A study on the vitrification of stage III zebrafish (Danio rerio) ovarian follicles

Leandro Cesar Godoy a,c,⁎, Danilo P. Streit Jr. b, Tiziana Zampolla c, Adriana Bos-Mikich b, Tiantian Zhang d

a Aquaculture Graduate Program, Nilton Lins University, Manaus 69058-040, Brazil
b Aquam Research Group, Department of Animal Science, Federal University of Rio Grande do Sul, Porto Alegre 91540-000, Brazil
c Institute of Biomedical and Environmental Science and Technology, University of Bedfordshire, Luton, Bedfordshire LU2 8DL, UK
d School of Applied Sciences, Bournemouth University, Dorset BH12 5BB, UK

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A B S T R A C T

Attempts to cryopreserve fish embryos have been conducted over the past three decades, nevertheless successful cryopreservation protocol for long-term storage still remains elusive. Fish oocytes offer some advantages when compared to embryos, which may help in improving the chances of cryopreservation. In the present study, a series of cryo-solutions were designed and tested for their vitrifying ability using different devices (0.25 ml plastic straw, vitrification block and fibreplug™). Toxicity of vitrification solutions was evaluated by assessing follicle membrane integrity with trypan blue staining. In addition, the effect of vitrification protocol on stage III zebrafish ovarian follicles was investigated by measuring the cytoplasmic ATP content and the mitochondrial distribution and activity using JC-1 probe and confocal microscopy. After vitrification, follicles showed membrane integrity of 59.9 ± 18.4% when fibreplug and V₂ (1.5 M methanol + 4.5 M propylene glycol) solution were employed. When vitrified in V₂ (1.5 M methanol + 5.5 M Me₂SO) the membrane integrity decreased to 42.0 ± 21.0%. It was observed that follicles located in the middle of the fragments were more protected from injuries and some of them showed good morphological appearance 2 h post-warming. Mitochondria integrity of granulosa cells layer was clearly damaged by the vitrification protocol and ATP level in the follicles declined significantly after warming. Vitrification of zebrafish follicles in ovarian tissue fragments and its effect at sub-cellular level is reported here for the first time. Information gained from this study will help in guiding development of optimal protocol for cryopreservation of fish oocytes.

Introduction

The importance of preserving animal genetic resources for wildlife conservation as well as animal reproduction has become more and more evident in recent years. Fish stocks are globally threatened mainly due to overfishing and environmental pollution [17,27]. Aquaculture has consequently become an important activity for high quality food production in order to meet the growing protein demand globally, and at the same time protects the wild fish populations from being overexploited [9]. Conservation programmes for wild and cultured fish have been established worldwide in order to protect them from becoming extinct [9]. Cryopreservation of aquatic germplasm brings the possibility of preserving the genome of endangered species, increasing the representation of genetically valuable animals for farming purposes and avoiding genetic losses through diseases and catastrophes [3,9].

For majority of animal species, cryopreservation of embryos at any developmental stages still represents major challenges. Whereas, according to Saragusty and Arav [29], thousands of offspring were born following the transfer of frozen-thawed embryos in humans, cattle and mice, success is very limited in many others, even closely related species. In fish, successful cryopreservation of semen from many species including salmonids, cyprinids, cichlids, silurids, acipenserids, anostomids and characids has been well documented [4,6,7,37,38] and cryopreserved semen has been used for reproduction of many wild and farmed species [11]. Attempts to cryopreserve fish embryos have been conducted over the past three decades, nevertheless successful cryopreservation protocol for long-term storage still remains elusive [5,8,14,15,47,48].

Fish embryos are multi-compartmentalized, and there are several barriers that have been identified as obstacles for successful cryopreservation: their high yolk content, large size, low permeability of the membranes and their high sensitivity to chilling [26].
It has recently been reported [39] that the use of oocytes may offer some advantages when compared to fish embryos, mainly due to the absence of a fully formed chorion, their smaller size resulting in higher surface-to-volume ratio and higher membrane permeability, therefore improving the chances of successful cryopreservation.

