Running title: Cryopreservation and chimerism in fish

Title: Production of loach (*Misgurnus anguillicaudatus)* germ-line chimera using transplantation of primordial germ cells isolated from cryopreserved blastomeres[1]

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ABSTRACT

An efficient procedure for cryopreservation of fish blastomeres followed by restoration through germ-line chimera formation was established. Blastomeres of the loach (*Misgurnus anguillicaudatus)* were cryopreserved in 250-µL straws in Eagle's minimum essential medium (MEM) with various concentrations of dimethyl-sulfoxide (DMSO; 0, 5, 10, 15, and 20%), and the best concentration was combined with glycerol (1, 2, and 4%) and external cryoprotectants (1 or 2% sucrose; 2, 5, or 10% fetal bovine serum; 1 or 2% bovine serum albumin). Post-thaw viability of the blastomeres was used to optimize cryopreservation conditions. Donor blastomeres were injected with zebrafish GFP-*nos1* 3'UTR mRNA and biotin dextran prior to cryopreservation in the optimal freeze medium. Host embryos were injected with zebrafish DsRed-*nos1* 3'UTR mRNA and reared to the blastula stage. Donor blastomeres were thawed at 25°C for 10 s and transplanted to the host embryos either immediately or after incubation for 16 h at 20°C. Donor and host primordial germ cell migration was visualized with fluorescent imaging during the early stages of embryogenesis, and also by histology in 4-d-old embryos. Transplantation of blastomeres immediately after thawing gave lower hatching rates (~3%) and generated a low percentage of germ-line chimeras (~1.1%). In contrast, incubation of cryopreserved sample for 16 h followed by transplantation of the GFP-positive blastomeres improved the hatching rate to 90%, and successfully produced presumable germ-line chimeras at a rate of 16.5%. The improved survival rates and germ-line chimerism may be an effective method for gene banking and subsequent reconstitution of endangered fish genotypes.

Keywords: aquaculture, cryobank, embryo, gamete, germ cell, germplasm, loach, teleost.

INTRODUCTION

Genebanking is of importance for both farmed and wild species as a means to preserve endangered populations and reconstitute unique genotypes. In fish, a large number of species or strains are endangered (Hiemstra et al., 2006), and farmed stocks are derived from few genotypes from wild populations. Thus, germplasm preservation may represent a useful tool to increase genetic diversity in broodstock.

Liquid nitrogen cryopreservation is effective for long-term gene banking, but is only applicable for sperm preservation in most species. Cryopreserved sperm is useful for breeding programs and maintenance of genetic diversity, but the reconstitution of already extinct fish using cryopreserved sperm is difficult at present because sperm gives rise to inviable haploid progeny after induced androgenesis. Moreover, maternally inherited genotypes and cytoplasmic mitochondrial DNA are not preserved by sperm cryopreservation and thus never restored by androgenesis.

The loach (*Misgurnus anguillicaudatus)* is a fish widely distributed in Japan, and the species includes rare genotypes including natural diploid clones and polyploids (Arai, 2001, 2003). Previously, we cryopreserved loach sperm (Yasui et al., 2008, 2009) and reconstituted viable progeny by combining cryopreserved diploid sperm and artificially-induced androgenesis (Yasui et al., 2010). However, the resultant androgenotes exhibited low survival rates and were predominantly males due to unknown mechanisms (Fujimoto et al., 2010a). These androgenotes result presumably in reduced genetic diversity.

Technologies to produce germ-line chimeras were developed recently in loach (Nakagawa et al., 2002) by transplantation of primordial germ cells (PGC) (Saito et al., 2006; Fujimoto et al., 2006, 2010b). Targeted genotypes may be reconstituted by this method because the host should produce donor gametes in the germ-line chimeras (Yamaha et al., 2006). In a similar manner, cryopreservation of blastomeres followed by production of germ-line chimeras is considered a useful repository strategy. In the present study, we established a protocol for cryopreservation of blastomeres and subsequent production of germ-line chimeras in the loach.

