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Development of cryopreservation protocols for early stage zebrafish (*Danio rerio*) ovarian follicles using controlled slow cooling

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Abstract

Cryopreservation of germplasm of aquatic species offers many benefits to the fields of aquaculture, conservation and biomedicine. Although successful fish sperm cryopreservation has been achieved with many species, there has been no report of successful cryopreservation of fish embryos and late stage oocytes which are large, chilling sensitive and have low membrane permeability. In the present study, cryopreservation of early stage zebrafish ovarian follicles was studied for the first time using controlled slow freezing. The effect of cryoprotectant, freezing medium, cooling rate, method for cryoprotectant removal, post-thaw incubation time and ovarian follicle developmental stage were investigated. Stages I and II ovarian follicles were frozen in 4 M methanol and 3 M DMSO in either L-15 medium or KCl buffer. Ovarian follicle viability was assessed using trypan blue, FDA + PI staining and ADP/ATP assay. The results showed that KCl buffer was more beneficial than L-15 medium, methanol was more effective than DMSO, optimum cooling rates were 2–4 °C/min, stepwise removal of cryoprotectant improved ovarian follicle viability significantly and stage I ovarian follicles were more sensitive to freezing. The results also showed that FDA + PI staining and ADP/ATP assay were more sensitive than TB staining. The highest follicle viabilities after post-thaw incubation for 2 h obtained with FDA + PI staining were 50.7 ± 4.0% although ADP/ATP ratios of the cryopreserved follicles were significantly increased indicating increased cell damage. Studies are currently being carried out on *in vitro* maturation of these cryopreserved ovarian follicles.

Keywords: Zebrafish ovarian follicle; Cryopreservation; Controlled slow cooling; ADP/ATP ratio; FDA + PI; Trypan blue

1. Introduction

Cryopreservation of fish gametes has been studied extensively in the last three decades and successful cryopreservation of the spermatozoa from many species has been achieved including salmonid, cyprinids, silurids and acipenseridae [1–3]. Cryopreservation of fish embryos has not been successful mainly due to their low membrane permeability and chilling sensitivity [4,5]. Systematic studies on cryopreservation of fish oocytes have only recently been undertaken [6]. The ability to preserve both maternal and paternal gametes would provide a reliable source of fish genetic material for scientific and aquaculture purposes as well as for conservation of biodiversity. Cryopreservation of oocytes offers several advantages when compared to fish embryos, such as their smaller size, and the absence of a fully formed chorion that may render them more permeable to water and solutes. However, these follicles will need to undergo *in vitro* maturation, ovulation and

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fertilization after cryogenic storage. Development of cryopreservation procedures for early stage ovarian follicles would need to be companied by the development of *in vitro* techniques for follicle vitellogenesis after freeze-thawing.

Cryopreservation of stage III zebrafish oocytes has been studied in our laboratory [6] and the results showed that although oocytes viability obtained immediately after freeze-thawing was relatively high ($88.0 \pm 1.7\%$) using TB staining, oocytes viability decreased to $29.5 \pm 5.1\%$ after 2 h incubation at 22 °C. The results also showed that the ATP level in oocytes decreased significantly after thawing and all oocytes became translucent. Early stage oocytes (ovarian follicles) are smaller in size resulting in higher surface to volume ratios and therefore may be more permeable to water and solute [7]. Early stage zebrafish ovarian follicles have also been shown to be less sensitive to chilling than late stage ovarian follicles [8], they are therefore investigated. In the present study, two different cryopreservation media, the effect of cryoprotectant, ovarian follicle developmental stage, cooling rate, post-thaw incubation time and methods for ovarian follicle viability assessment after controlled slow freezing were investigated.

2. Materials and methods

Zebrafish was kept in filtered and aerated 40 L tanks with a light/dark cycle of 12/12 h and fed twice a day with ''TetaMin'' (Tetra, Germany) dry fish food and fresh brine shrimp. Experiments were conducted with stage I (primary growth, 90–150 μm) and stage II (cortical alveolus, 200–350 μm) ovarian follicles. To obtain ovarian follicles, zebrafish were anaesthetised with a lethal dose of tricaine (0.6 mg/ml) and then decapitated. Ovaries were removed after decapitation and follicles were put either in 50% L-15 medium supplemented with L-glutamine (Sigma) or KCl buffer immediately at 22 °C. The composition of KCl buffer is: 55 mM KCl; 55 mM $C_2H_3O_2K$; 1 mM $MgCl_2$; 2 mM $CaCl_2$; 10 mM HEPES; pH adjusted to 7.4 by 1 M KOH [6]. Stages I and II ovarian follicles were separated by using enzymatic treatment. The ovaries was immersed in 1.6 mg/ml hyaluronidase (made up in KCl buffer or 50% L-15 medium) at 22 °C for 10 min immediately after removal the ovaries. Ovarian follicles were separated by repeated gentle pipetting to remove the interstitial cells and the ovaries were separated into single follicles [6]. After isolation, ovarian follicles was washed three times in KCl buffer or 50% L-15 medium. Stages I and II ovarian follicles were selected based on their size (stage I, 90–150 μm and stage II, 200–350 μm) and randomly distributed in wells of 6-well plates in either KCl buffer or 50% L-15 medium. Ovarian follicle developmental stages were examined under light microscope according to the criteria described by Selman et al. [9]. Zebrafish oocytes are larger when compare with mammalian oocytes and filled with membrane-bound yolk bodies and covered externally by a single layer of follicles cells (granulosa) overlaid with a vascularised connective tissue compartment or theca [9]. Stage I (primary growth) ovarian follicles are transparent and stage II (cortical alveolar) ovarian follicles are translucent (Fig. 1). Three replicas were used for each experiment with 35–55 follicles (stage I 20–30 and stage II 15–25) in each replica. The experiments were repeated at least three times.

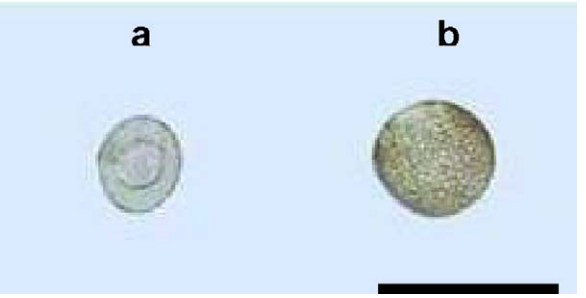

Fig. 1. Developmental stages of zebrafish ovarian follicles. (a) Primary growth stage (stage I), (b) early cortical alveolar stage (stage II). Stage numbers are marked above the relevant follicles. Scale bar = 1 mm.

2.1. Cryopreservation procedures

The no observed effect concentrations (NOECs) for methanol and DMSO for stages I and II zebrafish ovarian follicles were identified as 4 M and 3 M, respectively in a previous study [10]. The NOECs are the lowest concentrations used in the experiments (4 M methanol and 3 M DMSO) which had no effect on follicle viability statistically and when compared to controls. 4 M methanol and 3 M DMSO were therefore used in the controlled slow cooling experiments. Cryoprotectant solutions were made up in L-15 medium or KCl buffer. Ovarian follicles were exposed to cryoprotectant solutions for 30 min at 22 °C and then were loaded into 0.25 ml plastic straws before placing in a programmable cooler (Planer KRYO 550). Ovarian follicles incubated in cryoprotectant-free L-15 medium or KCl buffer were used as controls. The following cooling protocols were used: cooling at 2 °C/min from 20 °C to seeding temperature (−12.5 °C for 4 M