

23 Abstract

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

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

46

47 **Introduction**

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

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

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

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

80 **Materials and Methods**

81 **Chemicals**

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

84 **Animals maintenance and ethics statement**

85 Zebrafish (*Danio rerio*) were maintained in filtered and aerated 40 L aquaria at
86 27°C under a 14/10 h (light/dark) photoperiod. Fish were fed four times a day with
87 TetraMin® dry flake fish food (Tetra, Germany).

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

91 **Ovaries collection**

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

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

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

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

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

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

125 Control fresh ovaries were maintained in L-15 medium (pH 9.0) at RT (23-
126 25°C) until staining. Cryopreserved and control follicles were isolated by gentle
127 pipetting in Leibovitz L-15 medium and washed three times in phosphate buffered
128 saline (PBS) (pH 9.0). Five developmental stages were selected: Stage I (primary
129 growth); Stage II (cortical alveolus); Stage III (vitellogenic); Stage IV (maturation) and
130 Stage V (mature) according to Selman et al. [29]. Ovarian follicle viability was assessed
131 by the method described by Jones and Senft [15]. A stock solution of fluorescein
132 diacetate (FDA) was prepared by dissolving 5 mg/mL FDA in acetone. The FDA
133 working solution was prepared by adding 20 μ L of stock solution in 5 mL of PBS. The
134 propidium iodide (PI) solution was prepared by dissolving 1 mg of PI in 50 mL PBS.
135 Ovarian follicles were incubated in drops containing 100 μ L (2 μ g) FDA working
136 solution and 30 μ L (0.6 μ g) PI solution in the dark for 3-4 min at RT. Cell viability
137 assessment was performed on an inverted fluorescence microscope (Opton, TNI-51-
138 IMU). The bright green fluorescent follicles were considered viable (Figure 2A) and the
139 bright red stained follicles were considered non-viable (Figure 2B). Experimental and
140 control groups were composed by at least 100 ovarian follicles in each group, and the
141 experiments were repeated three times.

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

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

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

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

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

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

163 After vitrification/rewarming, Stage I follicles were isolated and classified as
164 described before (Experiment 1) and the follicles were selected according to their
165 diameter (between 90-140 μm). Using an *in vitro* culture protocol based on previously
166 published protocols [1,26,34] with some modifications, the selected follicles were
167 washed three times with L-15 medium 90% (pH 9.0), before being randomly distributed
168 into 96 well plates containing 200 μL of L-15 medium 90% (pH 9.0) supplemented with
169 0.5 mg/mL follicle stimulating hormone (FSH), 20% fetal bovine serum (FBS) and 100

170 $\mu\text{g/mL}$ gentamycin. All follicles, including fresh controls were individually cultured in
171 the wells for 24 h at 28°C. Survival was assessed by vital staining (FDA + PI) after *in*
172 *vitro* culture (IVC). Furthermore, follicular diameter increase was evaluated by
173 measuring diameters of viable follicles (bright green stained follicles), before and after
174 IVC, on an ocular micrometer under an inverted light microscope (Carl Zeiss, Axiovert
175 135, Germany).

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

178 **Histological analysis of ovarian follicles**

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

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

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

185 **Statistical Analysis**

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

189 **Results**

190 **Ovarian follicles viability at five difference stages after vitrification using** 191 **four different vitrification solutions.**

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

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

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

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

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

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

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

218 **Figure 4.** Average diameter growth of vitrified/rewarmed Stage I follicles after
219 24 h *in vitro* culture. Group 1 (whole ovary in plastic cryotube), Group 2 (whole
220 ovary in metal container), Group 3 (fragments in plastic cryotube), Group 4
221 (fragments in metal container). Bars labeled with common letters do not differ
222 significantly ($P < 0.05$) from each other (mean \pm SD).