MATERIALS AND METHODS

Embryo Management and Isolation of Blastomeres

This study was carried out in accordance with the Guide for the Care and Use of Laboratory Animals in Hokkaido University. Induced maturation of broodstock, dechorionation of eggs, and incubation of embryos followed procedures reported in previous studies (Fujimoto et al., 2004, 2007). Briefly, final maturation was induced with a single dose of hCG followed by gamete collection after 12 h at 27°C. Fertilization was achieved by the dry method in a Petri dish covered by a plastic film. Five min after fertilization, the water was removed from the dish and the remaining adhered eggs were fully covered by dechorionation medium (0.1% trypsin and 0.4% urea in Ringer's solution - 7.5 g/L NaCl, 0.2 g/L KCl, and 0.2 g/L $CaCl_2$, Yamaha et al., 1986). The dechorionated eggs were transferred to an agar-plated Petri dish filled with culture medium (Ringer solution with 1.6% albumen, 100 μg/mL streptomycin and 100 IU penicillin, Fujimoto et al., 2006).

At the blastula stage (512 to 1024 cells), blastoderms were removed mechanically using a fine needle. Control embryos were left intact. When controls reached 30% epiboly stage (Fujimoto et al., 2006), we carefully transferred 180 isolated blastoderms using a glass Pasteur pipette to a 2-mL microtube. The isolated blastoderms were rinsed in Ringer's solution to eliminate residues of yolk, and the supernatant was eliminated. Isolation of blastomeres was achieved by addition of 300 µL of 0.25% sodium citrate (diluted in Ringer solution) following by gently pipetting using a wide-bore pipette tip.

Cryopreservation, Thawing, and Viability Assessment

Isolated blastomere suspension (in sodium citrate) was diluted 4-fold in a cryo-solution (1 part of blastomere suspension: 3 parts of cryo-solution) and mixed. This procedure resulted in a cell concentration of 356 ± 75 x 10^3 cells/mL. The cryo-solution was prepared with Eagle's minimum essential medium (MEM) and dimethyl-sulfoxide (DMSO) at 0, 5, 10, 15, and 20% (vol/vol). The tube content was packed in 250-µL cryogenic straws (IMV, L'Aigle, France), sealed with sealing powder and set in a programmable freezer (EYELA, MPF-1000, Tokyo, Japan) previously cooled at 5°C. As we used many treatments to optimize blastomere cryopreservation, the straw volume was reduced to 130 µL by cutting, in order to reduce blastomere usage, but for transpantation procedures, we used the whole straw (250 µL).

The samples were frozen using the following freezing procedure: hold at 5°C for 5 min followed by cooling at -0.75°C/min until -40°C and then directly plunging into liquid nitrogen for storage. Fresh (not cryopreserved) and cryopreserved blastomeres in MEM (without cryoprotectant) were used as control groups.

Samples were thawed in a water bath at 25°C for 10 s. The straw content was immediately transferred to a microtube with 200 µL of MEM containing 0.8 µL of propidium iodide (2.4 mM, Sperm viability kit, Invitrogen, Eugene, OR). After 5 min, the cells were placed in a hemocytometer and observed under a fluorescence microscope (Nikon Eclipse E800, Tokyo, Japan) with a specific filter for propidium iodide excitation (536 nM). Dead cells were identified by intense intracellular fluorescent staining (red), whereas live cells maintained their translucent-like appearance. The viability was assessed twice for each sample by counting the number of dead cells in a total of 250 to 300 cells.

Di-Methyl-Sulfoxide (DMSO) Concentration

Blastomeres were cryopreserved following the above-mentioned procedure, but we used different cryo-solutions containing MEM solution and DMSO concentrations at 0, 5, 10, 15, or 20% (vol/vol). Viability of post-thaw blastomeres was evaluated 3 d afterwards.

Glycerol Additions

Blastomeres were cryopreserved in Eagle's MEM solution with 10% DMSO and glycerol at 0, 1, 2, and 4% (vol/vol). Cryopreservation was performed as mentioned above. Viability of post-thaw blastomeres was evaluated 1 d afterwards.

