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REVIEW ARTICLE

IN VITRO CULTURE OF ZEBRAFISH EARLY STAGE OVARIAN FOLLICLES AND VARIOUS APPLICATIONS

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ABSTRACT

Zebrafish ovarian follicle is developed in five stages. The best method of isolationg follicle from ovary is enzymatic treatment with collagenase and hyaluronidase. 90% L-15 media (pH 9.0, temperature 28°) supplemented with hCG (for stage I and II follicle) for 24 hour or 60% L-15 media (pH 9.0, temperature 26°) supplemented with DHP (for stage III follicle) for 8-24 hour for can be used to mature ovarian follicle in vitro. But follicles cultured this way cannot be fertilized. Follicle of third stage cultured in 90% L-15 media supplemented with DHP or BSA for 270 minute can be fertilized successfully. The follicle can be cryopreserved by slow-cooling method in cryoprotectant solution of DMSO and methanol prepared in KCl buffer. The viability of cryopreserved follicle can be tested by TB, FDA+PI and GVBD assay and by calculating ADP/ATP ratio.

INTRODUCTION:

Zebrafish (*Danio rerio)* is a freshwater teleost species of Cyprinidae family. It is native to India and Pakistan though originally found in slow stream and rice paddies of East India and Myanmar (Axelrod H. R. 1955). It has 4-5 cm long silvery cylindrical body with 7-9 dark blue horizontal stripes. It can withstand 15.5 to 43.3 temperature range and 6.6 to 9.2 pH range. It is popular in fish industry since it can be maintained and breed with little expense. Zebrafish became a good model of studying vertebrate development and genetics since first recognized by Sterisinger *et al* (Streisinger G. 1981). Zebrafish has many advantages which made it an ideal experimental animal for biological research. In laboratory environment zebrafish provides large fertilized egg on daily basis. The embryonic development is also fast resulting in hatching after 72 hour of fertilization at 26° C temperature (Hisoaka K. K. 1958). The most important advantage of using it to study vertebrate developmental biology is its transparent embryo which allows to observe embryonic development visibly. Zebrafish is also used for genetic study since its generation time is also short, only several months. Finally, the responsiveness of zebrafish to mutagens, carcinogens and toxins (Keizer 1991) made it an ideal candidate for assaying the effect of various chemicals on vertebrates (Wannemacher 1992). Zebrafish genome has also great importance since it is revealed and has many conserved segments with human genome (Postlethwait J. H. 1998).

In vitro culture of zebrafish ovarian follicle refers to maintaining it in laboratory condition out from the mother's body in such way they can undergo maturation. It is done for toxicity assessment of various compounds, identifying a way to preserve maternal genome and revealing stage of oocyte development as well as various substances and pathways involved in oocyte development. Extensive work has been done to identify suitable methods for isolating, culturing, cryopreserving and finally fertilizing cryopreserved oocytes. This review summarizes all the methods that have been revealed by previous studies so that it can be used to culture early stage zebrafish oocyte *in vitro* for various purposes.

STAGE OF DEVELOPMENT OF ZEBRAFISH OVOARIAN FOLLICLE:

The most recent and complete study (Selman 1993) divides the development process of zebrafish ovarian follicle into four stages (figure 2).

First Stage:

This stage is called "primary growth stage". The primary stage of follicle development is further subdivided into two stages. In the prefollicle phase, which is the initial part of the first stage, the oocyte is surrounded by a shell of oocytes. These oocytes have high nuclei to cytoplasm ratio. Chromosome becomes increasingly visible as the oocytes proceed through prophase up to pachytene stage. By then, the oocytes become completely enveloped

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by a sheath of pre-follicle cells which separates individual oocytes from the shell of oocytes. In the follicle phase, which is the later phase of first stage, the oocyte enters into diplotene stage, nuclei proliferates, decreases in size and moves toward the periphery of enlarging germinal vesicle. A thin layer of follicle cells surrounds the oocyte and the whole complex is surrounded by a theca which is composed of vascular connective tissue and a thin surface epithelium.

