Viability of zebrafish (*Danio rerio*) ovarian follicles after vitrification in a metal container

Lis S Marques[1], Adriana Bos-Mikich[2], Leandro C Godoy[3], Laura A Silva[1], Daniel Maschio[1], Tiantian Zhang[4] and Danilo P Streit Jr[1]

[1] Aquam Research Group, Department of Animal Science, Federal University of Rio Grande do Sul, Porto Alegre, RS, Brazil

[2] Department of Morphological Sciences, ICBS, Federal University of Rio Grande do Sul, Porto Alegre, RS, Brazil,

[3] Aquaculture Graduate Program, Nilton Lins University, Manaus, AM, Brazil.

[4] Faculty of Science and Technology, Bournemouth University, Dorset, UK.

Correspondence should be addressed to Lis S Marques, e-mail: lismarx@gmail.com.

Abstract

Cryopreservation of ovarian tissue has been studied for female germline preservation of farm animals and endangered mammalian species. However, there are relatively few reports on cryopreservation of fish ovarian tissue and especially using vitrification approach. Previous studies of our group has shown that the use of a metal container for the cryopreservation of bovine ovarian fragments results in good primordial and primary follicle morphological integrity after vitrification. The aim of this study was to assess the viability and *in vitro* development of zebrafish follicles after vitrification of fragmented or whole ovaries using the same metal container. In Experiment 1, we tested the follicular viability of five developmental stages following vitrification in four vitrification solutions using fluorescein diacetate and propidium iodide fluorescent probes. These results showed that the highest viability rates were obtained with immature follicles (Stage I) and VS1 (1.5 M methanol + 4.5 M propylene glycol). In Experiment 2, we used VS1 to vitrify different types of ovarian tissue (fragments or whole ovaries) in two different carriers (plastic cryotube or metal container). In this experiment, Stage I follicle survival was assessed following vitrification by vital staining after 24 h *in vitro* culture. Follicular morphology was analyzed by light microscopy after vitrification. Data showed that the immature follicles morphology was well preserved after cryopreservation. Follicular survival rate was higher ($P<0.05$) in vitrified fragments, when compared to whole ovaries. There were no significant differences in follicular survival and growth when the two vitrification devices were compared.

Keywords: cryopreservation, follicle, ovary, fish, female infertility.

Introduction

Cryopreservation of fish sperm is relatively common in fish breeding programs and fish farming, however, successful protocols for cryopreserving oocytes and embryos of most aquatic species remains absent. Factors limiting fish embryo cryopreservation include their complex structure, large size, high yolk content, low membrane permeability and high chilling sensitivity [37]. Ovarian tissue cryopreservation is a promising alternative to preserve the maternal genome, even after the death of the animal [25]. Ovarian tissue contains multiple immature follicles that have a smaller size resulting in higher surface area to volume ratio, higher membrane permeability, as well as the absence of a fully formed chorion [36].

To date, the two mainly used cryopreservation methods are slow-rate freezing and vitrification. Slow-rate freezing is the most commonly used method on the cryopreservation of fish gametes [10,33] and embryos [21,12,20]. Slow-rate freezing requires relatively slow cooling rates in a controlled manner which may not be ideal for chilling sensitive materials such as fish oocytes and ovarian follicles. There is the risk of ice crystal formation inside the cell, which may be correlated with membrane damage and cell death. In contrast, vitrification uses highly concentrated cryoprotectant solutions, allowing fast rates of cooling and resulting in a glassy state, therefore avoiding ice crystals formation inside the cells. It has been successfully applied to chilling sensitive materials such as Drosophila embryos [23]. In mammals, including humans, studies have suggested that vitrification could be more effective than slow-rate freezing for the cryopreservation of ovarian tissues, in terms of morphological integrity, particularly the ovarian stroma [5,16,30]. However, a few studies have investigated the vitrification technique on cryopreservation of fish ovarian follicles [9,11,27] and there has been no reported information on vitrification of early stage ovarian follicles of fish.

In order to accelerate temperature reduction in the sample and avoiding its contact with the liquid nitrogen (LN2), our group has developed a vitrification technique in a metal closed system [3]. The metal container was manufactured with stainless steel and this closed system is a good thermal conductor and also avoids direct contact of the tissue sample with vapor or liquid nitrogen.