Although several studies have been carried out on fish oocytes cryopreservation [16,21,23,24,39], all of them used controlled slow cooling protocol and success remains elusive as for embryos. Only one study, carried out by Guan et al. [12] reported the use of vitrification for isolated stage III zebrafish ovarian follicles, however the ovarian follicles were severely damaged during the process.

Despite the successful use of vitrification technique for oocytes cryopreservation in humans [19] and some domestic mammals [41], very limited studies on vitrification of fish oocytes has been carried out to date. Vitrification is an ice-free cryopreservation method using high concentrations of cryoprotectants (CPAs) and ultra-rapid cooling rates [25] which offers advantages that may contribute to overcome some of the difficulties associated with the slow cooling protocols.

The present study aimed to develop a cryopreservation protocol for stage III zebrafish ovarian follicles in tissue fragments using vitrification. A series of cryo-solutions were designed and tested for their vitrifying ability employing different vitrification devices. Toxicity of the vitrification solutions was evaluated by assessing ovarian follicle membrane integrity (immature oocytes at late stage III) were used, based on criteria described by Selman et al. [32]. In each experiment, one study, carried out by Guan et al. [12] reported the use of vitrification for isolated stage III zebrafish ovarian follicles, however the ovarian follicles were severely damaged during the process.

To obtain ovarian follicles, female zebrafish with fully grown ovaries were anesthetized with a lethal dose of tricaine (22 °C) by suction with a 5 ml syringe. The loaded straws were plunged directly into liquid nitrogen, held for 1 min and then the warming was performed by plunging the straws into a water bath maintained at 28 °C.

Vitrification Block™: by using a pipette, a 5 μl droplet was transferred to the hook at the end of a custom designed fibre named Fibreplug™ (CryoLogic Ltd, Melbourne, Australia). The vitrification block was chilled to liquid nitrogen temperature and the fibreplug holding a microdrop was placed on the chilled surface directly, where it was held for 1 min. Warming was carried out by placing the droplet on the fibreplug into pre-warmed L-15 medium at 28 °C.

Fibreplug™: the fibreplug (CryoLogic Ltd, Melbourne, Australia) holding a 5 μl droplet was plunged directly into liquid nitrogen, held for 1 min, and the warming procedure was performed as detailed for the vitrification block.

The transparent glassy appearance during cooling and warming was used to identify vitrified solution, and a milky appearance was used to identify crystallization or devitrification. Six replicates were used for each cryoprotectant concentration for each vitrification device tested, and the experiments were repeated three times.

**Vitrifying ability of vitrification solutions**

Twenty-four vitrification solutions (VS) containing combinations of cryoprotectants at different concentrations were prepared in 90% L-15 medium for testing. Vitrifying ability of the single cryoprotectant solutions was taken into account when choosing the combinations to formulate the vitrification solutions (Table 2).

Methanol was used at 1.5 M based on our previous studies which showed no negative effect on zebrafish ovarian follicles viability after 30 min incubation [unpublished results]. Furthermore, sucrose and glucose were added as non-permeating CPAs in order to increase the solution’s viscosity and therefore, aiding vitrification.

The transparent glassy appearance during cooling and warming was also used to identify vitrified solutions. Six replicates were used for each VS tested for each vitrification device, and the experiments were repeated three times.

**Toxicity of vitrification solutions**

Following isolation, ovarian tissue fragments (3 × 2 × 1 mm) containing approximately 15 stage III follicles were randomly distributed in 6-well plates (3 fragments in each well). First, follicles were exposed to L-15 medium containing 1.5 M methanol for 30 min at room temperature. Subsequently, follicles were exposed to vitrification solutions for 3 min in a stepwise manner: 1.5 min at 50% of the final VS concentration + 1.5 min at 100% VS concentration. Afterwards the CPAs were gradually removed in 3 steps.

**Vitrifying tendency of single cryoprotectant**

Leibovitz L-15 was chosen as the base medium for preparing all cryoprotectant solutions tested in our experiment, based on previous studies carried out by Guan et al. [12] and by Seki et al. [30]. To make the medium, Leibovitz L-15 (Sigma) was diluted to 90% and the pH was adjusted to 9.0 using NaOH.