Second Stage:

The second stage is called "cortical alveolus stage". In this stage, membrane bound vesicles of variable size called cortical alveoli (yolk vesicles) appears within oocyte. The number of cortical alveoli as well as its size increase with the growth of oocyte. The oocyte become opaque in this stage and centrally located germinal vesicle becomes indistinguishable. An initial layer was formed in first stage around oolema. Inside the first layer, a second layer and eventually inside it, the thickest third layer is formed around the oolemma. The first layer is called "zona radiata externa", the second layer-"the zona radiata interna 1" and the third layer-"the zona radiata interna 2" in a study (Ulrich 1969). The follicle cells around vitelline envelope continue dividing by remaining attached through desmosomes and other intercellular junction (Kessel 1988). Specialized cells appear within thecas which are suspected to be cells that secretes steroid for the growth of oocyte (Yamamoto 1968).

Third Stage:

This stage is called "vitellogenesis stage"**.** The ocyte sequesters a female specific protein of hepatic origin called vitellogenin via endocytosis. In this stage, the vitellogenin protein is converted into yolk proteins and accumulated in membrane-bound sacks called yolk bodies (Wallace 1985). As the follicle grow, number and size of yolk bodies increases with increasing protein content. Some yolk bodies are displayed as dark masses containing crystalline inclusion. They are referred as "main bodies" (Yamamoto 1967). Shape of follicle is still cuboidal throughout the stage and at the later part of the stage become competent to respond to hormonal signal.

Forth Stage:

This stage is called "oocyte maturation stage". At this stage, germinal vesicle migrate toward oocyte periphery and the nuclear envelope breaks down. The egg reinters into meosis, proceed, become arrested at second meotic prophase and converted into egg (Selman 1989). During the maturational stage, follicles become translucent, which is the visible evidence of oocyte maturation. Oocytes keep on enlarging, and some yolk proteins are processed through proteolysis though not in an extensive amount (Wallace n.d.). Crystalline main bodies are lost from yolk bodies and thus the yolk bodies become relatively more homogenous.

Fifth Stage:

This fifth and last stage is called "mature egg stage". In this stage matured eggs are released into the ovarian lumen. The vitelline envelope is still tripartite and the thickness is less than 3.0 µm. In this stage the envelope remain perforated by micropyle (Hart 1992). In the external surface aggregate of dense material are present and now covered by unknown flocculent material. Processes containing bundle of actin filaments are projected toward and into vitelline envelope. In the egg cortex there is a layer of cortical alveoli surrounded by variably sized yolk bodies.

CURRENT STATUS OF *IN VITRO* CULTURE OF ZEBRAFISH OVARIAN FOLLICLES:

Isolation of Zebrafish Follicle:

For *in vitro* culture of zebrafish oocyte, large number of oocyte is needed to be isolated. By using mechanical method, female zebrafish were anaesthetized and sacrificed, ovaries are removed and placed into Hank's solution. Using forceps and scissors, oocytes are removed from the overy (Plachinta M 2004). Recently enzymatic method has become more popular for isolating zebrafish embryo since enzymatic isolation has several advantages over mechanical method including its ability to remove more interstitial cells (Pang Y 1999), less labor consuming and quicker. M. Guan *et al* developed a successful protocol for enzymatic isolation of large number of zebrafish oocyte (T. Zhang 2008). The zebrafish ovaries were removed and placed immediately in Hanks solution. Then using forceps and scissors oocytes were separated manually (Plachinta M 2004). The ovarian cumulus was immersed into three enzymes (trypsin, hyaluronidase and collagenase) at different concentrations for different time durations. Oocytes were then pipette repeatedly so that single oocytes are separated (figure 3) and then washed in Hanks solution. Though three enzymes were investigated, optimal treatment conditions determined were- treatment with collagenase (0.4 mg per ml concentration) for 10 minutes and treatment with hyaluronidase (1.6 mg per ml concentration) for 10 minutes at 22^0 C temperature. More than 90% early stage (first stage to third stage) oocyte can be recovered by this process.

