1 **Evaluation of Zebrafish *(Danio rerio)* PGCs viability and DNA damage using**

2 **different cryopreservation protocols**

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11

12 **Abstract**

13 Cryopreservation of Primordial germ cells (PGCs) is a better alternative for the
14 conservation of the diploid genome in fish until embryo cryopreservation is achieved. A
15 good cryopreservation protocol must guarantee high survival rates but also absence of
16 genetic damage. In this study, a cell toxicity test using several internal and external
17 cryoprotectants was carried out. The best combination of cryoprotectants (DMSO 5 M,
18 EG 1 M, PVP 4%) was used with and without antifreeze proteins (AFP) at two different
19 concentrations (10 mg/mL and 20 mg/mL) for cryopreservation trials. Different
20 cryopreservation methods were used with single PGCs, genital ridges and whole
21 zebrafish embryos using cryovials, 0.5 mL straws, microcapsules and microdrops. All
22 embryos were obtained from the vasa EGFP zf45 transgenic line and viability was
23 evaluated using trypan blue. High cell viability rates after cryopreservation in 0.5 mL
24 straws were obtained (around 90%) and a decrease in viability was only observed when
25 cells were cryopreserved in microcapsules and when AFP at 20 mg/mL was added to the
26 freezing media. Genetic damage was determined by comet assay and was compared in
27 cells cryopreserved in 0.5 mL straws and microcapsules (lowest viability rate). There
28 were significantly more DNA strand breaks after cryopreservation in the cells
29 cryopreserved without cryoprotectants and in those cryopreserved in microcapsules.
30 Genetic damage in the cells cryopreserved with cryoprotectants in 0.5 mL straws was
31 similar to fresh control samples, regardless of the concentration of AFP used. The
32 decrease in PGC viability with the addition of AFP 20 mg/mL did not correlate with an
33 increase in DNA damage. This study reported a successful method for zebrafish PGC
34 cryopreservation that not only guarantees high cell survival but also the absence of
35 DNA damage.

36 **Keywords:** Zebrafish PGCs, Genital Ridges, Comet Assay, AFPI, microcapsule,
37 cryopreservation.

38 **1. Introduction**

39 Embryo cryopreservation has yetto be achieved in fish , due to the particular structure
40 and characteristics of fish embryos [1,2]. Cryopreservation of blastomeres [3,4], or
41 primordial germ cells [5] are possible alternatives for the preservation of the fish diploid
42 genome. Among all these alternatives, Primordial germ cells (PGCs) represent the most
43 promising option for germplasm banking. These cells retain their migration capacity
44 after transplantation in a receptor embryo and can easily give rise to functional sperm
45 and oocytes via germline chimerism [6,7].

46 Different biological samples required different methods for cryopreservation. In
47 particular, in fish embryos, vitrification rather than cryopreservation has been
48 recommended to increase the probability of success [1]. However, higher concentrations
49 of cryoprotectants are required for vitrification protocols [8]. Vitrification solutions are
50 usually formulated as a combination of internal and external cryoprotectants. This
51 combination enables the vitreous state of the solution to be reached at low
52 temperatures, but also allows the concentration of each individual cryoprotectant to be
53 reduced, therefore decreasing the toxic effects on cells [9]. Some authors have reported
54 the benefits of adding antifreeze proteins to the traditional cryoprotectant cocktail in
55 samples exposed to low temperatures or cryopreserved. Robles and colleagues found
56 that type I antifreeze protein (AFP I) increases seabream (*Sparus aurata*) embryo
57 tolerance to low temperatures [10], and improves zebrafish blastomere viability after
58 cryopreservation at the concentration of 10 mg/mL [11]. These proteins have also been
59 successfully used in other species: ovine embryos can be stored in AFPI at 4º C for 4
60 days, yielding similar pregnancy and embryo survival rates as fresh embryos [12].

61 PGC cryopreservation has been studied in some fish species [5] and transplantation
62 assays have been successfully achieved in some of them [13]. However, the evaluation
63 of genetic damage has not been studied in these cells yet. It is known that during
64 cryopreservation reactive oxygen species (ROS) can be produced provoking DNA
65 damage in samples [14,15,16,17]. Cryopreservation protocols must guarantee good
66 viability rates but also provide a protection at this level. Different levels of DNA
67 damage can be reported with the use of different cryopreservation protocols [18].
68 Absence of DNA damage is particularly important for gene banking purposes. Unlike
69 PGCs, DNA damage in spermatozoa has been studied in several species: rainbow trout
70 [19], sea bass [20], gilthead sea bream [21], Pacific oyster [22] and loach [23]. One of
71 the main possible damages that can occur during cryopreservation is DNA breakage.
72 The Comet assay technique measures DNA breakage in individual cells [24] combining
73 electrophoresis with fluorescence microscopy to visualize DNA migration from the
74 individual cells in an agarose microgel [25].

