopeika, J., Zhang, T., Rawson, D. M. and Elgar, G. (2005) 'Effect of cryopreservation on mitochondrial DNA of zebrafish (Danio rerio) blastomere cells', *Mutat Res*, 570(1), 49-61.

Kopeika, J., Zhang, T. and Rawson, D. (2006), 'Preliminary study on modification of yolk sac of zebrafish embryos (*Danio rerio*) using microinjection', *CryoLetters*, 27 (5), 319-328.

Kopeika, E., Kopeika, J., Zhang, T. (2007) 'Cryopreservation of fish sperm.' *Methods Mol. Biol.*; 368: 203-17.

Koleske, A. J., and Young, R. A. (1995). 'The RNA polymerase holoenzyme and its implications for gene regulation.' *Trends in Biochemical Sciences* 20:113-116.

Kreeger, P.K., Deck, J., Woodruff, T.K., Shea, L.D (2006). The in vitro regulation of ovarian follicle development using alginate-extracellular matrix gels. *Biomater.*, 27: 714-723.

Krishan, A. (1975) 'Rapid flow cytofluorometric analysis of mammalian cell cycle by propidium iodide staining.' *J Cell Biol.* 66(1):188–193.

Kumar, R.S., Ijiri, S., Trant, J.M. (2000) ' Changes in the expression of genes encoding steroidogenic enzymes in the channel catfish ovary throughout a reproductive cycle' *Biol Reprod* 63: 1676-1682.

Kumar, G.S., Singh,I.S.B., Philip, R. (2001). Development of a cell culture system from the ovarian tissue of African catfish(Clarias gariepinus). *Aquaculture,* 194:51-62.

Kuwayama, M., Vajta, G., Kato, O., Leibo, S.P. (2005) 'Highly efficient vitrification method for cryopreservation of human oocytes.' *Reprod Biomed Online*, 11(3): 300-308.

Kuwayama, M. (2007) 'Highly efficient vitrification for cryopreservation of human oocytes and embryos: The cryotop method' *Theriogenology* 67(1), 73-80.

Ksiazkiewicz, L.K. (2006) 'Recent achievements in invitro culture and preservation of ovarian follicles in mammals' *Reprod Biol* 6(1): 3-16.

Kwok, H.K., So, W.K., Wang, Y., Ge W.(2005). Zebrafish gonadotropins and their receptors: Cloning and characterization of zebrafish follicle stimulating hormone and

luteinizing hormone receptors-evidence for their distinct functions in follicle development. *Bio Reprod.* 72, 1370-1381.

Lahnsteiner, F. (2000) ‘Cryopreservation protocols for sperm of salmonid fishes. In: Cryopreservation in aquatic species Eds. Tiersch T.T and Mazik, P.M. *The World Aquaculture society*, Baton Rouge, pp91-100.

Lahnsteiner, F. (2008) 'The effect of internal and external cryoprotectants on zebrafish (Danio rerio) embryos', *Theriogenology*, 69(3), 384-396.

Lakra, W.S., Goswami, M., Rajaswaminathan, T., Rathore, G. (2010) ‘Development and characterization of two new cell lines from common carp, Crprinus carpio (Linn). *Biol Res*. 43(4): 385-92.

Larcher, W. (2001) ‘Physiological plant ecology’, 4th edn. Berlin:*Springer*.

LaFleur, G.J., Byrne, B.M., Kanungo, J., Nelson, L.D., Berg, R.M.G., Wallace, R.A., (1995) ‘Fundulus heteroclitus vitellogenin: the deduced primary structure of a piscine precursor to noncrystalline, liquid phase yolk protein.’ *J. Mol. Evol.* 41, 505-521.

Lee, B.H., Lim, E.H., Lam, T.J., Ding, J.L., 1994. Two major groups of vitellogenin c DNA clones from Oreochromis aureus (Steindachner). Biochem. Mol. Biol. Int. 34, 75-83.

Legault, R. (1958). ‘A technique for controlling the time of daily spawning and collecting of eggs of the zebra fish, Brachydanio rerio (Hamilton-Buchanan).’ *Copeia*, 328-330.

Levi, L., Ziv, T., Admon, A., Sivan, B.L., Lubzens, E. (2011) ‘Insight into molecular pathways of retinal metabolism, associated with vitellogenesis in zebrafish’ *Am J Physiol Endocrinol Metab* 302: E626–E644.

Levitt, J. (1980) ‘Responses of plants to environmental stresses’ *Chilling, freezing and high temperature stresses*, Vol 1, Academic Press, New York, 2nd Edn.

Leung, L.K.P (1991) ‘Principles of biological cryopreservation’ In: *Fish evolution and systematic*: evidence from spermatozoa Ed. Jamieson B.G.M.

Lewis, J.G., Learmonth, R.P., Watson, K. (1994) ‘Cryopreservation of yeast by alcohols during rapid freezing’. *Cryobiology* 31 (2): 193-8.

Li, X.L., Su, T., Li, W.Ji., Dinneys, A. (2002) ‘Vitrification of Yunnan yellow cattle oocytes’ *Theriogenology,* 58:1253-1260.

Li, S., Mao, Z., Han, W., Sun,Z., Yan, W., Chen,H., Yan, S. (1993) ‘ In vitro oocytes maturation in the zebrafish and the fertilization and development of the mature egg.’ *Chin J Biol* 19(4): 247-255.

Lieschke, G. J., and Currie, P. D. (2007). ‘Animal models of human disease: Zebrafish swim into vie.’ *Nature Rev Genet* 8, 353–67.

Lin, C., Spikings, E., Zhang, T. and Rawson, D. (2009) 'Housekeeping genes for cryopreservation studies on zebrafish embryos and blastomeres', *Theriogenology,* 71(7), 1147-1155.

Liu, X.-H. (2000) Studies on limiting factors relating to the cryopreservation of fish embryos, *unpublished thesis*, University of Luton.

Liu, X. H., Zhang, T. and Rawson, D. M. (2001) 'Effect of cooling rate and partial removal of yolk on the chilling injury in zebrafish (Danio rerio) embryos', *Theriogenology,* 55(8), 1719-1731.

Lovelock J.E. (1953) ‘The mechanism of the protective action of glycerol against haemolysis by freezing and thawing’. *Biochim Biophys Acta;* 11: 28-36.

Lovelock, J.E. (1957) ‘The denaturation of lipid-protein complexes as a cause of damage by freezing’. *Proc. Roy. Soc.* London, B, 147, 427.

Lubzens, E., T. Gattegno, et al. (2006). "Proteomic analyses on the effect of cryopreservation procedures on fish oocytes." *Cryobiology* 53(3): 398-399.

Lubzens, E., Young, G., Bobe, J., Cerda, J. (2010) ‘Oogenesis in teleosts: How fish eggs are formed’ *General and comparative endocrinology* 165(3): 367-389.