In vitro culture of early stage ovarian follicle:

The most widely used incubation media used for incubation of zebrafish ovarian follicle cell is 60% Leibovitz

L-15 medium (Gibco) (W 1999). Various substances have been used as additive with the media to promote maturation of the follicle. Substances found to have potentiality to promote *in vitro* maturation include transforming growth factor-β1 (Kohli G 2005), transforming growth factor-α (W 2002a), gonadotropin, activin (Pang Y 1999), epidermal growth factor (W 2002b) and cyclin B (Kondo T 1997). But 90% L-15 medium adjusted at pH 9 and supplemented with human chorionic gonadotropin (hCG) (10 IU per ml concentration) at 28° C for 24 hour for early stage (first and second stage) zebrafish ovarian follicles (S. Tsaia 2010) and 60% Leibovitz L-15 medium adjusted at pH 9.0 and supplemented with 17a,20P-dihydroxy-4-pregnen-3-one (DHP) (1 pg per ml concentration) at 26°C for 8-24 hour for third stage follicles (Selman K 1994) were found to be the optimum *in vitro* culture protocols

In vitro culture method for in vitro fertilization:

Many substances have found to have the potential to promote maturation of Zebrafish ovarian cell *in vitro*. But the ability of being fertilized and hatched after *in vitro* maturation of oocytes have either not been examined or was found to be absent since those substances were responsible for only nuclear maturation not cytoplasmic maturation. But when Zebrafish oocyte is cultured with the objective to preserve genetic material, it must be capable of being fertilized. An study by Seki *et al* revealed that, if stage III follicles are incubated in 90% L-15 medium supplemented with DHP and 0.5 mg/ml BSA and adjusted at pH 9.0 for 270 minutes, up to 70% cleavage and 63% hatching can be obtained (Shinsuke Seki 2008) . Thus this culture method can be used if the oocytes to be fertilized after culturing.

CRYOPRESERVATION OF CULTURED ZEBRAFISH OVARIAN FOLLICLE:

Genetic material of species of interest can be stored in gamete crybank for unlimited period. The species having commercial value can be recovered from cryopreserved embryo, oocyte or sperm. Cryopreservation of oocyte is more preferred over cryopreservation of embryo since it is smaller in size, it is devoid of completed chorion and its higher permeability to solute and water. But the limitation of zebrafish oocytes for being cryopreserved is its chilling sensitivity. Oocytes of third and fourth stage are very susceptible to chilling (A. Isayeva 2004) where oocytes of first two stages are more resistant (S. Tsai 2009a). S. Tsai *et al* developed a successful cryopreservation protocols using controlled slow cooling for early stage zebrafish ovarian follicle (S. Tsai 2009b). Cryoprotectant solutions (4 M methanol and 3 M dimethyl sulfoxide or DMSO) were prepared in KCl buffer or L-15 medium. KCl buffer was prepared by mixing 1 mM $MgCl_2$; 55 mM KCl; 2 mM $CaCl_2$; 55 mM K acetate; 10 mM HEPES; using 1 M KOH pH was adjusted at 7.4 (M. Guan 2006). Ovarian follicles were immersed into cryoprotectant solutions for 30 min at 22°C and then placed in a programmable cooler. The solution is then cooled to seeding temperature at 2°C/min rate and held for 5 minute. For methanol, seeding temaperature is -12.5° where for DMSO it is -10° C. Temperature is then decreased at 4° C/min rate until it is decreased to -40 C and then again decreased at 10° C/min rate until it is decreased to -80° C. After holding for 10 minutes at -80° C it is stored in liquid nitrogen at least for 10 minutes. Water bath (28° C) was then used to thaw sample and cryoprotectant removed in 4 step or 1 step. For 4-step removal, cryoprotectant was removed by diluting the sample in three solutions with decreasing concentration of cryoprotectant in KCl buffer. Sample was kept in each solution for 10 minutes. For 1-step removal, sample was mixed with KCl buffer or L-15 media devoid of cryoprotectant for 10 minutes. The study found KCl buffer better over L-15 medium, follicle of first stage over second stage , four-step removal of cryoprotectant over one-step removal and methanol over DMSO as cryoprotectant for cryopreservation. Other cryoprotectant tested include propylene glycol (PG), ethanol and ethylene glycol (EG) and cryoprotectant toxicity increased methanol/ethanol, DMSO, PG and EG, in order (Anil et al. 2011).