75 In this study, the toxicity of the cryoprotectant solution used, which includes external
76 (PVP 4%) and internal cyoprotectants (DMSO 5 M, EG 1 M) with and without AFP (10
77 and 20 mg/mL) was determined. PGC viability after cryopreservation was tested using
78 single cells, genital ridges and embryos loaded in different containers and DNA damage
79 was evaluated using the comet assay. Results demonstrated that PGC viability after
80 genital ridge and embryo cryopreservation in 0.5 mL straws using DMSO 5 M, EG 1
81 M, PVP 4% was very high (90%). Moreover, no genetic damage was detected in
82 frozen/thawed cells that were cryopreserved using this protocol.

83 **2.Materials and methods**

84 *2.1.Zebrafish maintenance*

85 Zebrafish (*Danio rerio*) vasa EGFP zf45 strain, were maintained in tanks with a re-
86 circulating water system (AquaticHabitats) under standard conditions (Westerfield,
87 1995). Fish were fed twice daily with dry food(200 mg) at 9 AM and 6 PM and live
88 artemia(100 mg) at 1 PM.

89 *2.2.Embryo collection and Genital Ridges dissection*

90 For embryo collection, males and females were transferred to a breeding tank at a rate
91 of 1 male to 2 females. The embryos were collected and washed for 2 min with a 0.5%
92 bleach solution , rinsed twice with embryo medium (EM) (NaCl 13.7mM, KCl 0.54
93 mM, $NaHPO_4$ 0.025 mM, KH_2PO_4 0.044 mM, $CaCl_2$ 1.3mM, $MgSO_4$ 1.0mM, $NaHCO_3$
94 4.2mM) and kept in fresh EM at 28ºC until they reached the 24 h post fertilization (hpf).
95 The genital ridges (GRs) were manually excised from earlier than 24 hpf embryos using
96 fine watchmaker´s forceps under a dissecting microscope (Nikon, Tokio, Japan), as
97 described by Kobayashi et al [26]. The migration capacity of PGCs obtained from later
98 stages could decrease, as was reported by Saito and colleagues [7]. …The excised GRs
99 and embryos were collected and placed in modified Leibovitz medium (L15)
100 supplemented with 5% fetal bovine serum (FBS) in a culture dish at room temperature
101 (RT) until further use. Each experiment was done in triplicate using ten embryos or GRs
102 in each replicate (approximately 200 PGCs in each replicate).

103 *2.3. Cryoprotectant exposure and toxicity assay*

104 Samples (embryos or GRs) were exposed to cryoprotectants in a three-step manner.
105 First, they were placed in a pretreatment solution with DMSO 2 M and EG 0.5 M in
106 Hank´s premix (NaCl 138.28 mM, KCl 5.42 mM, Na_2HPO_4 0.255 mM, KH_2PO_4 0.455
107 mM, $CaCl_2$ 1.3 mM, $MgSO_4$ 1.0 mM) for 10 min. In those samples treated with AFP, the
108 protein was incorporated from the first step at two different concentrations (10 mg/mL
109 or 20 mg/mL). Secondly, samples were exposed to DMSO 5 M and EG 1 M in Hank´s

110 premix for 2 minutes. In the last step, samples were exposed to the vitrifying solution:
111 DMSO 5 M, EG 1 M, PVP 4% for 2 min.

112 *2.4. Assessment of PGC viability*

113 Ten GR or embryos, fresh (control) and cryopreserved, were dissociated with
114 collagenase 0.1% (in Hank´s premix solution) by pipetting. PGCs were identified by
115 their bright fluorescence and relatively large size. For PGC viability assessment 0.5%
116 trypan blue was added and the number of EGFP-positive and negative cells for trypan
117 blue were counted under an inverted and fluorescence microscope (Nikon, Tokyo,
118 Japan). Results were expressed related to the fresh control. The experiment was done in
119 triplicate. All procedures were carried out at RT.