Lucena, E., Bernal, D.P., Lucena,C., Rojas, A., Moran, A., Lucena, A. (2006) ' Successful ongoing pregnancies after vitrification of oocytes.' *Fertil Steril*, 85(1), 108-111.

Lucci, C.M., Kacinskis, M.A., Lopes, L.H.R., Rumpf, R., Bao, S.N. (2004) 'Effect of different cryoprotectants on the structural preservation of follicles in frozen zebu bovine (Bos indicus) ovarian tissue', *Theriogenology*, 61, 1101–1114.

Magyary, I., Dinnyes, A., Varkonyi, E., Szabo, T., Varadi, L. (1996) 'Cryopreservation of fisu embryos and embryonic cells. *Aquaculture*, 137:103-108.

Magalhaes, D.M., Araujo, V.R., Lima-Verde, I.B., Matos, M.H.T., Silva, R.C., Lucci, C.M., Bao, S.N., Campello, C.C., Figueiredo, J.R. (2009) 'Impact of pituitary FSH purification on *in vitro* early folliculogenesis in goats' Biocell, 33, pp. 91–97.

Mandelbaum, J., Junca, A.M., Plachot, M. et al. (1987) Human embryo cryopreservation, intrinsicand extrinsic parameters of success. *Hum. Reprod*., 2, 709-715.

Mandelbaum, J., Junca, A.M., Plachot, M., Alnot, M.O. *et al.* (1988) 'Cryopreservation of human embryos and oocytes' *Human reprod.* 3: 117-119.

Martinez-Madrid B., Dolmans, M.M., Van Langendonckt ,A., Defrère, S., Donnez, J. (2004) 'Freeze-thawing intact human ovary with its vascular pedicle with a passive cooling device', Fertil Steril. 82(5), 1390-4.

Marco-Jiménez, F., Garzon, D.L., Penaranda, D.S., Perez, L., Viudes-de-Castro, M.P., Vicente, J.S., Jover, M., Asturiano, J.F. (2006)' Cryopreservation of European eel (Anguilla anguilla) spermatozoa: effect of dilution ratio, foetal bovine serum supplementation, and cryoprotectants.' *Cryobiology* 53, 51–57.

Matsubara, H., Kazeto, Y., Ijiri, S., Hirai, T., Adachi, S., Yamauchi, K. (2003) 'Changes in mRNA levels of ovarian steroidogenic enzymes during artificial maturation of Japanese eel' *Fish Sci* 69: 979-988.

Maurer, H.R. (1992) ‘Towards serum-free, chemically defined media for mammalian cell culture’. In: Freshney RI (ed.), *Animal Cell Culture*: A Practical Approach (Second Edition). Oxford: Oxford University; 15-46.

Mazur, P. (1963). ‘Kinetics of water loss from cells at subzero temperatures and the likelihood of intracellular freezing’. *J Gen Physiol*, 47:347-369

Mazur, P. (1965) 'Causes of Injury in Frozen and Thawed Cells', *Fed Proc*, 24(82), S175-82.

Mazur, P. (1977) 'The role of intracellular freezing in the death of cells cooled at supraoptimal rates', *Cryobiology*, 14(3), 251-72.

Mazur, P., Rall, W.F., Rigopoulos, N. (1981) ‘Relative contributions of the fraction of unfrozen water and of salt concentration to the survival of slowly frozen human erythrocytes’. *Biophysics J,* 36:653-665.

Mazur, P. (1984) 'Freezing of living cells: mechanisms and implications', *American Journal of Physiology - Cell Physiology*, 247(3), C125-C142.

Mazur, P., Rall, W. and Leibo, S. (1984) 'Kinetics of water loss and the likelihood of intracellular freezing in mouse ova', *Cell Biochemistry and Biophysics*, 6(3), 197-213.

Mazur, P. (1990) 'Equilibrium, quasi-equilibrium, and nonequilibrium freezing of mammalian embryos', *Cell Biochemistry and Biophysics*, 17(1), 53-92.
Mazur, P., Schneider, U. and Mahowald, A. P. (1992) 'Characteristics and kinetics of subzero chilling injury in Drosophila embryos', *Cryobiology*, 29(1), 39-68.

Mazur, P. (2004). Principles of cryobiology. In: Life in the Frozen state Ed. Fuller, B.J., Lane, N., Benson, E.E. *CRC press*, 214-315.

Mazur, P., Cole, K.W. (1989). ‘Roles of unfrozen fraction, salt concentration and changes in cell volume in the survival of frozen human erythrocytes.’ *Cryobiology,* 26:1-29.

Mazur, P., Leibo, S.P., Chu, E.H.Y. (1972). ‘A two-factor hypothesis of freezing injury’. *Exp Cell Res,* 71:345-355.

Mazur, P. (1985). ‘Basic concepts of freezing cells’ *L.Johnson and K.Larsson eds.* P 91-112.

Mazur, P., Schneider, U., Mahawald, A.P. (1992) ‘Characteristics and kinetics of subzero chilling injury in Drosophila embryos’, *Cryobiology.* 29: 39-68.

McAndrew, B. J. (1993) In Recent Advances in Aquaculture IV, edited by J. Muir and R. J. Roberts., *Blackwell Scientific*, Oxford.

McLaughlin, M., Telfer, E.E. (2010) ‘Oocyte development in bovine primordial follicles is promoted by activin and FSH within a two-step serum-free culture system.’ *Reproduction* 139, 971–978.

McCurley, A. and Callard, G. (2008) 'Characterization of housekeeping genes in zebrafish: male-female differences and effects of tissue type, developmental stage and chemical treatment', *BMC Molecular Biology,* 9(1), 102.

Meduri G., Charnaux N., Driancourt M.A., Combettes, L., Granet, P., Vannier B., Loosfelt H., Milgrom, E.(2008). Follicle stimulating hormone receptors in oocytes? *J Clin Endocr Metab* 87:2266-76.

Menke, A., Spitsbergen, J.M., Wolterbeek, A.P.M., Woutersen, R.A. (2011) ‘Normal Anatomy and Histology of the Adult Zebrafish’ *Toxicologic Pathology,* 39: 759-775.

Melamed, P., Rosenfeld, H., Elizur, A., Yaron. Z.(1998) ‘Endocrine regulation of gonadotropin and growth hormone gene transcription in fish.’ *Comp. Biochem. Physiol. C Pharmacol. Toxicol. Endocrinol.* 119(3):325-338.

Meryman, H.T. (1970). The Frozen Cell (*G.E.W.Wolstenholme and M.O’Connor,Eds.*),51-67

Meryman, H.T. (1971) ‘Osmotic stress as a mechanism of freezing injury.’ *Cryobiology,* 8(5): 489-500

Meryman, H.T.; Williams, R.T.; Doughlas, M.St.J. (1977) ‘Freezing injury from solution effects and its prevention by natural or artificial cryoprotection’. *Cryobiologu* 14. 287-302.

