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Studies on chilling sensitivity of early stage zebrafish (*Danio rerio*) ovarian follicles ☆

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ABSTRACT

Cryopreservation of fish gametes is of great importance in aquaculture, conservation and human genomic research. The creation of gamete cryobanks allows the storage of genetic material of targeted species for almost unlimited time periods. Cryopreservation has been successfully applied to fish sperm of many species, but there has been no success with fish embryos and oocytes. One of the obstacles to fish oocyte cryopreservation is their high chilling sensitivity and especially at subzero temperatures. Although studies on late stage oocyte cryopreservation has been carried out, there have been no reported studies on cryopreservation of early stage ovarian follicles. The aim of this study is to investigate the chilling sensitivity of early stage zebrafish ovarian follicles before developing protocols for their cryopreservation. Experiments were conducted with stage I (primary growth), stage II (cortical alveolus) and stage III (vetillogenesis) ovarian follicles, which were chilled in KCl buffer and L-15 medium for up to 144 h at −1 °C in a low temperature bath. Ovarian follicles were also exposed to 2 M methanol or 2 M DMSO in L-15 medium for up to 168 h at −1 and −5 °C, respectively. Control follicles were kept at 28 °C. Ovarian follicle viability was assessed using trypan blue staining. The results showed that stage I and II ovarian follicles are less sensitive to chilling than stage III follicles. These results were also confirmed following *in vitro* maturation of the chilled ovarian follicles. The results also showed that L-15 medium is more beneficial than KCl buffer for ovarian follicles at all stages. The presence of both methanol and DMSO reduced chilling sensitivity of ovarian follicles at all stages with methanol being the most effective. The study indicated that stage I and II follicles are less sensitive to chilling than stage III follicles, and that early stage zebrafish ovarian follicles may be better candidates for cryopreservation.

Cryopreservation offers a viable alternative for maintaining active breeding colonies while safeguarding the genetic integrity of scientifically valuable strains. The ability to generate banks of cryopreserved sperm, oocytes and embryos that retain full viability following recovery from the frozen state would be the most powerful facilitating tool in species conservation and commercial applications such as aquaculture. The creation of gamete cryobanks allows the storage of genetic materials of targeted species for almost unlimited time periods.

One of the obstacles affecting zebrafish oocytes cryopreservation is their high chilling sensitivity which was shown to increase rapidly at subzero temperatures in studies carried out using late stage large oocytes [15]. Five main stages of zebrafish oocyte development are recognized [42]: stage I (primary growth stage), stage II (cortical alveoli stage), stage III (vitellogenic stage), stage IV (maturation stage), stage V (mature eggs). Each developmental stage has specific characteristics of membrane composition, protein content, lipid distribution, and organelle organization [20,22], and the nature of chilling injury may vary from stage to stage. It is therefore, important to assess chilling sensitivity of oocytes at different developmental stages [15]. Previous studies on zebrafish oocyte chilling sensitivity were only carried out with oocytes at later developmental stages (stage III to stage V oocytes) and the study showed that these oocytes are very sensitive to chilling and their survival at low temperature depends on the developmental stage, exposure temperature, exposure period, and individual female [15]. The aim of the present study was to investigate the sensitivity of early stage zebrafish ovarian follicles to subzero temperatures at different developmental stages, and the effectiveness of cryoprotectant in protecting ovarian follicles from chilling injury.

Material and methods

All procedures were approved by the Ethics Committee of the University of Bedfordshire.

Isolation of zebrafish ovarian follicles

Zebrafish were kept in filtered and aerated 40L tanks with a light/dark cycle of 12/12 h and fed twice a day with "TetaMin" (Tetra, Germany) dry fish food and live brine shrimp. To obtain

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ovarian follicles, zebrafish were anaesthesied with a lethal dose of tricaine (0.6 mg/ml for 10 min), fish were decapitated before ovaries were removed. Ovarian cumulus were immediately put into KCl buffer (pH 7.4, 198 mOsm) or 50% Leibovitz L-15 medium (pH 7.4, 166 mOsm) supplement with L-glutamine (Sigma) at 22 °C. KCl buffer was made up with 55 mM KCl; 55 mM K acetate; 1 mM $MgCl_2$; 2 mM $CaCl_2$; 10 mM HEPES; pH was adjusted to 7.4 using 1 M KOH [13]. Ovarian follicles of various sizes (stage I, 0.09–0.14 mm, stage II, 0.26–0.34 mm and stage III, 0.58–0.69 mm in diameter) were separated by using enzymatic treatment (1.6 mg/ml hyaluronidase 10 min at 22 °C). Ovarian follicle developmental stages were determined by light microscopy according to the criteria described by Selman et al. [37]. Three replicas were used for each experiment with 45–75 follicles (stage I 20–30, stage II 15–25 and stage III 10–20) in each replica. The experiments were repeated at least three times.

Chilling sensitivity of zebrafish ovarian follicles at subzero temperatures

To investigate the effect of chilling, ovarian follicles were exposed to −1 °C in KCl buffer or 50% Leibovitz L-15 medium for 4, 8, 16, 24, 48, 72, 96, 120 and 144 h. Ovarian follicles were chilled rapidly by placing them in 2 ml of KCl buffer or 50% Leibovitz L-15 medium in test tubes in a pre-cooled low temperature bath (Grant LTD 20). After chilling, the test tubes containing ovarian follicles were warmed in a water bath at 28 °C for 30 min. Control ovarian follicles were kept at 28 °C. In total, ovarian follicles from more than 100 females were used in these experiments.