Vitrifying tendency of methanol, ethanol, dimethyl sulfoxide (Me₂SO), propylene glycol and ethylene glycol solutions made up in L-15 medium was tested at the following range of concentrations (Table 1).

Cryo-solutions were tested for vitrification by using three different devices:

- **Plastic straw**: 0.25 ml plastic straws (IMV Technologies, L’Aigle, France; reference 005565) were filled at room temperature (22 °C) by suction with a 5 ml syringe. The loaded straws were plunged directly into liquid nitrogen, held for 1 min and then the warming was performed by plunging the straws into a water bath maintained at 28 °C.

- **Fibreplug™**: the fibreplug (CryoLogic Ltd, Melbourne, Australia) holding a 5 μl droplet was plunged directly into liquid nitrogen, held for 1 min, and the warming procedure was performed as detailed for the vitrification block.

- **Vitrification Block™**: by using a pipette, a 5 μl droplet was transferred to the hook at the end of a custom designed fibre named Fibreplug™ (CryoLogic Ltd, Melbourne, Australia). The vitrification block was chilled to liquid nitrogen temperature and the fibreplug holding a microdrop was placed on the chilled surface directly, where it was held for 1 min. Warming was carried out by placing the droplet on the fibreplug into pre-warmed L-15 medium at 28 °C.

**Materials and methods**

**Fish care and ovarian follicles collection**

Zebrafish were maintained in aerated and temperature-regulated (27 °C) water in 40 L aquarium under a light/dark photoperiod of 12/12 h. Fish were fed twice a day with TetraMin® dry flake food (Tetra, Germany) and live brine shrimp (Artemia franciscana) nauplii.

To obtain ovarian follicles, female zebrafish with fully grown ovaries were anaesthetized with a lethal dose of tricaine (0.6 mg/ml) followed by decapitation. Ovaries were immediately removed after decapitation and were gently placed into a Petri dish containing 90% Leibovitz L-15 medium (pH 9.0) supplemented with L-glutamine (Sigma). Ovarian tissue fragments containing stage III ovarian follicles were obtained manually by using forceps and fine needles under a dissecting microscope.

In this study, follicles of 0.50–0.69 mm in diameter, having an intrafollicular oocyte with a dark ooplasm and a well-marked cell outline (immature oocytes at late stage III) were used, based on the criteria described by Selman et al. [32]. In each experiment, ovarian tissue fragments obtained from three females were randomly distributed to experimental groups. All procedures reported here were approved by the Ethics Committee of iBEST.

<table>
<thead>
<tr>
<th>Cryoprotectant</th>
<th>Concentration (mol/L)</th>
</tr>
</thead>
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<tr>
<td>Methanol</td>
<td>8.0 8.5 9.0 9.5 10.0 10.5 11.0</td>
</tr>
<tr>
<td>Ethanol</td>
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</tr>
<tr>
<td>Dimethyl sulfoxide</td>
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<tr>
<td>Propylene glycol</td>
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</tr>
<tr>
<td>Ethylene glycol</td>
<td>4.0 4.5 5.0 5.5 6.0 6.5 7.0</td>
</tr>
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Table 2