Montaldo,H., Herrera, M., Cesar, A. (1998) ‘Use of molecular markers and major genes in the genetic improvement of livestock’ *Electron. J. Biotechnol.* vol.1, n.2, pp. 15-16.

Morris, G.J., Watson, P.F. (1984) ‘Cold shock injury-A comprehensive bibliography’ *Cryoletters,* 5: 352-372.

Morris, G.J. ,Clarke, A. (1987) ‘Cells at low temperatures. In G.J.Morris; A. Clarke, ed: *Effect of low temperatures on biological systems Eds.* 72-119.

Moles, G., Gomez, A., Rocha, A., Carrillo, M., Zanuy, S. (2008) ‘Purification and characterization of follicle-stimulating hormone from pituitary glands of sea bass (Dicentrarchus labrax).’ *Gen Comp Endocrinol.* 158, 68-76.

Muldrew, K. and McGann, L. E. (1994) 'The osmotic rupture hypothesis of intracellular freezing injury', *Biophysical Journal*, 66(2, Part 1), 532-541.

Mullis, K., Faloona, F., Scharf, S., Saiki, R., Horn, G., and Erlich, H. (1986) ‘Specific enzymatic amplification of DNA in vitro: the polymerase chain reaction.’ *Cold Spring Harb. Symp. Quant. Biol.* 51: 263-273.

Muchlisin, Z.A., Siti-Azizah, M.N.(2009) ‘Influence of cryoprotectants on abnormality and motility of baung(Mystus nemurus) spermatozoa after long-term cryopreservation’, *Cryobiology*, 58, 166-169.

Muncke,J and Eggen, R.L.(2006) ‘Vitellogenin 1 mRNA as an early molecular biomarker for endocrine disruption in developing zebrafish (*Danio rerio*).’ *Envt Toxi & Chem*. 5(10): 2734-2741.

Nagahama, Y., Kagawa, H., Tashiro, F. (1980) 'The in vitro effects of various gonadotropins and steroid hormones on oocyte maturation in amago salmon and rainbow trout.' *Jpn Soc Sci Fisheries* 46: 1097-1102.

Nagahama, Y. (1994) 'Endocrine regulation of gametogenesis in fish.' Int. J. Dev. Biol., 38 pp. 217–229.

Nakamura, T., Takagi, H., Shima, J. (2009) 'Effects of ice-seeding temperature and intracellular trehalose contents on survival of frozen saccharomyces cerevisiae cells'. *Cryobiology*; 58:170–4.

Narayan,R., Kenney, M.C., Kamjoo, S., Trinh, T.H., Seigel, G.M., Resende, G.P, Huppermann, B.D. (2005) 'Trypan blue: effect on retinal pigment epithelial and neurosensory retinal cells.' *Invest ophthalmol vis.Sci* 46(1) 304-9.

Newton, H., Aubard, Y., Rutherford, A., Sharma, V., Gosden, R. (1996) 'Low temperature storage and grafting of human ovarian tissue. *Hum.Reprod*., 11, 1487-1491.

Newton. H., Picton. H., Gosden. R.G. J. (1999) '*In vitro* growth of oocyte-granulosa cell complexes isolated from cryopreserved ovine tissue', *J Reprod Fert*, **115,** 141–150.

Ng, T.B., Idler, D.R. (1983) 'Teleosts gonadotropins: isolation, biochemistry and functions. In W.S. Hoar, D.J. Randall, E.M. Donaldson in *Fish Physiology*. Vol IXB, Academic press, 187-221.

Niimi, A.J., Laham, Q.N. (1974) 'Influence of breeding time interval on egg number, mortality, and hatching of the zebrafish Brachydanio rerio.' *Canadian Journal of zoology*, 52(4): 515-517.

Norton, W., Cuif, L.B. (2010) 'Adult zebrafish as a model organism for behavioural genetics' *BMC Neuroscience*, 11:90.

Oba, Y., Hirai, T., Yoshiura, Y., Yoshikuni, M., Kawauchi, H., Nagahama, Y. (1999) 'The duality of fish gonadotropin receptors: cloning and functional characterization of a second gonadotropin receptor cDNA expressed in the ovary and testis of amago salmon *Oncorhynchus rhodurus*.' *Biochemical and Biophysical Research Communications* 265: 366–371.

Oetome, Z.J., Nunes, R.N., Kouassi, C.K., Hem, S., Agnese, J.F. (1996) 'Testicular structure, spermatogenesis and sperm cryopreservation in the African clarid catfish.' *Aquaculture Research* 27 (11): 805-813.

Oktay, K. (2001) Ovarian tissue cryopreservation and transplantation: preliminary findings and implications for cancer patients. *Hum. Reprod.Update*, 7, 526-534.

Oktay, K.,Nugent, D., Newton, H., Salha, O., Gosden, R.G. (1997) ' Isolation and characterization of primordial follicles from fresh and cryopreserved human ovarian tissues' *Fert Steril.* 67:481-486.

Onions, V.J., Mitchell, M.R.P., Campbell, B.K and Webb, R. (2007) 'Ovarian tissue viability following whole ovine ovary cryopreservation: assessing the effects of sphingosine-1-phosphate inclusion' *Human Reproduction*, 23, 606-618.

Orvar, B.L., Sangwan, R.J., Omann F., Dhindsa, R.S.(2000), 'Early steps in cold sensing by plant cells: the role of cryoskeleton and membrane fluidity', *Plant J*,23, 785-794.

Otala,M., Erkkilla,K., Tuuri,T., Sjoberg,J., Suomalainen, L., Suikkari, A.M., Pentikainen,V., Dunkel, L. (2002). Cell death and its suppression in human ovarian tissue culture. *Molecular human reproduction.* 8(3):228-236.

Palmer, S., Wiegand, A. P., Maldarelli, F., Bazmi, H., Mican, J. M., Polis, M., Dewar, R. L., Planta, A., Liu, S., Metcalf, J. A., Mellors, J. W. and Coffin, J. M. (2003) 'New real-time reverse transcriptase-initiated PCR assay with single-copy sensitivity for human immunodeficiency virus type 1 RNA in plasma', *J Clin Microbiol*, 41(10), 4531-6.

Pan, M.L., Bell, W.J., Telfer, W.H. (1969) 'Vitelligenic blood protein synthesis by insect fat body' *Science* 164, 393.

Pang Y., Ge W.(2002). Gonadotropin and activin enhance maturational competence of oocytes in the zebrafish (Danio rerio). *Biol Reprod,* 66:259-265.