120 *2.5.Cryopreservation trials*.

121 After the toxicity test, the PGCs (for embryos and GRs earlier than 24 hpf) were
122 cryopreserved using three different cryoprotective solutions a) DMSO 5 M, EG 1 M,
123 PVP 4% b) DMSO 5 M, EG 1 M, PVP 4% and AFP (10 mg/mL) c) DMSO 5 M, EG 1
124 M, PVP 4% and AFP (20 mg/mL). Four different containers, providing different
125 freezing/thawing rates, were used: cryovials, 0.5 mL straws, microcapsules and
126 microdrops. PGCs from 20 dissociated GRs were cryopreserved in microcapsules, GRs
127 were cryopreserved in 0.5 mL straws, cryovials and vitrified in microdrops (5 GRs per
128 microdrop) (Fig.1A), and embryos were cryopreserved in 0.5 mL straws and cryovials
129 (10 embryos per replicate). Experiments were done in triplicate.

130

131 *2.6.Cell microencapsulation*

132 Microencapsulation procedures developed for bovine, porcine and canine sperm [27]
133 were used with minor modifications. Cell suspensions were digested with collagenase
134 0.1% in Hank´s premix solution by pipetting. They were then diluted in L15 with 1.5%

135 (wt/vol) sodium alginate (Sigma- Aldrich Co. St. Louis, MO, USA). The solution was
136 dissolved in physiologic saline to reach a final concentration of 0.75%. The cell
137 suspension was forced through a needle attached to a 1 mL syringe into a 60 mm plastic
138 dish containing 1.5% (wt/vol) calcium chloride dissolved in physiological saline. The
139 cell suspension suffered solidification immediately upon contact with the calcium
140 chloride solution forming a high-viscosity microgel (Fig.1B). The microgels were
141 swayed gently and allowed to react with calcium ions for 30 s. After freezing/thawing,
142 microcapsules were placed in sodium citrate solution and were vortexed to release cells
143 from the microcapsules.

144 *2.7.Freezing and Thawing*

145 The samples were exposed to cryoprotectants as previously described, loaded in the
146 above mentioned containers. Those cells loaded in 0.5 mL straws and cryovials were
147 expose to liquid nitrogen vapor during 20 min and then plunged into liquid nitrogen,
148 whereas microdrops were vitrified in liquid nitrogen over a metal surface and then were
149 stored in a cryovial to be plunged into liquid nitrogen.

150 Cryovials and 0.5 mL straws were thawed in a water bath for 20 sec at 25 º C and 6 sec
151 at 63ºC respectively and washed with Hank premix solution. After thawing, cell
152 viability was analyzed as it was previously described and DNA fragmentation damage
153 was determined by comet assay.

154 *2.8. DNA damage determination by Neutral Comet assay*

155 DNA fragmentation damage was determined in fresh and cryopreserved samples (in 0.5
156 mL straws and microcapsules). As a negative control, genital ridges frozen without
157 cryoprotective agents were processed. Fresh and cryopreserved genital ridges were
158 dissociated and the resulting pellets were resuspended in L15 medium supplemented
159 with 5% FBS.

160 The comet assay was performed according to Olive and Bánath (2006)[24]with the
161 following modifications. Cell suspensions were mixed with 1% low melting point
162 agarose in PBS, cell suspension was rapidly placed onto a precoated slide and
163 immediately covered with a coverslip. The slides were left for 20 min at 4 º C to allow
164 the agarose to solidify and to ensure that it was fully set before submerging in lysis
165 solution.
166 After removing the coverslips, the slides were submerged in freshly prepared lysis
167 solution at 4º C (sarkosyl 2%, Na_2EDTA 0.5 M, proteinase K 25 μg/mL pH=8) for DNA
168 separation in the dark. After overnight lysis, the slides were submerged in neutral rinse
169 and electrophoresis solution (Tris buffer 90 mM, Boric Acid 90 mM, Na2EDTA
170 (pH=8.5)) for 30 min at RT. Electrophoresis was conducted at 25V/300A 25 min. After
171 electrophoresis, the slides were neutralized in distilled water and fixed with methanol
172 for 3 min. Finally, the slides were stained with DAPI, covered with a coverslip and
173 analyzed using a fluorescence microscope (Nikon, Tokyo, Japan). At least 50 comet
174 images were analyzed from each slide, three slides from each replicate were done and
175 three replicates from each treatment or cryopreservation method were done.
176 Approximately 100 cells per slide were photographed. Comet analysis was performed
177 using the software Tritek Comet Score Freeware v.1.5. The percentage of tail DNA (%
178 DNA_t) was obtained.
179 *2.9.Data analysis*
180 Viability data was subjected to logarithm transformation and statistical differences were
181 analyzed by one way ANOVA followed by Tukey HSD as a *post hoc* test. Significant
182 differences in DNA fragmentation damage (percentage of DNA in tail (% DNA_t) were
183 analyzed using the Tukey HSD test. All the statistics were conducted using the R
184 software 2.12.