Effect of cryoprotectants on ovarian follicle chilling sensitivity

The effect of ovarian follicle survival at subzero temperatures (−1 and −5 °C) in the presence of cryoprotectants were studied. Cryoprotectants were added to prevent ice formation in the bulk solutions. The effect of two cryoprotectants (methanol and dimethy sulfoxide) on ovarian follicle chilling sensitivity were investigated. These cryoprotectants were chosen because they were less toxic to zebrafish ovarian follicles [40]. Cryoprotectants were added and removed in one step. Cryoprotectants were made up in 50% Leibovitz L-15 medium, and ovarian follicles were exposed to 2 M methanol and 2 M DMSO for 30 min at 22 °C. Ovarian follicles in test tubes containing cryoprotectant solutions were placed in a low temperature bath at −1 or −5 °C, where the follicles were allowed to chill. Ovarian follicles were exposed to 2 M methanol or 2 M DMSO for 4, 8, 16, 24, 48, 72, 96, 120, 144, and 168 h at −1 or −5 °C. After chilling, ovarian follicles were warmed in a water bath at 28 °C for 30 min. Control ovarian follicles were kept in L-15 medium at −1 °C or −5 °C. In total, follicles from more than 100 females were used in these experiments.

Viability assessment

Trypan blue (TB) staining

TB staining was used to assess membrane integrity. Ovarian follicles were stained for 3–5 min with 0.2% TB at room temperature (22 °C) immediately after warming. Unstained Ovarian follicles were considered viable, while ovarian follicles stained blue were considered non-viable (Fig. 1). Total ovarian follicle and viable ovarian follicle (not stained) counts were carried out under the microscope.

In vitro culture of ovarian follicles

Ovarian follicles were chilled in 50% L-15 medium, 2 M methanol or 2 M DMSO at −1 °C for 4 h. After chilling, stage I, II and III ovarian follicles were transferred to fresh 50% L-15 medium. Ovarian follicles vary in size (stage I, 0.09–0.14 mm, stage II, 0.26–0.34 mm and stage III, 0.58–0.69 mm in diameter). Stage I, II and III follicles were washed twice in 50% L-15 medium before culturing and twenty ovarian follicles were individually cultured in culture plate. For assessing stage I and II ovarian follicle viability, follicles were cultured using a newly developed method in our lab: follicles were cultured for 24 h in 90% L-15 medium (pH 9.0, 280 mOsm) supplemented with 10 IU/ml human chorionic gonadotropin (hCG; Sigma) in 24-well plates at 28 °C. It has been reported that hCG can activate *follicle-stimulating hormone* (FSH) and the expression of FSH receptor is associated with follicle growth. FSH receptors have been reported to appear in the oocytes of primary follicles during follicular development and persist up to the preovulatory stage [18,28]. For assessing stage III ovarian follicle viability, stage III follicles were incubated for 24 h in 50% L-15 medium supplemented with 0.1 mg/ml 17α, 20β-dihydroxy-4-pregnen-3-one (DHP; Sigma) at 28 °C [15]. After culturing, the diameters of stage I and II ovarian follicles were measured with an ocular micrometer. Stage III follicles were also measured in size followed by observation of germinal vesicle breakdown (GVBD). GVBD occurs during oocyte maturation, at this stage the nucleus migrates to periphery, nuclear envelope breaks down and at the same time the follicle became translucent. The translucent appearance of oocyte cytoplasm after incubation *in vitro* was used as the indication of viable oocytes that successful went through GVBD process [37]. Three replicas were used for each experiment. The experiments were repeated at least three times.

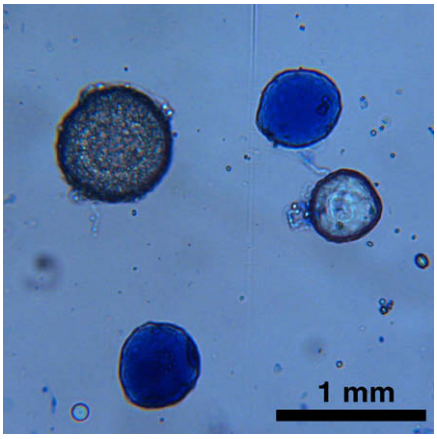

Fig. 1. Stained and non-stained ovarian follicles after TB staining. Unstained ovarian follicles were considered viable while ovarian follicles stained blue were considered non-viable. Scale bar = 1 mm.

Statistical analysis

Statistical analysis was carried out using SPSS (Version 12.01). One-Sample Kolmogorov–Smirnov test was used to insure the data was normally distributed. One-way ANOVA was then 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. In this case, the homogeneity of variance was also tested using Levene's Test. All data were expressed as means ± SEM across the three replicas and *p* values of less than 0.05 were considered to be significant.

Results

Effect of temperature and exposure period

Survival of stage I, II and III follicles after exposure in L-15 medium or KCl buffer to −1 and 28 °C for up to 144 h are