Patra, M., Salonen, E., Terama, E., Vattulainen, I., Faller, R., Lee, B. W., Holopainen, J. and Karttunen, M. (2006) 'Under the Influence of Alcohol: The Effect of Ethanol and Methanol on Lipid Bilayers', *Biophysical Journal,* 90(4), 1121-1135.

Parkes, A.S. and Smith, A.U. (1953) 'Regeneration of rat ovarian tissue grafted after exposure to low temperatures', *Proc. R. Soc. Lond., Ser. B,* 140,455-470.

Parkes, A.S. (1957) 'Viability of ovarian tissue after freezing', *Proc. R. Soc.Lond., Sen B,* 147**,** 520-528.

Pearl, M. and Arav, A. (2000) 'Chilling sensitivity in zebrafish (Brachydanio rerio) oocytes is related to lipid phase transition', *Cryo Letters,* 21(3), 171-178.

Pegg, D.E., Diaper, M.P. (1990) 'Freezing versus vitrification; basic principles.' *Boston, Kluwer Academic Publisher,* p: 55-69.

Pegg, D.E. (2007) 'Principles of cryopreservation' *Methods Mol Biol,* 368:39-57.

Perchec, G., Jeulin, C., Cosson, J., Andre, F., Billard, R. (1995) 'Relationship between sperm ATP content and motility of carp spermatozoa' *Journal of cell science*. 108(2) 747-753.

Picton, H.M., Danfour, M.A., Harris, S.E., Chambers, E.L., Huntriss, J.(2003) 'Growth and maturation of oocytes invitro'. *Reprod Suppl*, 61: 445-462.

Picton, H.M., Gosden, R.G., Leibo, S.P (2002) 'In Current Practices and Controversies in Assisted Reproduction', Vayena E, Rowe P & Griffin PD (eds), World Health Organization, Geneva. 142-151.

Pitt, R.E., Steponkus, P.L. (1989). 'Quantitative analysis of the probability of intracellular ice formation during freezing of isolated protoplast', *Cryobiology,* 26:44-63.

Pitt, R.E., Chandrashekaran, M., Parks, J.E. (1992) 'Performance of a kinetic model for intracellular ice formation based on the extent of supercooling.' *Cryobiology,* 29(3): 359-373.

Pfaffl, M. W. and Hageleit, M. (2001) 'Validities of mRNA quantification using recombinant RNA and recombinant DNA external calibration curves in real-time RT-PCR', *Biotechnology Letters,* 23(4), 275-282.

Pfaffl, M. W. (2004) 'Chapter 3: Quantification strategies in real-time PCR' in Bustin, S. A., ed. *A-Z of quantitative PCR,* La Jolla, USA: International University Line (IUL), 87 - 112.

Plachinta, M., Zhang, T., Rawson, D.M. (2004), 'Studies on cryoprotectant toxicity to zebrafish(Danio rerio) oocytes', *Cryoletters*, 25(6), 415-424

Plachinta, M. (2007) 'Studies on cryopreservation of zebrafish oocytes using controlled slow cooling' *PhD Thesis.* University of Bedforshire.

Peters, T. (1995) 'All about albumin: biochemistry, genetics and medical application'. *Academic press*, San Diego.

Potts, M. (1994) 'Desiccation tolerance of prokaruotes' *Microbiological Review* 58: 755-805.

Pringle, M.D., Chapman. (1981) ' Biomembrane structure and effects of temperature' in G.J.Morris; A. Clarke, ed. *Effects of low temperature on biological membranes,* London: Academic Press, 21-37.

Quinn, P.J. (1985). A lipid phase serration model of low temperature damage to biological membranes. *Cryobiology,* 22:128-146.

Rauen, U., Polzar, B., Stephan, H., Mannherz, H.G., Groot, H. (1999) ' Cold-induced apoptosis in cultured hepatocytes and liver endothelial cells:mediation by reaction oxygen species' *Faseb J* , 13:155-168.

Rojas, R. R., Leopold, R. A. (1996). 'Chilling injury in the housefly: Evidence for the role of oxidative stress between pupariation and emergence'. *Cryobiology,* 33: 447-458.

Rall, W.F., Mazur, P., McGrath, J.J. (1983) 'Depression of the ice-nucleation temperature of rapidly cooled mouse embryos by glycerol and dimethyl sulfoxide' *Biophysics,* 41(1): 1-12.

Rall, W., Fahy, G. (1985) ‘Ice-free cryopreservation of mouse embryos at -196°C by vitrification’ *Nature,* 313: 573.

Rall, W. (1987) ‘Factors affecting the survival of mouse embryos cryopreserved by vitrification’ *Cryobiology,* 24: 387-402.

Rall, W. (1991) 'Advances in Cryopreservation of Embryos and Prospects for Application to the Conservation of Salmonid Fishes' in Cloud, J. G. and Thorgaard, G. H., eds., *Genetic Conservation of Salmonid Fishes*, New York: Plenum Press, 137-158.

Rasmussen, D.H., Macaulay, M.N., Mackenzie, A.P. (1975) ‘ Supercooling and nucleation of ice in single cells’ *Cryobiology*, 12(4): 328-339.

Rajarajan, K., Rao, B.S., Vagdevi,R., Tamilmani.G., Arunakumari, G, Sreenu, M., Amarnath, D., Naik,B.R.,Rao,V.H. (2006). Effect of various growth factors on the in vitro development of goat preantral follicles. *Small ruminant research*, 63:204-212.

Rodrigues, A.P.R., Amorim, C.A., Costa, S.H.F., Matos, M.H.T., Santos, R.R., Lucci, C.M., Bao, S.N., Ohashi, O.M., Figueiredo, J.R. (2004) ‘Cryopreservation of caprine ovarian tissue using dimethylsulphoxide and propanediol.’ *Anim. Reprod. Sci.*, 84 (2004), pp. 211–227.

Rodrigues, A.P., Amorim, C.A., Costa, S.H., Matos, M.H., Santos, R.R., Lucci, C.M., Bao, S.N., Ohashi, O.M., Figueiredo, J.R. (2004). ‘Cryopreservation of caprine ovarian tissue using glycerol and ethylene glycol.’ *Theriogenology* 61, 1009–1024.

Rotman, B., Papermaster, B.W. (1966) ‘Membrane properties of living mammalian cells as studied by enzymatic hydrolysis of fluorogenic esters’ *Proc Natl Acad Sci*. 55:134.

Rossetto, R., Lima-verde,I.B., Matos,M.H.T., Saraiva M.V.A., Martins,F.S., Faustino,L.R., Arajo, V.R., Silva,C.M.G., Name,K.P.O., Bao,S.N., Campello, C.C., Figueiredo,J.R., Blume,H. (2009). Interaction between ascorbic acid and follicle-

stimulating hormone maintains follicular viability after long-term in vitro culture of caprine preantral follicles. *Domestic animal endocrinology,* 37:112-123.

