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Cryobiological properties of immature zebrafish oocytes assessed by their ability to be fertilized and develop into hatching embryos ☆

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ABSTRACT

As a step to develop a cryopreservation method for zebrafish oocytes, we investigated the cryobiological properties of immature oocytes at stage III by examining their ability to mature and to develop into hatching embryos after fertilization. When oocytes were chilled at −5 °C for 30 min, the maturation rate decreased, but the rates of fertilization and hatching were not significantly different from those of controls. When oocytes were exposed to hypotonic solutions for 60 min at 25 °C, the rates of maturation, fertilization, and hatching decreased in a solution with 0.16 Osm/kg or below. When oocytes were exposed to hypertonic solutions (containing sucrose) at 25 °C for 30 min, the maturation rate decreased in solution with 0.51 Osm/kg, whereas the hatching rate decreased with lower osmolality (0.40 Osm/kg). In an experiment on the toxicity of cryoprotectants (~10%, at 25 °C), it was found that glycerol and ethylene glycol were toxic both by the assessment of maturation and hatching. Propylene glycol, DMSO and methanol were less toxic by the assessment of maturation, but were found to be toxic by the assessment of hatching. Methanol was the least toxic, but it was less effective to make a solution vitrify than propylene glycol. Therefore, a portion of methanol was replaced with propylene glycol. The replacement increased the toxicity, but could be effective to reduce chilling injury at −5 °C. These results clarified the sensitivity of immature oocytes to various cryobiological properties accurately, which will be useful for realizing cryopreservation of zebrafish oocytes.

Introduction

Cryopreservation of embryos is useful for the management of various stocks of model organisms and has been used in several mammals. In fish, however, a reproducible method for cryopreservation of embryos has not yet been reported.

Since the 1990's, many studies have been conducted on the cryobiological properties of zebrafish (*Danio rerio*) embryos [7,9,10,30,31]. However, the cryopreservation of zebrafish embryos has not succeeded, probably because they have a large volume, a large amount of egg-yolk and a thick chorion, and form complex compartments including a multinucleated yolk syncytial layer which is a barrier to permeation of cryoprotectants [7,8,20].

An alternative strategy is the cryopreservation of oocytes, because fish spermatozoa can be cryopreserved and oocytes can be easily fertilized by cryopreserved sperm [28]. Fish oocytes are constituted from a single compartment and do not have permeability barriers such as a multinucleated yolk syncytial layer. In fish, therefore, oocytes appear to have advantages over embryos for cryopreservation. In zebrafish [21,22,32] and in medaka (*Oryzias latipes*) [26], it has been reported that the permeability of immature oocytes to water and cryoprotectants is markedly higher than that of mature oocytes. Therefore, long-term preservation of zebrafish variants could be realized if the cryopreservation of immature oocytes becomes possible.

To help cells survive after cryopreservation, it is essential to prevent the formation of intracellular ice. To prevent this, the cytoplasm must be concentrated enough, usually with the aid of a cell-permeating cryoprotectant. However, cryoprotectants can injure cells via their chemical toxicity and by hypotonic stress during removal of the permeated cryoprotectant after warming. In addition, cells are at risk of injury due to chilling during cooling and by hypertonic stress during concentration of the cytoplasm by dehydration. All of these obstacles must be circumvented for successful cryopreservation of cells.

In immature zebrafish oocytes at stage III, Zhang and Rawson's group has already examined the sensitivity of oocytes to chilling, hypertonic stress, and cryoprotectant toxicity [12,19]. However, they assessed the viability of oocytes by trypan blue (TB) staining

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and the nuclear maturation. In mammals, it has been recognized that the nuclear maturation of oocytes does not guarantee their ability to be fertilized and develop to term, and that the maturation of the cytoplasm is essential to evaluate the viability [6]. To assess the viability of matured zebrafish oocytes accurately, it is necessary to examine their ability to be fertilized and develop into hatching embryos after insemination.

Until recently, it was not possible to mature immature zebrafish oocytes normally *in vitro*. In a previous study, however, we developed a reliable *in vitro* maturation method for immature zebrafish oocytes at late stage III, before the LH surge, by which they can be fertilized and develop into hatching embryos [23].

To develop a cryopreservation method for immature zebrafish oocytes, it will be important to clarify their cryobiological properties accurately by assessing their ability to be fertilized and develop into hatching embryos. The present study examined the sensitivity of immature zebrafish oocytes to chilling, to hypotonic stress, hypertonic stress, and the toxicity of cryoprotectants using a reliable method.

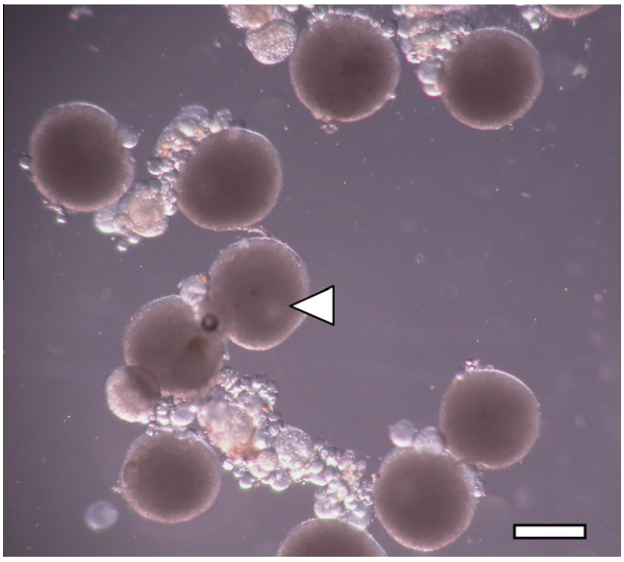

Fig. 1. Immature zebrafish oocytes at late stage III used in the present study. The open triangle shows the germinal vesicle and the open bar indicates 0.5 mm.