<table>
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<th>MT</th>
<th>PG</th>
<th>Me$_2$SO</th>
<th>EG</th>
<th>SU</th>
<th>GLU</th>
</tr>
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<tbody>
<tr>
<td>V1</td>
<td>1.5</td>
<td>4.0</td>
<td>–</td>
<td>–</td>
<td>–</td>
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</tr>
<tr>
<td>V2</td>
<td>1.5</td>
<td>–</td>
<td>5.5</td>
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<td>–</td>
</tr>
<tr>
<td>V3</td>
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<td>–</td>
<td>6.5</td>
<td>–</td>
<td>–</td>
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<tr>
<td>V4</td>
<td>1.5</td>
<td>3.0</td>
<td>–</td>
<td>–</td>
<td>0.25</td>
<td>–</td>
</tr>
<tr>
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<td>1.5</td>
<td>–</td>
<td>4.5</td>
<td>–</td>
<td>0.25</td>
<td>–</td>
</tr>
<tr>
<td>V6</td>
<td>1.5</td>
<td>–</td>
<td>–</td>
<td>5.5</td>
<td>0.25</td>
<td>–</td>
</tr>
<tr>
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<td>1.5</td>
<td>3.0</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>0.25</td>
</tr>
<tr>
<td>V8</td>
<td>1.5</td>
<td>–</td>
<td>4.5</td>
<td>–</td>
<td>–</td>
<td>0.25</td>
</tr>
<tr>
<td>V9</td>
<td>1.5</td>
<td>–</td>
<td>–</td>
<td>5.5</td>
<td>–</td>
<td>0.50</td>
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<tr>
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<td>–</td>
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<td>–</td>
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<td>–</td>
</tr>
<tr>
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<td>3.0</td>
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<td>–</td>
<td>–</td>
<td>0.50</td>
</tr>
<tr>
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<td>–</td>
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<td>–</td>
<td>–</td>
<td>0.50</td>
</tr>
<tr>
<td>V15</td>
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<td>–</td>
<td>5.5</td>
<td>–</td>
<td>0.50</td>
</tr>
<tr>
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<td>4.5</td>
<td>–</td>
<td>–</td>
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<td>1.5</td>
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<td>–</td>
<td>–</td>
<td>0.50</td>
<td>–</td>
</tr>
<tr>
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<td>5.0</td>
<td>–</td>
<td>0.50</td>
<td>–</td>
</tr>
<tr>
<td>V21</td>
<td>1.5</td>
<td>–</td>
<td>–</td>
<td>6.0</td>
<td>0.50</td>
<td>–</td>
</tr>
<tr>
<td>V22</td>
<td>1.5</td>
<td>3.5</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>0.50</td>
</tr>
<tr>
<td>V23</td>
<td>1.5</td>
<td>–</td>
<td>5.0</td>
<td>–</td>
<td>–</td>
<td>0.50</td>
</tr>
<tr>
<td>V24</td>
<td>1.5</td>
<td>–</td>
<td>–</td>
<td>6.0</td>
<td>0.50</td>
<td>–</td>
</tr>
</tbody>
</table>

MT, methanol; PG, propylene glycol, Me$_2$SO, dimethyl sulfoxide; ET, ethylene glycol; SU, sucrose; GLU, glucose.

(2 min for each step), and ovarian follicles were washed three times in L-15 medium. Control ovarian follicles were kept in L-15 medium for 30 min at room temperature. In order to test the ovarian follicles viability after exposure to VS, trypan blue (TB) staining medium for 30 min at room temperature. Subsequently the follicles were washed three times with L-15 medium. Control ovarian follicles were kept in L-15 (2 min for each step), and ovarian follicles were washed three times in 90% L-15 medium. Those unstained were considered as membrane intact ovarian follicles, while the blue stained ones were considered as membrane damaged follicles [24,46]. Total and membrane intact ovarian follicles counts were carried out under a light microscope.

**Assessing ovarian follicles viability after vitrification**

**Membrane integrity assay**

Immediately after warming, ovarian follicles membrane integrity was assessed by using trypan blue (TB) staining. To carry out the TB assay, a 0.4% TB stock solution (Sigma–Aldrich, Dorset, UK) was diluted to 0.2% in 90% L-15 medium. Ovarian follicles were stained for 3 min with 0.2% TB solution at room temperature, and then washed three times in 90% L-15 medium. Those unstained were considered as membrane intact ovarian follicles, while the blue stained ones were considered as membrane damaged follicles [24,46]. Total and membrane intact ovarian follicles counts were carried out under a light microscope.
Statistical analysis

Statistical analysis was carried out using the software STATISTICA 6.0 (Statsoft 2001). Homogeneity of variances (Levene’s test) and normality of the data distribution (Kolmogorov–Smirnov test) were tested. When data were normally distributed, comparisons among groups were tested by one-way ANOVA. Where differences were found Tukey’s post hoc test was performed in order to identify which groups differ. For data not normally distributed, comparisons among groups were made by nonparametric Kruskal–Wallis test. Data were expressed as mean ± standard deviation (SD) across the three replicates and P < 0.05 was considered significant.

Results

Vitrifying tendency of single cryoprotectants

The minimum vitrifying concentration of each cryoprotectant is presented in Table 3.