The main objective of the present study was to assess the viability and *in vitro* development of vitrified-rewarmed zebrafish ovarian follicles after ovarian tissue cryopreservation in a stainless steel container.

Materials and Methods

Chemicals

Unless otherwise stated, all chemicals used were purchased from Sigma Chemical (St. Louis, MO, USA).

Animals maintenance and ethics statement

Zebrafish (*Danio rerio*) were maintained in filtered and aerated 40 L aquaria at 27°C under a 14/10 h (light/dark) photoperiod. Fish were fed four times a day with TetraMin® dry flake fish food (Tetra, Germany).

All the experimental procedures and protocols described in this study were approved by the Ethics Committee of our Institution (Federal University of Rio Grande do Sul). Permit Number: 25676.

Ovaries collection

Ovaries were collected from six-month-old adult females, euthanized in a lethal dose of tricaine methane sulfonate (0.6 mg/mL) for 5 min and decapitated. Ovaries were collected and placed in a Petri dish containing 90% Leibovitz L-15 medium (pH 9.0) supplemented with L-glutamine.

Experiment 1 - Ovarian follicles viability at five difference stages after vitrification using four different vitrification solutions.

The aim of Experiment 1 was to test the effects of four different vitrification solutions on ovarian follicle viability at different stages.

The cryopreservation procedures are an adaptation of two published vitrification protocols [2,9]. All cryoprotectants and warming solutions were prepared in Leibovitz L-15 medium (pH 9.0) supplemented with L-glutamine. Ovaries were carefully removed from the Leibovitz L-15 medium and exposed to the equilibrium solution (ES) for 15 minutes at room temperature (24±2 °C) (RT). Vitrification procedures are illustrated in Figure 1. The ES1 contained 1.5 M of methanol and 2.25 M of propylene glycol, and in ES2 contained 1.5 M of methanol and 2.75 M of dimethyl sulfoxide (Me_2SO). Ovaries were then divided into four different vitrification solutions (VS): VS1 (1.5 M of methanol and 4.5 M of propylene glycol), VS2 (1.5 M of methanol and 5.5 M of Me_2SO), VS3 (1.5 M of methanol, 4.5 M of propylene glycol and 0.5 M of sucrose) and VS4 (1.5 M of methanol, 5.5 M of Me_2SO and 0.5 M of sucrose). The ovaries were kept in different vitrification solutions for 90 sec and then gently transferred with a minimum volume of medium to the metal container [2]. The bottom of the metal container was placed in contact with LN2, tightly sealed and immediately plunged in LN2 for storage. This container has similar dimensions to a conventional plastic cryotube, however, it is manufactured in stainless steel, which is an excellent thermal conductor. Moreover, it is a closed system that prevents direct contact to vapor or liquid nitrogen, avoiding microbiological contamination risk. After two to seven days, the metal container was removed from the LN2 for rewarming. The metal container remained at RT for 30 sec, and immersed into a water bath at 28°C for 30 sec and then opened. Ovaries were removed and transferred to the first warming solution

containing 1 M sucrose for 1 min, then to the second solution containing 0.5 M sucrose for 3 min and finally to the third solution of 0.25 M sucrose for 5 min at RT.

Figure 1. Vitrification procedures for zebrafish ovaries. Equilibrium solution (ES) and vitrification solutions (VS).

Control fresh ovaries were maintained in L-15 medium (pH 9.0) at RT (23-25°C) until staining. Cryopreserved and control follicles were isolated by gentle pipetting in Leibovitz L-15 medium and washed three times in phosphate buffered saline (PBS) (pH 9.0). Five developmental stages were selected: Stage I (primary growth); Stage II (cortical alveolus); Stage III (vitellogenic); Stage IV (maturation) and Stage V (mature) according to Selman et al. [29]. Ovarian follicle viability was assessed by the method described by Jones and Senft [15]. A stock solution of fluorescein diacetate (FDA) was prepared by dissolving 5 mg/mL FDA in acetone. The FDA working solution was prepared by adding 20 μL of stock solution in 5 mL of PBS. The propidium iodide (PI) solution was prepared by dissolving 1 mg of PI in 50 mL PBS. Ovarian follicles were incubated in drops containing 100 μL (2 μg) FDA working solution and 30 μL (0.6 μg) PI solution in the dark for 3-4 min at RT. Cell viability assessment was performed on an inverted fluorescence microscope (Opton, TNI-51-IMU). The bright green fluorescent follicles were considered viable (Figure 2A) and the bright red stained follicles were considered non-viable (Figure 2B). Experimental and control groups were composed by at least 100 ovarian follicles in each group, and the experiments were repeated three times.