Rugh, R. (1948) 'Experimental embryology, a manual of techniques and procedure.' *Burgess Pub.Co..,* New York.

Santos, R.R., Amorimc, C., Cecconid, S., Fassbendere, M., Imhoff, M., Lornageg, J., *et al.* (2010) 'Cryopreservation of ovarian tissue: An emerging technology for female germline preservation of endangered species and breeds' *Animal Reproduction* 4213.

Schneider, U., Mazur, P. (1987). 'Relative influence of unfrozen fraction and salt concentration on the survival of slowly frozen eight-cell mouse embryos.' *Cryobiology*, 24: 17-41.

Schmidt, K.L.T., Ernst,E., Byskov, A.G., Andersen, A.V., Andersen, C.Y.(2003) 'Survival of primordial follicles following prolonged transportation of ovarian tissue prior to cryopreservation' *Hum. Reprod*, 18(12), 2654-2659.

Schiewe, M.C., Rail, W.F., Stuart, I.D., Wildt, D.E. (1991) 'Analysis of cryoprotectant, cooling rate and in situ dilution using conventional freezing or vitrification for cryopreserving sheep embryos. *Theriogenology* 36 279—293.

Schroeder, A.C., Champlin, A.K., Mobraaten, L.E., Eppig, J.J. (1990) 'Developmental capacity of mouse oocytes cryopreserved before and after maturation in vitro.' *J Reprod Fertil* ; 89:43–50.

Scott, A.P., Baynes, S.M. (1980) 'A review of the biology, handing and storage of salmonid spermatozoa.' *J. Fish Biol.* 17: 707-739.

Shaw, P.W., Fuller, B.J., Bernard, A., Shaw, R.W. (1991) 'Vitrification of mouse oocytes: improved rates of survival, fertilization and development to blastocysts.' *Mol Reprod Dev*, 29(4) 373-378 .

Shaw,J.M., Jones, G.M. (2003) ' Terminology associated within vitrification and other cryopreservation procedures for oocytes and embryos', *Human Reprod Update,* 9(6): 583-605.

Shepard, M. L., Goldston, C. S. and Cocks, F. H. (1976) 'The H2O-NaCl-glycerol phase diagram and its application in cryobiology', *Cryobiology*, 13(1), 9-23.

Seki, S., Kouya, T., Valdez, D.M., Jin, B., Hara, T., Saida, N., Kasai, M., Edashige, K. (2007a) 'The permeability to water and cryoprotectants of immature and mature oocytes in the zebrafish' *Cryobiology*, 54(1): 121-124.

Seki, S., Kouya, T., Hara, T., Valdez, D.M. Jr., Jin, B., Kasai, M., Edashige, K. (2007b) 'Exogenous expression of rat aquaporin-3 enhances permeability to water and cryoprotectants of immature oocytes in the zebrafish (Danio rerio).' *Journal of Reproduction and Development* 53 597–604.

Seki, S., Kouya,T., Tsuchiya, R, Valdez D.M., Jin,B., Hara,T., Saida N., Kasai M., Edashige,K. (2008). Development of a reliable in vitro maturation system for zebrafish oocytes. *Reproduction*,125:285-292

Selman, K., Wallace, R.A., Sarka. A., Qi X. (1993). 'Stage of oocyte development in the zebrafish Brachy danio rerio.' *Journal of Morphology*, 218:203-224

Selman, K., Petrino, T., Wallace, R. (1994) 'Experimental conditions for oocytes maturation in the zebrafish Brachydanio rerio.' *J Exp Zool* 269: 538-550.

Shaw, J.M., Jones, G.M. (2003) 'Terminology associated with vitrification and other cryopreservation procedures for oocytes and embryos' *Human Reproduction* 9: 583-605.

Si, W., Hildebrandt, T.B., Reid, C., Krieg, R., Ji, W.Z., Fassbender, M., Hermes, R. (2006) 'The successful double cryopreservation of rabbit (Oryctolagus cuniculus) semen in large volume using the directional freezing technique with reduced concentration of cryoprotectant'. *Theriogenology* 65:788–798.

Simon, A.M., Goodenough, D.A., Li, E., Paul, D.L. (1997) 'Female infertility inmice lacking connexin.' *Nature* ; 385:525–529.

Silva, J.R., Tharasanit, T., Taverne, M.A., Van der Weijden, G.C., Santos, R.R., Figueiredo, J.R., Van den Hurk, R. (2006) 'The activin-follistatin system and in vitro early follicle development in goats' *J. Endocrinol*. 189, 113–125.

Simpson, E.R., Mahendroo, M.S, Means, G.D., Kilgore, M.W., Hinshelwood, M.M *et al.* (1994) ' Aromatase cytochrome P450, the enzyme responsible for estrogen biosynthesis' *Endocr.Rev.*,15: 342-355.

So, W.K., Kwok, H.F., Ge, W. (2005) 'Zebrafish gonadotropins and their receptors: II. Cloning and characterization of zebrafish follicle-stimulating hormone and luteinizing hormone subunits their spatial-temporal expression patterns and receptor specificity.' *Biology of Reproduction* 72 1382–1396.

Son, J.H; Ha, Y.M; Kim, Y.I; Kim, K.M; Park, J.K; Kim, S.K. (2006) 'Immobilization of Cryopreserved Primary Rat Hepatocytes for the Development of a Bioartificial Liver System' Biotechnology Letters; Vol. 28 Issue 1, p51.

Spence, R., Gerlach, G., Lawrence, C., Smith, C. (2008) 'The behaviour and ecology of the zebrafish, Danio rerio.' 83(1): 13-34.

Specker, J.L., Sullivan, C.V., (1994) 'Vitellogenesis in fishes: status and perspectives.' In:
Davey, K.G., Peter, R.E., Tobe, S.S. (Eds.), *Perspectives in Comparative Endocrinology*. National Research Council Canada, Ottawa, pp. 304-315.

Stachecki, J.J., Willadsen, S.M. (2000) 'Cryopreservation of mouse oocytes using medium with low sodium content: effect of plunge temperature. *Cryobiology*, 40: 4-12.

Stewart, D.L. (1951) 'Storage of bull spermatozoa at low temperatures' *Veterinary Record*, 63: 65–66.

Stein, A., Fisch, B., Tadir, T., Ovadia, J., Kraicer, P.F. (1993) 'Cryopreservation of rat blastocysts: A comparative study of different cryoprotectants and freezing thawing method' *Cryobiology*, 30: 128-134.

Stein, G. S., Stain, J. L., van Wijnen, A. J. and Lian, J. B. (1996). 'The maturation of a cell.' *American Scientist* 84: 28-37.