The results showed that methanol vitrified at 10.0 M only when the fibreplug was used. Ethanol did not vitrify at any concentration with any vitrification device tested. Me₃SO vitrified at 5.5 M in both plastic straw and fibreplug. Propylene glycol reached vitrification at 4.0 M in straws and at 5.0 M using fibreplug; and ethylene glycol vitrified at 6.5 M only in straw.

In the present study, the use of the vitrification block did not allow to achieve vitrification with any of the cryo-solutions tested. Based on these results, the vitrification block was not used for subsequent experiments. Likewise, as ethanol did not vitrify with any of the vitrification devices and concentrations tested, it was not used for subsequent experiments.

Vitrifying ability of vitrification solutions

The results from these experiments are presented in Table 4, where each shade represents the appearance of the solution evidenced throughout the experiments. Crystallization of the solution (in light gray) was more frequently recorded when 0.25 ml plastic straw was used. Most of the solutions vitrified during cooling; however devitrification was frequently evidenced during warming (in dark gray). Among the 24 vitrification solutions, three of them remained vitreous (Table 4, in black color) during both cooling and warming procedures. V₁₆, V₂₁ and V₂₇ solutions were therefore selected for toxicity studies.

Toxicity of vitrification solutions

The effect of toxicity of the vitrification solutions on membrane integrity of zebrafish ovarian follicles is shown in Fig. 1. When ovarian follicles were exposed to V₂₁ solution the membrane integrity (77.9 ± 12.9%) did not differ (P > 0.05) from results obtained in the control group (91.0 ± 6.1%). Ovarian follicles exposed to V₁₆ and V₂ showed a decrease (P < 0.05) in membrane integrity compared to the control group.

Viability of zebrafish ovarian follicles after vitrification

Membrane integrity

There was significant difference in membrane integrity of ovarian follicles between the room temperature control group and the vitrified groups (Fig. 2). Ovarian follicles showed membrane integrity of 59.9 ± 18.4% when fibreplug and V₁₆ solution were employed. When ovarian follicles were vitrified in V₂ the membrane integrity decreased to 42.0 ± 21.0%, using fibreplug as vitrification device (P < 0.05). After vitrification in V₂₁ solution using plastic straw the largest decrease in membrane integrity was recorded, with a value of only 2.1 ± 3.6%. Based on these results, V₂₁ solution was not used for the subsequent experiments.

Cytoplasmic ATP concentration in the ovarian follicles

The ATP concentration in the follicles declined significantly (P < 0.05) after vitrification. To make the comparisons clearer we normalised the data considering the ATP measured in the control group as 100% (Fig. 3). Soon after warming, the ATP in the follicles vitrified in V₂ declined to 22.0 ± 4.23%. Likewise, the ATP in ovarian follicles vitrified in V₁₆ dropped to 6.9 ± 0.6% (Fig. 3). Nevertheless, when measured 120 min post-warming the ATP in the ovarian follicles vitrified in V₂ (15.1 ± 2.8%) did not differ (P > 0.05) to the ATP concentration recorded immediately after warming. In contrast, a decrease over time was observed in the follicles vitrified in V₁₆ (3.5 ± 0.7%).

Mitochondrial membrane potential (ΔΨₘ) and distribution

The photomicrographs shown in Fig. 4 are representative examples of ovarian follicles obtained by confocal microscopy after exposure to JC-1 fluorescent probe. JC-1 was unable to penetrate deep inside the oocytes, therefore the fluorescence remained concentrated at the margins of the granulosa cells layer (Fig. 4A, and A₁). Ovarian follicles from the control group displayed a contiguous peripheral aggregation of mitochondria in the granulosa cells that surround the oocytes, with a well-organized distributional arrangement and red fluorescence emission (Fig. 4A, and A₁). Images from the vitrified groups showed a complete loss of mitochondrial structure pattern as well as loss of fluorescence, indicating that the vitrification protocol caused loss of mitochondrial membrane potential (ΔΨₘ) (Fig. 4B and C).

Discussion

Assessment of the minimum concentration of a cryoprotectant required to vitrify is the very first step in designing cryo-solutions to be used for a vitrification protocol.