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

226 **Histological analysis of ovarian follicles**

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

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

234 Cortical, vitellogenic and mature stage follicles presented the major cytoplasmic
235 alterations mainly concerning the distribution and integrity of lipid droplets after
236 cryopreservation. Follicle membrane layers appeared detached in few instances (Figura
237 5B). However, in higher resolution we observed that the membrane remained intact
238 (Figure 6B, arrowhead).

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

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

247 **Discussion**

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

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

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

271 Follicle viability obtained from VS1 (1.5 M of methanol and 4.5 M of propylene
272 glycol) and VS4 (1.5 M of methanol, 5.5 M of Me₂SO and 0.5 M of sucrose) did not
273 significantly differ from the control group in terms of follicular viability for Stage I.
274 Seki et al. [27] had already suggested that the combination of methanol and propylene
275 glycol might be more effective in reducing damage caused by cryopreservation on
276 zebrafish immature follicles. In addition, in an extensive survey on the toxicity of
277 several cryoprotectants, it was found that methanol and propylene glycol were the least
278 toxic agents for zebrafish follicular survival after cryopreservation [9].

279 On the other hand, it has already been shown that dimethyl sulfoxide (Me₂SO) is
280 a more permeable [28] and less toxic [19] cryoprotectant than propylene glycol to
281 zebrafish follicles and embryo cryopreservation. Also, it was reported that the addition
282 of sucrose enhanced the Me₂SO cryoprotectant action [19]. This observation is in
283 agreement with our data, wherein sucrose addition to the cryoprotectant solution
284 containing methanol and Me₂SO (VS4), increased immature follicle survival (Stages I
285 and II). Unlike Me₂SO, sucrose does not penetrate the cell membrane and acts only in
286 the extracellular medium by increasing its osmolarity. Sucrose, as a hydrophilic
287 compound, binds to water molecules, increasing viscosity of the solution and resulting
288 in cellular dehydration by water retention in the extracellular medium. Consequently,
289 decreasing the osmotic stress and ice crystals formation [14,8]. Both cryoprotectant,
290 intracellular and extracellular, interact with the cell membrane phospholipids conferring
291 greater stability to the membrane [7]. Therefore, it may be suggested that sucrose

292 supplementation in cryoprotectant solutions containing Me₂SO enhances follicular
293 viability. However, sucrose addition into vitrification solution containing propylene
294 glycol did not increase the follicular viability. Propylene glycol is highly hydrophilic
295 and the H-bond formation between the ether group and H₂O is supposed to contribute to
296 its affinity to water [35]. Chauvigné et al. [6] reported that membrane permeability of
297 zebrafish oocytes to propylene glycol and water varies according to osmolarity. Thus,
298 sucrose concentration may interfere on propylene glycol influx, enabling a better or
299 worse dehydration. Probably for this reason, follicular viability decreases by sucrose
300 addition into vitrification solutions containing propylene glycol.

301 In order to increase the efficacy of ovarian cryopreservation protocols in
302 different mammalian species, cryopreservation of whole ovary has been suggested as an
303 alternative option [22]. After avascular transplantation of ovarian tissue fragments, the
304 tissue undergoes significant damage caused by ischemia [31,32]. Thus, vascular
305 transplant of intact ovaries would be an answer to this problem [13,18]. Moreover,
306 when the whole ovary is cryopreserved, a larger pool of follicles is transplanted back to
307 the donor or recipient of the tissue, unlike the situation that occurs when small
308 fragments are used. However, vascular transplantation of whole cryopreserved ovary
309 has two major limitations: the technical difficulty in proceeding reanastomosis [31] and
310 the challenge to cryopreserve an intact organ [18]. These issues must be addressed in
311 studies with experimental animal models, before they may be put through human
312 clinical trials. Therefore, the effect of the tissue dimension (ovarian fragment or whole
313 ovary) was also evaluated here in terms of follicular survival after vitrification. We
314 observed that the survival rate in terms of *in vitro* growth, after vitrification was higher
315 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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