FERTILIZATION OF CULTURED OOCYTE:

The zebrafish oocyte can be fertilized by direct injection of sperm in oocyte cytoplasm. This method is called ICSI, which stands for intra-cytoplasmic sperm injection. Fertilization is most effective when injection within one hour of egg collection. The fertilization can be confirmed by using flow cytometry which is capable of detecting diploid genome. Another method of confirmation includes dominant phenotypic expression of a pigmentation gene provided by parental genome (Germa´n A. Poleo 2001).

VIABILITY ASSESMENT METHOD AFTER CRYOPRESERVATION:

There must be efficient viability assessment method of *in vitro* cultured zebrafish ovarian follicle cell. There are four methods reported up to yet-1. Trypan Blue (TB) Assay 2. Fluorescein Diacetate (FDA) and Propidium Iodide (PI) assay 3. ADP/ATP ratio assay and 4. Germinal Vesicle Breakdown (GVBD) Assay.

Trypan Blue (TB) assay:

Trypan Blue assay tests viability by assaying membrane integrity as it stains the cytoplasm of cells that have a

damaged membrane. But the limitation associated with this method include its inability to distinguish between damaged and dead cell and difficulty to interpret result after 3-5 minutes since dye is taken up by more cells with time (Hudson L. 1989). But it is still a fast and reliable method for viability testing and used successfully to test viability of cryopreserved zebrafish oocyte (Plachinta 2007). Trypan blue test involve incubating ovarian follicles in trypan blue solution (0.2%, in Hanks solution) for 3-5 minutes at 22° C temperature. The cells are then observed under microscope and viable (non-stained) and non-viable (stained blue) cells are counted (figure 4). The viability is calculated by following formula

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

Fluorescein Diacetate (FDA) and Propidium Iodide (PI) assay:

Fluorescein Diacetate (FDA) passes through cell membrane and converted into fluorescein by the action of the enzyme esterase. Flurescein accumulated within living cell is detected as green fluorescent under blue light. Injured cells lost its ability to be detected by this way and fluorescein diacetate is non-toxic to living cell (Rotman B. 1966). Propidium iodide is a fluorescent dye that is passed through the membrane of damaged cell, intercalated with DNA and RNA and detected as red fluorescent complex (Loken M. R. 1982). Non-viable cell with membrane integrity remain unstained. Double staining with fluorescein diacetate and propidium iodide is more sensitive than trypan blue method for testing viability of all stage zebrafish oocyte (Zamplla T. 2006). Tsai S. (in his PhD thesis submitted to University of Bedfordshire) used 0.1 ml (2µg) of FDA and 0.03 ml (0.6 µg) of PI directly to stain zebrafish ovarial follicle cells. FDI+PI stained cells were examined by inverted microscope and viable (fluorescing bright green) and nonviable cells (bright red) were counted (figure 5). The viability is calculated by following formula-:

$$\text{Viability (\%)} = \frac{\text{Green cells}}{\text{Green+Red cells}} \times 100$$

ADP/ATP Ratio Assay:

This is a quantitative assay that reveals the energy state of cell. ATP level is decreased in proliferating cell whereas vice versa is a evidence of cell death. The only study to determine the ATP/ADP ratio was done by Tsai S. (PhD thesis submitted to University of Bedfordshire) where the frozen sample was thawed, transferred into luminometer plate and 100 µl nucleotide releasing agent was added followed by addition of 1 µl of ATP monitoring enzyme after 5 minutes. Then ATP was measured by luminometer (data A) and measured again after 10 minutes (data B). The ATP was then converted by 1 µl ADP converting enzyme and ADP was measured after 1 minute (data C). The ADP/ATP ratio is calculated by following formula-

$$\text{ADP/ATP ratio} = \frac{\text{Data C- Data B}}{\text{Data A}}$$

Germinal Vesicle Breakdown (GBVD) Assay:

Germinal vesicle breakdown assay is based on the ability of immature fish oocyte being matured *in vitro* in a L-15 medium supplemented with hormones. In stage iv of zebrafish oocyte development, germinal vesicle migrates toward the oocyte periphery, nuclear envelope is broken down and follicle becomes translucent. Cells that did not undergo maturation contain intact germinal vesicle and thus remain opaque (figure 6). After *in vitro* maturation, translucent oocytes are a indicator of viable oocyte that underwent maturation (Selman 1993) (Selman K 1994). In the study of Selman *et al* at (1994), oocytes incubated in 60% L-15 media supplemented with DHP showed germinal vesicle breakdown after 8 hour, where control follicle incubated without DHP remain immature and germinal vesicle breakdown was not evident. The viability is calculated by following formula-

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

APPLICATION OF *IN VITRO* CULTURE OF ZEBRAFISH OOCYTE:

Toxicity assessment:

Egg of female fish is susceptible to chemical. Since exposure to chemical affects gametogenesis, eggs can be used to identify chemicals which are toxic as well as which has therapeutic property.

Conservation of maternal genome:

Cryopreservation of gamete is a way of preserving genetic material to preserve species diversity, to preserve fish used in biomedical research and important for aquaculture. Maternal genome cryopreservation is important for preservation of the mitochondrial genetic materials. Cryopreservation of fish oocyte has several advantages over cryopreserving embryo such as small size, relatively lower water content, structural simplicity and absence of fully formed chorion, better tolerance of

cryprotectant toxicity of oocyte than embryo (Plachinta M 2004) and better permeability of membrane to water of oocyte than embryo (Zhang T. 2005)

Prevention of infertility:

Oncology patient can cryopreserve her oocyte before starting to take treatment, so that she can has her offspring if the treatment causes permanent ovarian damage (R. 1987). Cryopreservation of zebrafish oocyte can be used as a model to develop a method of cryopreserving human oocyte.

Assessing the stage of oocyte development:

In vitro culture of ovarian follicles to maturity can be used to assess follicle development. The five stages of zebrafish ovarian follicle development were determined by *in vitro* culture of zebrafish ovarian follicle (Selman 1993).

Determining various factors/pathways involve in oocyte maturation:

Various factors or pathways involved with zebrafish oocyte maturation have been determined in various studies by culturing ovarian follicles *in vitro.* Here are some examples-

- Germinal vesicle break down process is found independent of the occurrence of the second proteolytic cleavage of yolk protein (O. Carnevali 2006).
- In teleost fishes, GPR30, which is a G protein coupled receptor is found to be involved in maturation of oocyte (Yefei Pang 2009).
- An estrogen is found to be responsible for maintaining meotic arrest by acting through a G protein coupled receptor in zebrafish oocytes (Anna Chung-Kwan Tse 2009).
- An ovarian IGF system is revealed in zebrafish that is differentially regulated by gonadotropin and steroids (Sharon N. Nelson 2010).
- Arachidonic acid pathway is found to be involved in ovulation and the maturation of oocyte and a inhibitor of cyclooxygenase is found to be involved in disrupting these processes (A.L. Lister 2008).
- It was found that, a paracrine/autocrine role for activin controls the final oocyte maturation (W 1999).
- In the zebrafish ovary, gonadotropin increases the expression of activin and activin receptors and thus the activin system is activated (Yefei Pang 2002a).
- The activin system of ovary mediates EGF/TGF actions in a downstream portion in the zebrafish ovary (Yefei Pang 2002b).
- By acting on 20beta- HSD, LHR, and mPR-beta, TGF-β1 inhibit zebrafish oocyte maturation (Gurneet Kohli 2005).

Identification of disease causing recessive gene:

Cultured oocytes can be fertilized by ultraviolet ray (UV) exposed sperm which contains inactivated dominant genes. It allows recessive gene from mother to be expressed and disease causing recessive genes can be identified.

CONCLUSION:

Though culture method of Zebrafish oocyte is now well defined by various experiments, only a few scientists tried its application. We hope that in near future, more and more applications will be revealed by extensive research in this field.

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