185 **3.Results**

186 *3.1.Cryoprotectant toxicity*

187 Exposure to cryoprotectants did not affect PGCs survival (Fig. 2). There were no
188 significant differences among the 3 different solutions tested and PGCs survival rates
189 were, in some cases close to 100% (GRs treatment with CPA and AFP 10 mg/mL or
190 AFP 20 mg/mL plus internal and external cyorpotectants) and in all cases higher than
191 70% (Fig. 2).

192 *3.2.PGCs viability after cryopreservation*

193 PGC viability after cryopreservation (Fig.3) did not differ significantly among
194 treatments, except for the microcapsules and those samples cryopreserved with AFP 20
195 mg/ml. The use of whole embryos or GRs provided similar viability rates (higher than
196 90%) in the 0.5 mL straws method, as shown in Figure 4.
197 The addition of AFP 10 mg/mL did not have any positive or negative effect on viability.
198 However the use of AFP 20 mg/mL significantly decreased PGC viability after
199 cryopreservation (less than 50%). With regard to the obtained viability rates and the
200 manipulation requirements, 0.5 mL straws were chosen as the best loading container for
201 PGC cryopreservation (Fig.4) (Sup. Mat.Table.1). This method provided 90% of PGC
202 survival and significantly simplified genital ridge manipulation during the
203 cryopreservation protocol.

204 *3.3.DNA damage*

205 DNA fragmentation damage after cryopreservation, using the above mentioned three
206 different cryoprotectant combinations, was compared in samples that were
207 cryopreserved in 0.5 mL straws (chosen as the best cryopreservation protocol) and in
208 microcapsules (which reported the lowest viability rates in this study).

209 Cells cryopreserved in the absence of cryoprotectants showed the largest tails and
210 therefore the highest percentage of DNA damage (Fig.5). However, DNA fragmentation
211 did not differ significantly in the cells cryopreserved in 0.5 mL straws and fresh control
212 (less than 10% in all treatments) (Fig.6). The cells cryopreserved in microcapsules
213 showed greater damage (more than 25%), which was significantly different to the fresh
214 samples (less to 5%) and cryopreserved cells without CPAs (40%) (Fig.6). Regarding
215 DNA damage using the different combination of cryoprotectants, we observed that the
216 addition of AFP at any of the concentrations used did not significantly modify DNA
217 damage (Fig.6, Sup. Mat.Table. 2).

218 **4. Discussion**

219 PGC cryopreservation is a useful alternative for preserving the diploid fish genome
220 [28]. Some cryopreservation trials have been assayed with different fish species [5,13].
221 However, none of them have evaluated genetic damage. It is well known that ROS
222 production is induced during the cryopreservation. These radicals can cause an increase
223 in peroxidation, and DNA fragmentation occurs [29]. Taking into account that one of
224 the main advantages of cryopreservation is the possibility of preserving genetic material
225 (either for conservation purposes of endangered species or preservation of valuable
226 biotechnological lines), absence of genetic damage is a very important point to be
227 considered in the choice of a suitable cryopreservation protocol. The effect of
228 cryopreservation on sperm and oocytes DNA has been evaluated in several species [18].
229 Cabrita and colleagues observed that cryopreservation can induce DNA fragmentation
230 damage in rainbow trout and gilthead sea bream sperm, and that this fact should be
231 taken into account in the evaluation of cryopreservation protocols. Several authors have
232 detected sperm DNA damage using the comet assay, demonstrating that
233 cryopreservation sperm affected DNA stability through DNA fragmentation [30,19,20].