In this study, the vitrifying ability of cryo-solutions was examined by using five permeating CPAs and three different vitrification devices. The results showed that 0.25 ml plastic straw and fibreplug provided better results than the vitrification block. Whether vitrification occurs is dictated by the composition of the vitrification solution and other factors including the cooling and warming rates [22]. Thus, a solution which vitrifies in one device may form ice crystals when used under other conditions. Vitrification occurs most readily at high cooling and warming rates, and it is possible that the lower cooling rates on vitrification block surface resulted in the crystallization of all tested solutions when this device was employed.
The permeating CPAs used in the present study were chosen based on the previous studies carried out in our laboratory on cryopreservation of zebrafish embryos and oocytes by using controlled slow cooling protocols (2,16,23,24,39,44). Despite the recent report of Anil et al. [2] showing ethanol as a promising CPA to be used in zebrafish ovarian follicles cryopreservation, i.e. less toxic when compared to methanol, it did not vitrify at the maximum concentration (11 M) tested in our study. Thus, ethanol was not included when designing the vitrification solutions.

Methanol is well known for its rapid penetration through cell membranes and low toxicity for fish gametes and embryos [10,11,36]; however at the concentration required to achieve vitrification (10 M) it becomes very toxic. Zampolla et al. [44,46] reported that concentrations of methanol below 2 M do not affect viability of zebrafish ovarian follicles after incubation for 30 min at 22 °C. Therefore, we used 1.5 M methanol as an equilibrating CPA in the vitrification solutions. Among the vitrification solutions tested in 0.25 ml plastic straws, only V21 vitrified during cooling and remained vitreous when warmed. The CPAs concentration of 59.17% (w/v) in this solution, achieved by the combination of two permeating (methanol and ethylene glycol) and a non-permeating (sucrose) cryoprotectant contributed to its vitrification. The combination of two or three permeating CPAs and a non-permeating (normally sugars) cryoprotectant has been shown to be beneficial in increasing viscosity and glass transition temperature (Tg) of solutions, therefore improving the chance of vitrification as well as reducing the toxicity of a CPA. Kuleshova et al. [20] assessed the vitrification properties of ethylene glycol-based solutions by replacing ethylene glycol with an equimolar concentration of six different sugars. Their results showed that the Tg of the solutions rose as the proportions of these sugars in the vitrification solution increased.

The permeating CPAs used in the present study were chosen based on the previous studies carried out in our laboratory on cryo-

### Table 4

<table>
<thead>
<tr>
<th>Vitrification solutions</th>
<th>Vitrifying ability of the 24 vitrification solutions tested by using two different devices.a</th>
</tr>
</thead>
<tbody>
<tr>
<td>Straw</td>
<td>Fibreplug™</td>
</tr>
<tr>
<td>Light gray</td>
<td>Dark gray</td>
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<tr>
<td>Light gray</td>
<td>Dark gray</td>
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</table>

a Each shade in the table represents the appearance of the solution during cooling and warming procedures. Light gray: crystallization resulted in milky appearance of the solution during cooling. Dark gray: vitrification reached during cooling but devitrification was evidenced during warming. Black: vitrification was identified by transparent glassy appearance of the solution during cooling and no ice crystal formation during warming. The composition of each vitrification solution is given in Table 2.

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**Fig. 1.** Membrane integrity of stage III zebrafish ovarian follicles after 30 min at room temperature in 90% L-15 medium containing 1.5 M methanol followed by exposure to vitrification solutions for 3 min in a stepwise manner: 1.5 min [50%] + 1.5 min [100%]. Cryoprotectants were removed in three steps and membrane integrity was assessed by using trypan blue staining. V16 (1.5 M methanol + 4.5 M propylene glycol); V2 (1.5 M methanol + 5.5 M Me2SO); V21 (1.5 M methanol + 6.0 M ethylene glycol + 0.5 M sucrose). Bars labelled with different letters indicate difference (P<0.05) between control and treated groups (mean ± SD).