Steponkus, P.L., Myers,S.P., Lynch, D.V., Pitt, R.E., Lin, T.T., MacIntyre, R.J., Leibo, S.P., Rall,W.F. (1991) ' Cryobiology of Drosophila melanogaster embryos'.

In : *insects at low temperature Eds.* Lee.R.E and Denlinger, D.L. Chapman and Hall, Ltd. Pp 408-423.

Stoss, J., Donaldson, E.M. (1983) 'Studies on cryopreservation of eggs from rainbow trout (*Salmo gairdneri*) and coho salmon (*Oncorhynchus Kisutch*)'. *Aquaculture.* 31: 51-65.

Suquet, M., Dreanno, C., Fauvel, C., Cosson, J., Billard, R. (2000) 'Cryopreservation of sperm in marine fish'. *Aquaculture Res.* 31(3): 231-243.

Suwa, K., Yamashita, M. (2007) 'Regulatory mechanism of oocytes maturation and ovulation' *The Fish oocyte*, 323-347.

Synder,L., Champness, W. (2007) 'Bacterial gene expression: Transcription, Translation and Protein folding' In Molecular genetics of bacteria. 3rd Edn. *ASM Press*. 71-76.

Sztein, J.M., Noble, K., Farley, J.S., Mobraaten, L.E. (2001) 'Comparison of permeating and non-permeating cryoprotectants for mouse sperm cryopreservation' *Cryobiology,* 42(1):28-39.

Tanaka, M., Telecky, T.M., Fukada, S., Adachi, S., Chen, S., Nagahama, Y. (1992) 'Cloning and sequence analysis of the cDNA encoding P-450 aromatase(P450arom) from a rainbow trout (*Oncorhynchus mykiss*) ovary; relationship between the amount of P450arom mRNA and the production of oestradiol-17 in the ovary' *J. Mol. Endocrinol.,* 8, pp. 53–61

Tanaka,M., Fukada,S., Matsuyama, M., Nagahama, Y (1995) 'Structure and promoter analysis of the cytochrome P-450 aromatase gene of the teleost fish, medaka (Orzias latipes)' *J. Biochem*. 117:719–725.

Takahashi, T., Hirsh, A., Erbe, E. F., Bross, J. B., Steere, R. L. and Williams, R. J. (1986) 'Vitrification of human monocytes', *Cryobiology*, 23(2), 103-115.

Tchoudakova,A., Callard, G.V. (1998) ‘Identification of multiple CYP19 genes encoding different cytochrome P450 aromatase isozymes in brain and ovary.’ *Endocrinology* 139:2179–2189.

Telfer, E.E., Binnie, J.P., McCaffery, F.H., Campbell, B.K. (2000) ‘In vitro development of oocytes from porcine and bovine primary follicles.’ *Mol. Cell Endocrinol.* 163, 117–123.

Telfer, E.E., McLaughlin, M., Ding, C., Thong, K.J. (2008) ‘A two-step serumfree culture system supports development of human oocytes fromprimordial follicles in the presence of activin.’ *Hum. Reprod.* 23, 1151–1158.

Tichopad, A., Dilger, M., Schwarz, G. and Pfaffl, M. W. (2003) 'Standardized determination of real-time PCR efficiency from a single reaction set-up', *Nucleic Acids Research*, 31(20), e122.

Tiersch, T.R., Yang, H., Jenkins, J.A., Dong, Q. (2007) ‘Sperm cryopreservation in fish and shellfish.’ *Soc. Reprod. Fertil. Suppl.* 65: 493-508.

Trad, F.S., Toner, M., Biggers, J.D. (1998) ‘Effects of cryoprotectants and iceseeding temperature on intracellular freezing and survival of human oocytes.’ *Hum Reprod*.14:1569–1577.

Trant, J.M (1994) ‘Isolation and chracterization of the cDNA encoding the channel catfish (*Ictalurus punctatus*) form of cytochrome P450arom’ *Gen. Comp. Endocrinol.*, 95, pp. 155–168.

Trichet, V., Buisine, N., Mouchel, N., Moran, P., Pendas, A.M., Lepenne, J.P., Wolff, J., (2000). Genomic analysis of the vitellogenin locus in rainbow trout (Oncorhynchus mykiss) reveals a complex history of gene amplification and retroposon activity. Mol. Gen. Genet. 263, 828-837.

Trounson, A.O. and Mohr, L. (1983) Human pregnancy followingcryopreservation, thawing and transfer of an eight-cell embryo. *Nature*, 305, 707-709.

Tsai,S., Rawson, D.M., Zhang,T. (2008), 'Studies on cryoprotectant toxicity to early stage zebrafish ovarian follicles', *Cryoletters*, 29(6), 477-483

Tsai, S., Rawson, D.M., Zhang, T. (2009) 'Studies on chilling sensitivity of early stage zebrafish(Danio rerio) ovarian follicles'. *Cryobiology*; Vol 58: Issue.3.

Tsai,S., Rawson, D.M., (2009) 'Development of cryopreservation protocols for early stage zebrafish ovarian follicles using controlled slow cooling' *Theriogenology,* 71, 1226-1233.

Tsai,S., Rawson,D.M., Zhang.T. (2010) ' Development of in vitro culture method for early stage zebrafish(Danio rerio) ovarian follicles for use in cryopreservation studies', *Theriogenology,* 74,290-303.

Tsai, S., Lin,C. (2012) 'Advantages and applications of cryopreservation in Fisheries science' *Braz.arch.biol.technol.* 55(3).

Tsvetkova, L.I., Cosson, J., Linhart, O., Billard, R. (1996) 'Motility and fertilizing capacity of fresh and frozen-thawed spermatozoa in sturgeons *Acipenser baeri* and *A. ruthenus*.' *J. Appl. Ichthyol.* 12: 107-112.

Tyler,C.R., Sumpter J.P., Kawauchi,H., Swanson,P.(1991). Involvement of gonadotropin in the uptake of vitellogenin into vitellogenic oocytes of the rainbow trout, Oncorhynchus mykiss. *Gen Comp Endocrinol*, 84:291-299.

Tyler C.R., Sumpter J.P. (1996) Oocyte growth and development in teleosts *Reviews in fish biology and fisheries.* Vol 6, 287-318.

Tyler, C.R., Santos, E.M., Prat, F. (2000) 'Unscrambling the egg - cellular biochemical molecular and endocrine advances in oogenesis.' In: Norberg, B., Kjesbu, O.S., Taranger, G.L., Andersson, E., Stefansson, S.O. (Eds.), Proceedings of the 6th International Symposium of Reproductive Physiology of Fish. John Greig, Bergen.

Van den Abbeel, E., Van der Elst, J., and Van Steirteghem, A.C (1994). 'The effect of temperature at which slow cooling is terminated and thawing rate on the survival of one-cell mouse embryo's frozen in dimethylsulfoxide or 1,2-propanodiol solutions'. *Cryobiology* 31,423-433.