234 In previous studies on zebrafish PGCs cryopreservation, the exposure times to
235 cryoprotectants was too long (30 min) in order to increase their penetration. These long
236 exposure times caused a decrease in PGC viability [31]. To avoid this negative effect, a
237 combination of external and internal CPAs at lower doses were recommended for fish
238 embryo cryopreservation [8] and recently it has also been used for embryo
239 cryopreservation with the aim of recovering viable PGCs from them [13].
240 In this work, we compare PGC cryopreservation success using whole embryos, genital
241 ridges and single cells and comparing different cryopreservation protocols using three
242 vitrifyingt solutions and several containers that provided different freezing/thawing
243 rates. Our findings showed that the selected conditions (external and internal CPAs
244 combination, cryoprotectant concentrations, exposure times, temperature of exposure
245 and freezing/thawing rates) were optimal for zebrafish PGC cryopreservation in all
246 cases except for those samples supplemented with AFP 20 mg/mL and those
247 cryopreserved in microcapsules. Survival rates were close to 90% or 100% in samples
248 (GRs and embryos) cryopreserved in 0.5 mL straws and microdrops (GRs). In those
249 samples (GRs and embryos) cryopreserved in cryovials the survival was higher than
250 70%. When PGCs were microencapsulated, survival decreased significantly to 20%.
251 Microencapsulation has also been used in the preservation of canine sperm at 4ºC,
252 maintaining motility and viability for several days [27]. Our results showed a PGCs
253 survival rate decrease when microencapsulation was done prior cryopreservation
254 (Fig.4). This method was more laborious than the other methods used, and also
255 subjected the biological material to extra *in vitro* manipulations (for encapsulation and
256 decapsulation), which could have a negative effect on cell viability. Taking into account
257 that PGCs were not lost during the cryopreservation process when GRs were used, this
258 method does not have any real advantage over the other systems used for loading.

259 The addition of AFPs was not toxic for the cells (Fig.2) but did not improve PGCs
260 viability after cryopreservation, regardless of the concentration used (Fig.4). It is known
261 that AFPs adhere to ice crystals blocking their further growth, and therefore, under
262 certain conditions, they can produce a decrease in cell damage [32,33]. Our previous
263 experiments in zebrafish blastomere cryopreservation indicated that the addition of AFP
264 type I 10 mg/mL as a supplement in the cryoprotective solution, significantly increased
265 cell survival after freezing/thawing [10]. However, the same concentration of AFP had
266 no effect on zebrafish PGC cryopreservation (Fig.4). Moreover, when the concentration
267 was increased to 20 mg/mL, a negative effect on survival was observed (Fig.4). This
268 could be explained considering the dual effect of AFPs. It is known that, depending on
269 their concentration, AFPs could avoid ice crystal growth, or induce spicule-shaped
270 crystals growth when the concentration is increased [34]. These spicule shaped ice
271 crystals could be more detrimental for cell survival than the original ones. In fact, high
272 concentration of AFP have been used to induce this type of crystals in order to kill
273 tumor cells [35,36]. Although a cryomicroscopy study would be required to test this
274 hypothesis,, we think this could be a feasible explanation for what we have observed.
275 It is interesting to point out, that the decrease in survival when PGCs were
276 microencapsulated was associate with an increase in DNA damage (Fig.4 and Fig.6) but
277 surprisingly, this did not happens when AFP 20 mg/ml was used (Fig.4 and Fig.6). This
278 is a very relevant observation since it indicates that the selection of a cryopreservation
279 protocol could not be done taking into account only cell survival.
280 Viability rates and absence of DNA fragmentation are not the only parameters that
281 should be tested in order to guarantee that a protocol is adequate for PGC
282 cryopreservation. After cryopreservation, PGCs should retain their migration ability in
283 order to be able to colonize the gonad of the recipient embryo once transplanted. Some

284 important studies indicate a correlation of high viability rates, and in particular, of
285 pseudopodia emission with a good colonization ability [26] In this work, we have
286 confirmed that PGCs cryopreserved using the 0,5 ml straw method have an active
287 pseudopodia emission (see *Time Lapse* video in Supplementary Material).