Materials and methods

Collection of immature zebrafish oocytes at stage III

Mature zebrafish were purchased from a local fish dealer. They were maintained in aerated and temperature-regulated (28 °C) green water in 60-liter aquaria under 14-h light and 10-h dark periods. Green water is transparent yellowish green color water including plant plankton, but not moss, which is suitable for fish breeding. Fish were fed 3–5 times a day with Tetramin dry flake fish food (Tetra, Melle, Germany). To obtain immature oocytes at late stage III (stage III), female zebrafish with fully grown bellies at 0–3 h before the end of the light period were decapitated under anesthesia with 0.2 mg/ml tricaine in distilled water (tricaine solution). To handle oocytes, 90% Leibovitz L-15 medium containing 0.5% bovine serum albumin (BSA) and 100 μg/ml gentamycin was used. The pH of this medium was adjusted to 9.0 with NaOH, and the medium was referred to as "modified Leibovitz medium". This solution was isotonic (0.29 Osm/kg) [23]. Ovaries were collected and placed in 4 ml of modified Leibovitz medium at 25 °C in a culture dish (35 × 10 mm) [23]. Follicles were separated manually with fine needles, and follicles of good quality were collected; such follicles were separated easily because they were tough but not fragile. Follicles including an oocyte at stage III vary in size (0.34–0.69 mm). In this study, follicles 0.65–0.69 mm in diameter having an oocyte with a tough and dark ooplasm, a distinct cell outline, and a distinct germinal vesicle (visible under transmitted light) were collected and used as immature oocytes at late stage III (Fig. 1). In each experiment, oocytes derived from one female were randomly distributed to experimental groups (10–15 oocytes each), and each experimental group was derived from 4 to 8 replicates.

Sensitivity of immature zebrafish oocytes to chilling, hypotonic stress, hypertonic stress, and cryoprotectant toxicity

To examine the sensitivity to chilling, oocytes in modified Leibovitz medium at 25 °C were pipetted into 4 ml of the same medium precooled at 5, 0 or −5 °C and kept at these temperatures for 30 min. As a control, oocytes were kept at 25 °C for 30 min.

To examine the sensitivity to hypotonic stress, oocytes were suspended in 75%, 60%, 50% or 40% modified Leibovitz medium at 25 °C for 60 min. The solutions were modified Leibovitz medium diluted with water to 75/90, 60/90, 50/90, and 40/90, respectively. The osmolality of the solutions were 0.24, 0.19, 0.16, and 0.13 Osm/kg, respectively. As a control, oocytes were kept in modified Leibovitz medium (0.29 Osm/kg).

To examine the sensitivity to hypertonic stress, oocytes were suspended in modified Leibovitz medium containing various concentrations of sucrose (0.05, 0.10, 0.15, 0.20 or 0.30 M) at 25 °C for 30 min. The osmolalities of the solutions were 0.34, 0.40, 0.45, 0.51, or 0.63 Osm/kg, respectively. As a control, oocytes were kept in modified Leibovitz medium without sucrose (0.29 Osm/kg).

To examine the sensitivity to the chemical toxicity of cryoprotectants, oocytes were suspended in modified Leibovitz medium containing a low concentration of a cryoprotectant (5% v/v methanol, 8% v/v ethylene glycol, 10% v/v glycerol, 9.5% v/v DMSO, or 10% v/v propylene glycol) at 25 °C for 30 min; the concentration was close to the one used in the slow-freezing of mammalian embryos, and was varied slightly to prepare solutions with similar osmolalities (1.55–1.61 Osm/kg). In addition, modified Leibovitz medium containing 10% (v/v) methanol was used.

As a preliminary experiment to examine the sensitivity of oocytes to the toxicity of mixtures of methanol and propylene glycol, the concentration of the cryoprotectants required to make the solution vitrify was examined. Three microliter of distilled water containing various proportions of methanol + propylene glycol was placed on a cryotop (Kitazato Co., Fujinomiya, Japan) and was directly immersed in liquid nitrogen. The proportions (v/v) of methanol + propylene glycol were as follows; 0 + 22%, 0 + 23%, 0 + 24%, 12 + 15%, 13 + 15%, 14 + 15%, 18 + 10%, 19% + 10%, 20 + 10%, 25 + 5%, 26 + 5%, 27 + 5%, 31 + 0%, 32 + 0%, and 33 + 0%. The appearance of the solution was observed to examine whether it became opaque (crystallized) or remained transparent (vitrified).

To examine the sensitivity to the chemical toxicity of the mixture of methanol and propylene glycol, oocytes were suspended in modified Leibovitz medium containing methanol + propylene glycol (12.5 + 0%, 10 + 2.5%, 7.5 + 5%, 5 + 7.5%, 0 + 10%, v/v) for 30 min at 25 °C.

To examine the effect of the mixture of methanol and propylene glycol on chilling injury, immature oocytes were first equilibrated with modified Leibovitz media containing methanol + propylene glycol (12.5 + 0%, 10 + 2.5%, 7.5 + 5%, 5 + 7.5%, 0 + 10%, v/v) for 30 min at 25 °C, pipetted into the same media precooled at −5 °C, and kept there for 30 min.

The osmolalities of media used in this study are shown in Table 1.