Van der Straten, K.M., Leung, L.K., Rossini, R., Johnston, S.D. (2006) 'Cryopreservation of spermatozoa of black marlin, Makaira indica (*Teleostei: Istiophoridae*)'. *CryoLetters.* 27(4): 203-209.

Van der Elst, J., Nerinckx, S., Van, Steirteghem, A.C. (1992) 'In vitro maturation of mouse germinal vesicle-stage oocytes following cooling, exposure to cryoprotectants and ultra rapid freezing: limited effect on the morphology of the second meiotic spindle.' *Human Reprod,* 7:1440-1446.

Vandesompele, J., DePreter, K., Parryn, F., PPoppe, B., VanRoy, N., DePaepe, A., Speleman, F. (2002) 'Accurate normalization of real-time quantitative RT-RCR data by geometrc averaging of multiple internal control genes.' *Genome Biol* 3(7).

Veprintsev, B.N., Rott, N.N. (1980) Genome conservation, Academy of sciences of USSR, *Pushninology,* 1-49

Vischer, H.F., Granneman, J.C.M., Linskens, M.H.K., Schulz, R.W., Bogerd, J. (2003) 'Both recombinant African catfish LH and FSH are ableto activate the African catfish FSH receptor' *Journal of Molecular Endocrinology* 31, 133–140.

Walters, E.M., Men, H., Agca, Y., Mullen, S.F., Crister, E.S., Critser, J.K. (2005) ' Osmotic tolerance of mouse spermatozoa from various genetic backgrounds: Acrosome integrity, membrane integrity, and maintenance of motility.' *Cryobiology*, 50(2): 193-205.

Watson, P.E., Morris, G.J. Cold shock injury in animal cells. *Symp Soc Exp Biol*, 41:311-340.

Wandji, S.A., Srsen, V., Nahtanielsz, P.W., Eppig, J.J., Fortune, J.E. (1997) 'Initiation of growth of baboon primordial follicles in vitro.' *Hum. Reprod.* 12, 1993–2001.

Wang, T., Banker, M.C., Claydon, M., Hicks, G.L Jr., Layne, J.R Jr.(1992).' Freezing preservation of the mammalian cardiac explant. V. Cryoprotection by ethanol', *Cryobiology*, 29(4), 470-7.

Wang, H., Yan, T., Tan, J.T., Gong, Z. (2000) 'A zebrafish vitellogenin gene (vg3) encodes a novel vitellogenin without a phosvitin domain and may represent a primitive vertebrate vitellogenin gene.' *Gene* 256, 303–310.

Wang, Y., Ge, W. (2003) 'Gonadotropin regulation of follistatin expression in the cultured ovarian follicle cells of zebrafish, Danio rerio.' *Gen Comp Endocrinol*; 134:308–315

Wang, Y., Rippstein, P.U. and Tsang, B.K. (2003) 'Role and gonadotrophic regulation of X-linked inhibitor of apoptosis protein expression during rat ovarian follicular development *in vitro*.' *Biol. Reprod.* 68: 610-619.

Wang, Y., Ge, W. (2004) 'Developmental profiles of activin bA,bB and follistatin expression in the zebrafish ovary: Evidence for their differential roles during sexual maturation and ovulatory cycle' *Biol of Reproduction* 71, 2056-2064.

Wang, H., Tana, J.T.T., Emelyanovb, A., Korzhb, V., Gonga, Z. (2005) 'Hepatic and extrahepatic expression of vitellogenin genes in the zebrafish, Danio rerio' Gene 356 (2005) 91 – 100.

Wallace, R. A., Jared, D. W. (1969) 'Studies on amphibian yolk. VIII. The estrogen-induced hepatic synthesis of a serum lipophosphoprotein and its selective uptake by the ovary and trans- formation into yolk platelet proteins in Xenopus laevis.' *Dev. Biol.* 19:498.

Wallace, R.A., Selman, K. (1980) 'Oogenesis in fundulus heteroclitud II. The transition from vitellogenesis into maturation' *General and comparative endocrinology*, 42(3):345-354.

Wallace, R.A., Selman, K. (1981) ‘Cellular and dynamic aspects of oocyte growth in teleosts’ *Amer Zool*, 21:325-343.

Wallace, R.A. (1985) ‘Vitellogenesis and oocytes growth in nonmammalian vertebrates.’ In Developmental Biology: A Comprehensive Synthesis, Ed. RW Browder. New York: Plenum Press. vol. 1, pp 127–177.

Weil, C., Bougoussa-Houadec, M., Gallais, C., Sekine, S., Volotaire,Y. (1995). Preliminary evidence suggesting variations of GtH 1 and GtH 2 mRNA levels at different stages of gonadal development in rainbow trout, Oncorhynchus mykiss. *Gen Comp Endocrinol*, 100: 327-333.

Westerfield, M. (2000) ‘The zebrafish book: A guide for the laboratory use of zebrafish. Eugene: *University of Oregon press.*

Wessel, M.T., Ball, B.A. (2004) ‘Step-wide dilution for removal of glycerol from fresh and cryopreserved equine spermatozoa’ *Animal reproduction Science,* 84 (1-2): 147-156.

Wendling, N.C., Bencic, D.C., Nagler, J.J., Cloud, J.G., Ingermann, R.L. (2006) ‘Adenosine triphosphate levels in steelhead eggs: an examination of turnover, localization and role. *Comp Biochem Physiol. A. Mol. Intergr Physiol* 137(4): 739-48.

Whitfield, T.T. (2002) ‘Zebrafish as a model for hearing and deafness’ *Journal of neurobiology*, 53(2): 157-171.

Whittingham, D.G., Leibo, S.P., Mazur, P. (1972) ‘Survival of mouse embryos frozen to -196°C and -269°C. *Science,* 178: 411-414.

Widholm, J.M. (1972) ‘The use of fluorescein diacetate and phenosfranine for determining viability of cultured plant cells’ *Stain Technol*. 47: 189-194.

Wittwer, C. T., Herrmann, M. G., Moss, A. A. and Rasmussen, R. P. (1997) 'Continuous fluorescence monitoring of rapid cycle DNA amplification', *BioTechniques,* 22(1), 130-1, 134-8.

Wood, G.R., Walton, A.G. (1970) 'Homogeneous nucleation kinetics of ice from water'. *J Appl Physiol* 41: 3027-3036.

Wood,M.J., Barros,C., Candy, C.J., Carroll, J., Melendez, J., Whittingham, D.G. (1993) 'High rates of survival and fertilization of mouse and hamster oocytes after vitrification in dimethylsulphoxide' *Biol Reprod*, 49(3): 489-495.

Wood, C.E., Shaw, J.M., Trounson, A.O. (1997) 'Cryopreservation of ovarian tissue'. *MJA*, 166,366-369.