288 In conclusion, this study explored different cryopreservation methods for PGC
289 cryopreservation in zebrafish; most of these methods have never been used in these cells
290 before. The effect of the addition of antifreeze proteins in the cryopreservation media is
291 also considered for the first time in this work, and more importantly, this article has
292 reported the evaluation of DNA fragmentation in cryopreserved PGCs for the first time.
293 We have demonstrated that zebrafish PGCs can be successfully cryopreserved in 0.5 mL
294 straws using the cryoprotectants DMSO 5 M, EG 1 M, PVP 4%. This cryopreservation
295 method not only guarantees high PGC survival (90%) but also a DNA protection at the
296 fragmentation level. Furthemore, we have observed that a decrease in cell viability after
297 cryopreservation (PGCs cryopreserved in straws using AFP 20 mg/mL) does not
298 necessarily imply an increase in DNA damage. This evidence point out the importance
299 of analyzing DNA damage after cryopreservation, regardless of the cell viability
300 obtained, in order to choose the most adequate protocol for cryopreservation in a
301 specific cell type. In our laboratory, we are currently performing experiments that allow
302 quantification of the genetic damage caused by cryopreservation.

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307 6. References

308 1) Hagedorn M, Kleinhans FW, Freitas R, Liu J, Hsu EW, Wildt DE, Rall WF. Water
309 distribution and permeability of zebrafish embryos, Brachydanio rerio. J Exp Zool
310 1997;278:356-371.
311 2) Robles V, Cabrita E, Herráez MP. Germplasm cryobanking in zebrafish and other
312 aquarium model species. 2009;6(3):281-93.
313 3) Kopeika J, Zhang T, Rawson D.M., Elgar G. Effect of cryopreservation on
314 mitochondrial DNA of zebrafish (*Danio rerio*) blastomere cells. Mutat.
315 Res.2005;570(1): 49-61.
316 4) Lin C, Zhang T, Rawson D.M. Cryopreservation of Zebrafish (*Danio rerio*)
317 Blastomeres by Controlled Slow Cooling. Cryo Letters.2009;30(2):132-41.
318 5) Kobayashi T, Yutaka T, Takeuchi T, Yoshizaki G. Generation of viable fish from
319 cryopreserved primordial germ cells. Mol Reprod Dev. 2007;74(2):207-13.
320 6) Saito T, Goto-Kazeto R, Arai K, Yamaha E. Xenogenesis in teleost fish through
321 generation of germ-line chimeras by single primordial germ cell transplantation. Biol
322 Reprod. 2008;78(1):159-66.
323 7) Saito T, Goto-Kazeto R, Fujimoto T, Kawakami Y, Arai K, Yamaha E. Inter-species
324 transplantation and migration of primordial germ cells in cyprinid fish.Int J Dev Biol.
325 2010;54(10):1481-86.
326 8) Arakawa T, Carpenter J, Kita Y, Crowe J. The basis for toxicity of certain
327 cryoprotectants: A hypothesis. Cryobiology. 1990;27(4):401-415.
328 9) Cabrita E, Robles V, Chereguini O, Wallace JC, Herráez MP. Effect of different
329 cryoprotectants and vitrificant solutions on the hatching rate of turbot embryos
330 (*Scophthalmus maximus*). Cryobiology. 2003;47(3):204-13.

331 10) Robles V, Barbosa V, Herráez MP, Martínez-Páramo S, Cancela ML. The antifreeze
332 protein type I (AFP I) increases seabream (*Sparus aurata*) embryos tolerance to low
333 temperatures. Theriogenology. 2007;68(2):284-9.
334 11) Martínez-Páramo S, Barbosa V, Pérez-Cerezales S, Robles V, Herráez MP.
335 Cryoprotective effects of antifreeze proteins delivered into zebrafish embryos.
336 Cryobiology. 2009;58(2):128-33.
337 12) Arav A, Ramsbottom G, Baguisi A, Rubinsky B, Roche JF, Boland MP. Vitrification
338 of bovine and ovine embryos with MDS technique and antifreeze proteins. Cryobiology
339 1993;30:621-2.
340 13) Higaki S, Eto Y, Kawakami Y, Yamaha E, Kagawa N, Kuwayama M, Nagano M,
341 Katagiri S, Takahashi Y. Production of fertile zebrafish (*Danio rerio*) possessing germ
342 cells (gametes) originated from primordial germ cells recovered from vitrified embryos.
343 Reproduction. 2010;139(4):733-40.
344 14) Fuku E, Xia L, Downey BR. Ultrastructural changes in bovine oocytes
345 cryopreserved by vitrification. Cryobiology. 1995;32: 139–156.
346 15) Wu B, Tong J, Leibo SP. Effects of cooling Germinal vesicle stage bovine oocytes
347 on meiotic spindle formation following IVM. Mol Reprod Dev. 1999;54: 388–395.
348 16) Saunders KM, Parks JE. Effects of cryopreservation procedures on the cytology
349 and fertilization rate of IVM bovine oocytes. Biol Reprod. , 1999;61: 178–187.
350 17) Mullen SF, Agca Y, Broermann DC, Jenkins CL, Johnson CA, Critser JK. The
351 effect of osmotic stress on the methaphase II spindle of human oocytes, and the
352 relevance to cryopreservation. Hum Reprod. 2004;19: 1148–1154.
353 18) Stachowiak, K Papis M Kruszewski, T Iwanen´ko T Bartomiejczykand JA Modlin
354 ´ski. Comparison of the Level(s) of DNA Damage Using Comet Assay in Bovine