Figure 2. Fluorescein diacetate and propidium iodide assays. Bright green fluorescent follicles, Stage I, are viable (A) and bright red fluorescent follicles, Stage V, are non-viable (B). Bar=200 μm.

Experiment 2 – *In vitro* culture of ovarian follicles after vitrification using both tissue fragments and whole ovary in two different vitrification devices.

The aim of Experiment 2 was to evaluate the effects of vitrification on survival and *in vitro* growth of ovarian follicle in two different types of tissues (fragment or whole ovary) in two different devices (metal container or plastic cryotube).

Fragments containing Stage I follicles were carefully dissected from the ovaries and cut into thin slices (2 mm) using syringe needles. The ovarian pieces were stretched so that Stage I follicles could easily be observed. Dissections of ovarian fragments were performed within a maximum of 20 min at RT.

Vitrification and rewarming protocols were the same as described in Experiment 1. VS1 from Experiement 1, which showed the highest Stage I follicle viability was selected to be used in Experiment 2. In all groups, the samples were vitrified with minimum volume of vitrification solution.

Four vitrification treatments were tested: whole ovary in plastic cryotube (Minitüb, Tiefenbach, Germany) (Group 1), whole ovary in metal container (Group 2), ovarian fragments in plastic cryotube (Group 3) and ovarian fragments in metal container (Group 4). Groups 3 and 4 were performed with tissue fragments of 0.4-0.5 mm long and 2 mm thick containing Stage I ovarian follicles.

After vitrification/rewarming, Stage I follicles were isolated and classified as described before (Experiment 1) and the follicles were selected according to their diameter (between 90-140 µm). Using an *in vitro* culture protocol based on previously published protocols [1,26,34] with some modifications, the selected follicles were washed three times with L-15 medium 90% (pH 9.0), before being randomly distributed into 96 well plates containing 200 µL of L-15 medium 90% (pH 9.0) supplemented with 0.5 mg/mL follicle stimulating hormone (FSH), 20% fetal bovine serum (FBS) and 100

µg/mL gentamycin. All follicles, including fresh controls were individually cultured in the wells for 24 h at 28°C. Survival was assessed by vital staining (FDA + PI) after *in vitro* culture (IVC). Furthermore, follicular diameter increase was evaluated by measuring diameters of viable follicles (bright green stained follicles), before and after IVC, on an ocular micrometer under an inverted light microscope (Carl Zeiss, Axiovert 135, Germany).

The experimental and control groups were composed by 96 ovarian follicles and the experiment was repeated three times.

Histological analysis of ovarian follicles

The aim of the histological analysis was to assess morphological integrity of ovarian follicles after vitrification in VS1 using the metal container by light microscopy.

Whole ovaries were fixed in 10% buffered formalin and prepared for histological analysis, as described by Prophet et al. [24].

The histological evaluation of the control group and the vitrified ovaries was descriptive, and based on criteria described by Selman et al. [29].

Statistical Analysis

Variables between groups in Experiments 1 and 2 were analyzed using the statistical package SAS 9.2 (2009), passing the normality test, followed by analysis of variance, with mean comparison by Duncan test ($P<0.05$).

Results

Ovarian follicles viability at five difference stages after vitrification using four different vitrification solutions.

Table 1 shows follicular viability among the five Stages within each VS group. Stage I follicles showed the highest viability percentage in VS1 after rewarming.

Therefore, VS1 was chosen to be used in Experiment 2. Stage II follicular viability was higher than observed in Stages III, IV and V in groups VS1, VS3 and VS4 ($P<0.05$).

Table 1 also compares follicular viability of Stages I and II among groups. The results showed that there are no significant differences between viability in Stage I of the control group (95.74%), VS1 (76.84%) and VS4 (64.17%). In Stage II, there was significant difference among control and vitrified groups.

Experiment 2 – *In vitro* culture of ovarian follicles after vitrification using both tissue fragments and whole ovary in two different vitrification devices.