**Fig. 2.** Membrane integrity of stage III zebrafish ovarian follicles after vitrification in V16, V2 and V21 solutions. Trypan blue staining was used to assess membrane integrity immediately after warming. V16 (1.5 M methanol + 4.5 M propylene glycol); V2 (1.5 M methanol + 5.5 M Me2SO); V21 (1.5 M methanol + 6.0 M ethylene glycol + 0.5 M sucrose). Bars labelled with different letters indicate difference (P<0.05) between control and treated groups (mean ± SD).

**Fig. 3.** ATP concentration in stage III zebrafish ovarian follicles after vitrification. Measurement was made immediately after warming (time zero) and 120 min post-warming. V2 (1.5 M methanol + 5.5 M Me2SO); V16 (1.5 M methanol + 4.5 M propylene glycol). Groups with no common superscript letter differ significantly (P<0.05) from each other (mean ± SD). *Significantly different from control group.
The results from the present study showed that solutions were better vitrified using fibreplug when compared to 0.25 ml plastic straws. It has been shown in the literature that the most effective way for increasing cooling rates is to use the smallest possible volume of cryoprotectant solution in order to establish a direct contact (without any thermal insulating layer) between the solution and the liquid nitrogen [42]. A smaller volume may also offer a special advantage: it prevents heterogeneous ice formation.

In zebrafish, it has been shown that methanol and propylene glycol are less toxic to stage III oocytes than other cryoprotectants, such as ethylene glycol and Me2SO [24,31]. This explains the higher membrane integrity of ovarian follicles after exposure to V16 solution (1.5 M methanol + 4.5 M propylene glycol) when compared to the results recorded for the follicles exposed to V2 (1.5 M methanol + 5.5 M Me2SO). Me2SO at 5.5 M became toxic to stage III zebrafish ovarian follicles. Although ethylene glycol is considered to be the most toxic among the CPAs used in this experiment [43], ovarian follicles exposed to V21 (1.5 M methanol + 6.0 M ethylene glycol + 0.5 M sucrose) displayed the highest membrane integrity of all treated groups. The presence of sucrose may have lowered the toxicity of ethylene glycol and worked as an osmotic buffer stabilizing the follicles membrane and consequently preserved its integrity. Studies have shown that the use of sucrose as non-permeating CPA provides additional protection to membranes from the consequences of dehydration in fish embryos and optimizes the performance of permeable CPAs when used in combination [1,11,15,23,36].

The present study showed that the membrane integrity of ovarian follicles after vitrification, assessed by TB staining, was not preserved when using plastic straws. This result suggests that intracellular ice crystal formation may have taken place during vitrification process. No changes were observed in solution appearance in the straws during both cooling and warming procedures; however, even transparent solutions may contain countless ice nuclei and ice crystals, because the ice crystals only are detectable optically once they become larger than the wavelength of light [33].

The volume of the vitrification solution was minimized when fibreplug was used, increasing the probability of vitrification, which may have contributed to the higher membrane integrity of the ovarian follicles vitrified in V16 and V2.

Guan et al. [12] reported a slightly higher membrane integrity after vitrification of isolated stage III zebrafish ovarian follicles than the results obtained here using ovarian fragments, when assessed by TB staining. However, in the present study the membrane intact follicles found in V16 and V2 remained opaque and morphologically intact, when assessed immediately after warming. These results are different to those described by Guan et al. [12], where zebrafish follicles were observed to become swollen and translucent even during the warming process, with membrane ruptured within the following 10 min. Such phenomenon was also previously reported by Guan et al. [13] using controlled slow cooling protocol and by Isayeva et al. [16] in studies on chilling sensitivity of zebrafish ovarian follicles. In order to obtain more information relating to this phenomenon, we observed ovarian follicles appearance throughout the two hours following warming, under incubation in L-15 medium at room temperature. Thirty minutes after warming most of the follicles started to become semi-translucent and slightly swollen, indicating some changes in the structure of yolk. A translucent appearance of the follicle occurs naturally during its maturation in zebrafish (germinal vesicle breakdown – GVBD) and is associated with the proteolysis of yolk during stage IV. It is possible that the oocyte internal compartments were damaged during vitrification, releasing proteases (e.g. cathepsins) or affecting ion transport mechanisms that eventually change the physical structure of the yolk proteins.
It was observed that the follicles located in the middle of the fragments were more protected from injuries and some of them displayed good appearance (outlined cell membrane and opacity) even two hours after warming. This is a promising finding, however there is clearly a need for further investigation regarding the metabolic status and developmental capability of these follicles.