355 Oocytes. Subjected to Selected Vitrification Methods EM. Reprod Domest Anim.
356 2009;44(4):653-8.19
357 19) Labbe C, Martoriati A, Devaux A, Maisse G. Effect of sperm cryopreservation on
358 sperm DNA stability and progeny development in rainbow trout. Mol Reprod Dev.
359 2001;60(3):397-404.
360 20) Zilli L, Schiavone R, Zonno V, Storelli C, Vilella S. Evaluation of DNA damage in
361 *Dicentrarchus labrax* sperm following cryopreservation. Cryobiology. 2003;47(3):227-
362 35.
363 21) Cabrita E, Robles V, Rebordinos L, Sarasquete C, Herráez MP. Evaluation of DNA
364 damage in rainbow trout (*Oncorhynchus mykiss*) and gilthead sea bream (*Sparus*
365 *aurata*) cryopreserved sperm. Cryobiology. 2005;50(2):144-53.
366 22) Gwo JC, Wu CY, Chang WS, Cheng HY. Evaluation of damage in Pacific oyster
367 (*Crassostrea gigas*) spermatozoa before and after cryopreservation using comet
368 assay.Cryo Letters. 2003;24(3):171-80.
369 23) Kopeika J, Kopeika E, Zhang T, Rawson DM, Holt WV. Detrimental effects of
370 cryopreservation of loach (*Misgurnus fossilis*) sperm on subsequent embryo
371 development are reversed by incubating fertilised eggs in caffeine. Cryobiology.
372 2003;46(1):43-52.
373 24) Olive PL, Banáth JP. The comet assay: a method to measure DNA damage in
374 individual cells. Nat Protoc. 2006;1(1):23-9.
375 25) Ostling O, Johanson KJ. Microelectrophoretic study of radiation-induced DNA
376 damages in individual mammalian cells. Biochem Biophys Res Commun.
377 1984;123(1):291-8.

378 26) Kobayashi T, Takeuchi Y, Yoshizaki G, Takeuchi T. Isolation of highly pure and
379 viable primordial germ cells from rainbow trout by GFP-dependent flow cytometry. Mol
380 Reprod Dev. 2004;67:91–100.
381 27) Shah S, Nagano M, Yamashita Y, Hishinuma M. Microencapsulation of canine
382 sperm and its preservation at 4 degrees C. Theriogenology. 2010;73(5):560-7.
383 28) Harvey B. Cooling of embryonic cells, isolated blastoderms, and intact embryos of
384 the zebra fish *Brachydanio rerio* to -196 degrees C. Cryobiology. 1983;20(4):440-7.
385 29) Ahmadi A., Sonn-Chye N. Fertilizing ability of DNA damaged spermatozoa. J. Exp.
386 Zool. 1999;284:696–704.
387 30) Lee R.F, S. Steinert S, Use of the single cell gel electrophoresis/comet assay for
388 detecting DNA damage in aquatic (marine and freshwater) animals, Mutat. Res. 544
389 (2003) 43–64.
390 31) Higaki S, Mochizuki K, Baba H, Akashi Y, Yamaha E, Katagiri S &Takahashi Y.
391 Feasibility of cryopreservation of zebrafish (*Danio rerio*) primordial germ cells by
392 whole embryo freezing. Japanese Journal of Veterinary Research. 2009;57:119–128.
393 32) Fei YB, Wei LP, Gao SQ, Zhu HB, Luo YR, Zhang ZX. Additive of AFGPs in
394 enhancing survival rate of frozen swine embryos. Anim Reprod. 1995;3:21–3
395 33) Mugano JA, Wang T, Layne Jr R, DeVries AL, Lee RE. Antifreeze glycoproteins
396 promote lethal intracellular freezing of rat cardiomyocytes at high subzero temperatures.
397 Cryobiology. 1995;32:556–7.
398 34) Robles V, Cabrita E, Fletcher GL, Shears MA, King MJ, Herráez MP. Vitrification
399 assays with embryos from a cold tolerant sub-arctic fish species. Theriogenology.
400 2005;15;64(7):1633-46.