Wood, M.J., Candy, C.J., Holt, W.V. (2001) 'Gamete and embryo cryopreservation in rodents', In cryobanking the genetic resource. Wildlife conservation for the future, Watson, P.F. and Holt, W.V (Eds), *Taylor and Francis*, London, pp 229-266.

Wolfe SL. Molecular and Cellular Biology. Belmont, CA: Wadsworth Publishing Company; 1993: 209.

Wright, D.L., Eroglu, A., Toner, M., Toth, T.L.(2004) ' Use of sugars in cryopreservation' *Reprod Bio Med* 1328:179-186.

Wu, M.Ch. Lee, H.M. (1996) 'Vitrification of porcine oocytes.' *J Chinese Soc Animal Sci.* 25(1): 35-51.

Wu, T.T., Patel, H., Mukai, S., Garg, R., Ni, X.Y., Chang, J.B., Peng, C. (2000) ' Activin, inhibin and follistatin in zebrafish ovary: expression and role in oocyte maturation' *Biol Reprod.* 62(6): 1585-1592

Yang, G., Zhang, A., Xu, L. X.,He, X (2009), 'Modeling the cell-type dependence of diffusion-limited intracellular ice nucleation and growth during both vitrification and slow freezing', *Journal of applied Physics*, 105, 114701-114701-11.

Yamamoto, N. (1989) 'Effect of dimethyl sulfoxide on cytosolic ionized calcium concentration and cytoskeletal organization of hapatocytes in a primary culture.' *Cell struct Funct*; 14: 75-85.

Younis, A.I., Brackett. B.G., Fayrer-Hosken, R.A. (1989) 'Influence of serum and hormone on bovine oocyte maturation and fertilization in vitro.' *Gamete Res*., 23: 189-201.

York, W. S., Patino, R. and Thomas, P. (1993). ‘Ultrastructural changes in follicle cell–oocyte associations during development and maturation of the ovarian follicle in Atlantic croaker.’ *Gen.Comp. Endocr.* 92, 402–418.

YunHa, S., Jee, B.C., Suh, C.S., Kim, H.S., Oh, S.K., Kim, S.H, Moon, S.Y. (2006) ‘Cryopreservation of human embryonic stem cells without the use of a programmable freezer’ *Human Reproduction* 20 (7) pp. 1779–1785.

Zampolla, T., Rawson, D.M., Zhang, T. (2006) ‘Development of new viability assessment methods for zebrafish (Danio rerio) oocytes.’ *Cryobiology* 58:16.

Zampolla, T., Zhang, T., Rawson, D. M, (2008), ‘Evaluation of zebrafish (*Danio rerio*) ovarian follicle viability by simultaneous staining with Fluorescein Diacetate and Propidium Iodide, *CryoLetters*, 29 (6), 463-475.

Zampolla, T., Spikings, E., Zhang, T., Rawson, D. M. (2009). ’Effect of methanol and Me2SO exposure on mitochondrial activity and distribution in stage III ovarian follicles of zebrafish (Danio rerio)’. *Cryobiology*, 59, 188–194.

Zampolla,T. (2009) *Development of new methods to assess the quality of zebrafish ovarian follicles*, unpublished thesis University of Bedforshire.

Zhang, X. S., Zhao, L., Hua, T. C., Chen, X. H. and Zhu, H. Y. (1989) 'A study on the cryopreservation of common carp (*Cyprinus carpio*) embryos', *Cryoletters*, 10, 271-278.

Zhang, T., Rawson, D. M. and John Morris, G. (1993) 'Cryopreservation of pre-hatch embryos of zebrafish (Brachydanio rerio)', *Aquatic Living Resources*, 6(02), 145-153

Zhang, T. (1994) ‘Investigations into the cryopreservation of zebrafish (Brachydanio rerio) embryos. *PhD Thesis*, University of Luton.

Zhang, T. and Rawson, D. M. (1995) 'Studies on Chilling Sensitivity of Zebrafish (Brachydanio rerio) Embryos', *Cryobiology*, 32(3), 239-246.

Zhang, J., Liu, J., Xu, K.P., Liu, B., DiMattina, M. (1995). 'Extracorporeal development and ultrarapid freezing of human fetal ova', *J Assist Reprod Genet.*, 12(6), 361-8.

Zhang, T. and Rawson, D. M. (1996) 'Permeability of the vitelline membrane of zebrafish (Brachydanio rerio) embryos to methanol and propane-1,2-diol', *Cryoletters*, 17, 273-280.

Zhang, T. and Rawson, D.M. (1996), 'Feasibility studies on vitrification of intact zebrafish(Brachydanio rerio) embryos'. *Cryobiology*, 33, 1-13.

Zhang, T. and Rawson, D. M. (1998) 'Permeability of Dechorionated One-Cell and Six-Somite Stage Zebrafish (Brachydanio rerio) Embryos to Water and Methanol', *Cryobiology*, 37(1), 13-21.

Zhang, T., Isayeva, A., Adams, S. L. and Rawson, D. M. (2005) 'Studies on membrane permeability of zebrafish (Danio rerio) oocytes in the presence of different cryoprotectants', *Cryobiology*, 50(3), 285-293.

Zhang,J.M., Sheng, Y., Cao, Y.Z., Wang, H.Y., Chen, Z.J. (2011) **'**Effects of cooling rates and ice-seeding temperatures on the cryopreservation of whole ovaries'. *Journal of assisted reproduction and genetics*, 28(7):627-633.

Ziv, T., Gattegno, T., Chapovetsky, V., Wolf, H., Barnea, E., Lubzens, E., Admon, A. (2008) Comparative proteomics of the developing fish (zebrafish and gilthead seabream) oocytes. *Comp Biochem Physiol D.* 3(1):12–35.

Zon, L.I., Peterson, R.T. (2005) 'Invivo drug discovery in the zebrafish' *Nature reviews Drug discovery*, 4: 35-44.

APPENDIX

Anil S, Ghafari F, Zampolla T, Zhang T (2011). Studies on cryoprotectant toxicity to zebrafish (*Danio rerio*) ovarian tissue fragments. Cryoletters 32(1), 40-50

Anil S, Ghafari F, Zampolla T, Zhang T (2010). Studies on cryoprotectant toxicity to zebrafish (*Danio rerio*) ovarian tissue fragments. Cryobiology 61(3), 384-385.(abstract)

Anil S, Zampolla T, Zhang T (2011). Development of *in vitro* culture method for zebrafish ovarian tissue fragment. Cryobiology 63 (3), 311-312.(abstract)

Anil S, Zampolla T, Zhang T (2012). Development of molecular markers for stage II and stage III zebrafish ovarian follicles after *in vitro* culture. Cryobiology 65 (3), 360 (abstract)