Although TB staining is a fast and common method for assessing the viability of fish ovarian follicles, it only provides information on the membrane integrity and does not give information on follicle development capability. In order to provide a more accurate assessment of ovarian follicle viability after vitrification, and taking into account that mitochondria of cells are very vulnerable to low temperature injuries [40], measurement of ATP content in the ovarian follicles was performed. We carried out this assay immediately after warming and after 120 min incubation, taking into consideration the latent injury [34]. Results obtained immediately after warming can be misleading because injuries may be latent in character and, while escaping detection during initial tests of vital function, may be manifested later with the passage of time after warming, during which affected cells become altered sufficiently to reflect their earlier undetected or subthreshold injury [34].

While ovarian follicles vitrified in V16 showed higher membrane integrity compared to those vitrified in V2 solution, the ATP assay showed a lower concentration of ATP in the follicles which were vitrified using V16 solution. These results point out that despite 59.9 ± 18.4% of the ovarian follicles vitrified in V16 displayed intact membranes, the integrity of the mitochondria in most follicles was disrupted, explaining their reduced ATP content. The energy status of oocytes is critical for their maturation and ATP level has been suggested to be used as an indicator for the developmental potential [35]. The ATP levels in ovarian follicles determined in our study after vitrification were much higher than those reported by Guan et al. [13] for stage III zebrafish follicles using a controlled slow cooling protocol, either immediately after warming (1.7%) or 2 h later (0.4%).

Use of JC-1 allows both mitochondrial metabolic status and distribution to be determined at the same time. The negative charge established by the mitochondrial membrane potential allows the lipophilic dye, bearing a delocalized positive charge, to enter the mitochondrial matrix where it accumulates [18]. When the critical concentration is exceeded J-aggregates form, resulting in red fluorescence emission [28], which was evidenced in the ovarian follicles. The negative charge of oocytes is critical for their maturation and ATP level has been suggested to be used as an indicator for the developmental potential [35]. The ATP levels in ovarian follicles determined in our study after vitrification were much higher than those reported by Guan et al. [13] for stage III zebrafish follicles using a controlled slow cooling protocol, either immediately after warming (1.7%) or 2 h later (0.4%).

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Results from confocal microscopy were consistent with the data obtained by the ATP assay. The losses in mitochondrial spatial pattern as well as mitochondrial membrane potential (ΔΨm) evidenced that the granulosa cells layer of stage III zebrafish ovarian follicles are particularly sensitive to subzero temperature exposure. Mitochondria integrity of granulosa cells layer was clearly damaged by the vitrification protocol, which explains the significant decline of ATP level in the follicles after warming. These findings show that ATP bioluminescence assay combined with JC-1 staining provides an accurate assessment of ovarian follicles viability after vitrification.

Vitrification of stage III zebrafish follicles in ovarian tissue fragments with detailed cryobiological information at sub-cellular level is reported here for the first time. In this study, cryo-solutions were designed and tested for their vitrifying ability employing different devices. Toxicity of the vitrification solutions was evaluated by assessing ovarian follicle membrane integrity with trypan blue staining and the effect of vitrification protocol on the follicles was investigated by measuring the cytoplasmic ATP level and the mitochondrial distribution and activity using JC-1 molecular probe and confocal microscopy.

Mitochondrial integrity of granulosa cells layer was damaged by the vitrification protocol and ATP level in the follicles declined significantly after warming. Despite cryo-solutions have shown to achieve vitrification throughout the tests, it seems that the ovarian tissue fragments did not vitrify successfully. However, we observed that follicles located in the middle of the fragments were more protected from injuries and some of them showed good morphological appearance 2 h post-warming. In summary, fish ovarian follicles are a complex biological material to cryopreserve and a lot of studies are necessary. We believe that information gained from this study will be very useful to guide further studies and development of a successful protocol for cryopreservation of fish oocytes in the future.

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