401 35) Muldrew K, Rewcastle J, Donnelly BJ, Saliken JC, Liang S, Goldie S, Olson M,
402 Baissalov R, Sandison G. Flounder antifreeze peptides increase the efficacy of
403 cryosurgery. Cryobiology. 2001;42(3):182-9.

404 36) Larese A, Muldrew K, Acker J, McGann LE. Antifreeze proteins induce intracellular
405 nucleation. Cryoletters. 1997;17:175–182.

406 **7. Figure Captions**

407 Figure 1 PGCs vitrifyed in microdrops over a solid surface (metal cube) in liquid
408 nitrogen (A). PGCs microencapsuled in sodium alginate (B).

409 Figure 2 PGC survival rates (%) after embryo and GRs were exposed to cryoprotectants
410 (CPAs) and cryoprotectants supplemented with AFP 10 mg/ml (AFP 10) or 20 mg/ml
411 (AFP 20). Embryos or GRs per trial=10, n=3. Significant differences were not observed
412 among treatments.

413 Figure 3 Primordial germ cell from vasa EGFP zf45 zebrafish embryo after
414 freezing/thawing stained with trypan blue and observation under light microscope (A).
415 Primordial germ cell from vasa EGFP zf45 zebrafish embryo after freezing/thawing
416 stained with trypan blue and observed under fluorescence (B). PGC show green
417 fluorescence and pseudopodia emission (arrow)(C).

418 Figure 4 PGC survival rates (%) after Embryo and GR cryopreservation using different
419 loading containers. Embryos or GRs per trial=10, n=3. Significant differences among
420 different cryoprotectant solutions used in 0.5 straws are represented with one asterisk.
421 Significant differences among different cryopreservation protocols are represented with
422 two asterisks.

423 Figure 5. Comet gel electrophoresis of fresh sample (control) (A) samples treated with
424 cryoprotectant solution DMSO 5 M, EG 1 M, PVP 4%, without AFP addition (B),
425 cryoprotectant solution supplemented with AFP 10 mg/mL (C), cryoprotectant solution

426 supplemented with AFP 20 mg/mL (D), Microencapsulated samples (E) and samples
427 without cryoprotectants (F). The comet head is the high-molecular-weight DNA
428 (undamaged DNA) and the tail represent DNA fragments (damaged DNA).

429 Figure 6. Percentage of DNA in tail, (%DNAt) in control and cryopreserved PGCs.
430 Samples were cryopreserved in 0.5 mL straws and microcapsule, using DMSO 5 M, EG
431 1M, PVP 4% (CPAs) with or without AFP at 10 mg/ml (AFP 10) or 20 mg/ml (AFP 20).
432 GRs per trial=20, n=3. Letters represent significant differences among treatments.

433 Sup. Mat. Table 1. PGC survival rates (%) after Embryo and GR cryopreservation using
434 different loading containers. Embryos or GRs per trial=10, n=3. Significant differences
435 among different cryoprotectant solutions used in 0.5 straws are represented with one
436 asterisk. Significant differences among different cryopreservation protocols are
437 represented with two asterisks.

438 Sup. Mat. Table 2. Percentage of DNA in tail, (%DNAt) in control and cryopreserved
439 PGCs. Samples were cryopreserved in 0.5 mL straws and microcapsule, using DMSO 5
440 M, EG 1M, PVP 4% (CPAs) with or without AFP at 10 mg/ml (AFP 10) or 20 mg/ml
441 (AFP 20). GRs per trial=20, n=3. Letters represent significant differences among
442 treatments.

443 Sup. Mat. Time lapse of frozen/thawed Zebrafish PGCs showing pseudopodial
444 emission. PGCs were cryopreserved with the 0.5 mL straw method. PGC were recorded
445 for 2 hours using a Nikon microscope and NIS-Element software.

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