Follicular survival rate in control group (86%) was significantly higher compared with all four vitrified groups (Figure 3). Among the vitrified groups, Group 3 had the highest rate of follicle survival, however, there was no difference between Group 3 and Group 4 (31%; $P<0.05$). Figure 3 shows that Group 2 had the lowest follicular survival (12%) but, there is no significant difference between Group 2 and Group 1 (23%; $P<0.05$). These results suggest that vitrification of ovarian tissue fragments increase the chances of follicular survival in Stage I, when compared with whole ovary.

Figure 3. Survival rate of vitrified/rewarmed Stage I ovarian follicles after 24 h *in vitro* culture. Group 1 (whole ovary in plastic cryotube), Group 2 (whole ovary in metal container), Group 3 (fragments in plastic cryotube), Group 4 (fragments in metal container). Bars labeled with common letters do not differ significantly ($P<0.05$) from each other (mean ± SD).

Control group showed a greater increase in follicular diameter (40 µm), when compared with vitrified groups (Figure 4). However, there was no significant difference between the controls and Group 1 (29 µm; $P<0.05$).

Figure 4. Average diameter growth of vitrified/rewarmed Stage I follicles after 24 h *in vitro* culture. Group 1 (whole ovary in plastic cryotube), Group 2 (whole ovary in metal container), Group 3 (fragments in plastic cryotube), Group 4 (fragments in metal container). Bars labeled with common letters do not differ significantly ($P < 0.05$) from each other (mean ± SD).

Among the vitrified groups, there was no significant difference in diameter increase, suggesting that neither the device nor the dimension of the sample had an effect on *in vitro* follicular growth after vitrification/rewarming.

Histological analysis of ovarian follicles

Zebrafish follicular development was divided into four stages (primary growth, cortical alveolus, vitellogenic and mature, Figure 5A), based on morphological characteristics described by Selman et al. [29].

Figure 5. Control group (A) and vitrified ovarian tissue (B). Primary growth stage (Pg), cortical alveolus stage (Ca), vitellogenic stage (V) e mature stage (M). Lysed lipid droplet (arrow in B). Light microscope 10x. Stain: HE. Bar=150 µm.

Cortical, vitellogenic and mature stage follicles presented the major cytoplasmic alterations mainly concerning the distribution and integrity of lipid droplets after cryopreservation. Follicle membrane layers appeared detached in few instances (Figura 5B). However, in higher resolution we observed that the membrane remained intact (Figure 6B, arrowhead).

Figure 6. Control group (A) and vitrified ovarian tissue (B). In B condensed chromatin (arrow) and intact follicular membrane layer (arrowhead). Primary growth stage (Pg). Light microscope 100x. Stain: HE. Bar=50 µm.

The best preserved follicles were the primary growth stage. These follicles showed intact follicular layers and homogeneous cytoplasm. Condensed chromatin was the main alteration observed in primary growth stage (Figura 6B, arrow). These observations suggest that lipid droplets are the most affected cell components during vitrification, potentially making the follicle more susceptible to cryoinjuries.

Discussion

In Experiment 1, fluorescein diacetate (FDA) and propidium iodide (PI) assays showed that there were no significant differences in Stage I follicle viability between fresh controls and the cryopreserved groups after vitrification in VS1 or VS4. However, in Experiment 2, Stage I follicle survival was significantly lower in all four vitrification groups, when compared to fresh controls after 24 h *in vitro* culture (IVC). The FDA + PI assay is based on the living cells ability to eliminate the PI stain through their intact membranes and in the intracellular esterases to hydrolyze FDA. Therefore, FDA+ PI assay following 24 h IVC provides information on oocyte developmental competence (cell metabolic and growth competence).

High rates of primary growth (Stage I) follicular viability were obtained after vitrification of whole ovaries using the metal container (Experiment 1). These results are in agreement with other studies that used metal devices to vitrify ovarian tissue of mouse, caprine, human and bovine and reported a good rate of primordial and primary follicle recovery after rewarming [2,3,4,17]. Fish ovarian follicles, as well as mammalian follicles have a smaller size in early stages, which results in a higher surface / volume ratio. Therefore, early follicles are likely to be more permeable to water and solutes, increasing survival chances after cryopreservation. Histological analysis and FDA+PI assay showed very few vitellogenic (Stage III), maturing (Stage IV) and mature (Stage V) intact follicles. Possibly, the main reasons for follicular

damage at these stages were the high lipid content of the follicles and low membrane permeability, limiting cell dehydration and cryoprotectant penetration. During maturation, oocytes of freshwater fish become less permeable, acquiring resistance to hypotonic environment before being spawned in the water [28].

Follicle viability obtained from VS1 (1.5 M of methanol and 4.5 M of propylene glycol) and VS4 (1.5 M of methanol, 5.5 M of Me_2SO and 0.5 M of sucrose) did not significantly differ from the control group in terms of follicular viability for Stage I. Seki et al. [27] had already suggested that the combination of methanol and propylene glycol might be more effective in reducing damage caused by cryopreservation on zebrafish immature follicles. In addition, in an extensive survey on the toxicity of several cryoprotectants, it was found that methanol and propylene glycol were the least toxic agents for zebrafish follicular survival after cryopreservation [9].

On the other hand, it has already been shown that dimethyl sulfoxide (Me_2SO) is a more permeable [28] and less toxic [19] cryoprotectant than propylene glycol to zebrafish follicles and embryo cryopreservation. Also, it was reported that the addition of sucrose enhanced the Me_2SO cryoprotectant action [19]. This observation is in agreement with our data, wherein sucrose addition to the cryoprotectant solution containing methanol and Me_2SO (VS4), increased immature follicle survival (Stages I and II). Unlike Me_2SO, sucrose does not penetrate the cell membrane and acts only in the extracellular medium by increasing its osmolarity. Sucrose, as a hydrophilic compound, binds to water molecules, increasing viscosity of the solution and resulting in cellular dehydration by water retention in the extracellular medium. Consequently, decreasing the osmotic stress and ice crystals formation [14,8]. Both cryoprotectant, intracellular and extracellular, interact with the cell membrane phospholipids conferring greater stability to the membrane [7]. Therefore, it may be suggested that sucrose

supplementation in cryoprotectant solutions containing Me_2SO enhances follicular viability. However, sucrose addition into vitrification solution containing propylene glycol did not increase the follicular viability. Propylene glycol is highly hydrophilic and the H-bond formation between the ether group and H_2O is supposed to contribute to its affinity to water [35]. Chauvigné et al. [6] reported that membrane permeability of zebrafish oocytes to propylene glycol and water varies according to osmolarity. Thus, sucrose concentration may interfere on propylene glycol influx, enabling a better or worse dehydration. Probably for this reason, follicular viability decreases by sucrose addition into vitrification solutions containing propylene glycol.

In order to increase the efficacy of ovarian cryopreservation protocols in different mammalian species, cryopreservation of whole ovary has been suggested as an alternative option [22]. After avascular transplantation of ovarian tissue fragments, the tissue undergoes significant damage caused by ischemia [31,32]. Thus, vascular transplant of intact ovaries would be an answer to this problem [13,18]. Moreover, when the whole ovary is cryopreserved, a larger pool of follicles is transplanted back to the donor or recipient of the tissue, unlike the situation that occurs when small fragments are used. However, vascular transplantation of whole cryopreserved ovary has two major limitations: the technical difficulty in proceeding reanastomosis [31] and the challenge to cryopreserve an intact organ [18]. These issues must be addressed in studies with experimental animal models, before they may be put through human clinical trials. Therefore, the effect of the tissue dimension (ovarian fragment or whole ovary) was also evaluated here in terms of follicular survival after vitrification. We observed that the survival rate in terms of *in vitro* growth, after vitrification was higher on tissue fragments when compared to whole ovary. The reasons for these results may

316 be because the ovary is a bulky organ composed of different cell types, which hinders
317 the easy diffusion of cryoprotectants into cells and stromal components.

318 A point to emphasize from this study is the fact that after vitrification in the
319 metal container, primary Stage I follicles presented the highest survival rates in all
320 vitrification groups (Experiment 1). Zebrafish primary growth follicles are similar to
321 mammalian primordial or primary follicle, in the sense that it represents the female
322 gamete surrounded by a single layer of cells. Considering that the ovarian reserve and
323 fertility potential is directly related to the number of primordial and primary follicles,
324 the main concern when ovarian tissue cryopreservation is considered is the maintenance
325 of the integrity of this follicular population, together with the surrounding stroma. Our
326 results on zebrafish follicular survival and growth after warming suggest that ovarian
327 tissue vitrification is a promising technology to preserve the maternal genome in fish.

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