External Cryoprotectants

Isolated blastomeres were cryopreserved in cryo-solution containing Eagle's MEM with 10% DMSO and containing the following cryoprotectants: bovine serum albumin (BSA) at 1 and 2% (wt/vol); sucrose at 1 and 2% (wt/vol); fetal bovine serum (FBS) at 5 and 10% (vol/vol). Cryopreservation was performed as mentioned above. Viability of post-thaw blastomeres was evaluated 62 d afterwards.

Transplantation of Blastomeres Immediately After Thawing

Artificial green fluorescence protein (*GFP)* mRNA was produced combining the 3'UTR of germ-line specific mRNA (*nos-1*) from the zebrafish (Köprunner *et al.,* 2001) using the mMESSAGE MACHINE kit (Ambion, Austin, TX). A new batch of fertilized eggs was dechorionated and each embryo was injected with zebrafish GFP-*nos1* 3'UTR mRNA (200ng/µL) containing 5% biotin-dextran fixable (Sigma, St. Louis, MO) for PGC labeling.

Blastomeres from these embryos were cryopreserved using our optimized protocol (see above). About 1 mo afterward, 3 straws (250 µL) of blastomeres were thawed, transferred to a 1.5-mL microtube, and centrifuged at 800 × *g* for 4 min. Most of the supernatant was removed and the cells were resuspended in 500 µL of MEM solution with 100U/mL DNase (Invitrogen, Carlsbad, CA) and transferred to a 500-µL tube. The cells were maintained for 30 min at 20°C for removal of the cryo-solution. After incubation, the tube was centrifuged again and half of the supernatant was removed. The tube was observed under the stereoscopic microscope and the blastomeres were collected from the bottom using a capillary glass micropipette connected to the microinjector apparatus (Figure 1). The micropipette had an inside diameter of 70 µm and was beveled to a 30° angle.

For host embryos, another batch of eggs was obtained and the embryos were reared to the blastula stage. The collected blastomeres were injected at the lateral marginal part of host blastoderm and the transplanted embryos were maintained at 20°C.

Transplantation of GFP-Positive Blastomeres After Incubation

A new egg batch was obtained and blastomere labelling and cryopreservation were performed as mentioned above. Two straws were thawed and the content was placed in an agar-plated Petri dishes filled with culture medium. The Petri dishes were maintained at 20°C for 16 h. For host embryos, we obtained a new egg batch, each embryo was labeled by injection of zebrafish DsRed-*nos1* 3'UTR mRNA to identify host PGC. Donor blastomeres were observed under the fluorescence stereoscopic microscope and 1 to 3 GFP-positive cells were collected using a glass capillary micropipette (90- to 100-µm inside diameter). The cells were injected at the marginal part of each host blastoderm and cultured at 20°C.

Assessment of Induced Chimerism

Embryos were cultured at 20°C. Developmental stages (gastrulation, somite, and hatch) were determined following the criteria proposed by Fujimoto et al. (2006). Migration of PGC to the genital area was assessed in vivo by fluorescence microscopy: donor PGC exhibit GFP fluorescence and host PGC exhibit DsRed fluorescence. Four d postfertilization (dpf), the fish presenting donor PGC at the genital area were fixed in Bouin's fixative for 12 h, and then transferred to 80% ethanol. After paraffin embedding, serial 6-µm sections were collected and

the donor cells labeled with biotin were detected by biotin-streaptavidin-horseradish peroxidase complex and 3,3´-diaminobenzidine substrate followed by hematoxylin and eosin counterstaining.

Statistics

Data are shown as mean ± SD. All experiments were performed in triplicate using different egg sources. Data were checked for normality using the Kolmogorov-Smirnov test and then compared using one-way ANOVA. Based on the number of replications, normality, and homogeneity of the data, the Tukey's multiple-range test ($P < 0.05$) was used in order to establish a comparison within treatments. The software Minitab version 15 for Windows was used to make the statistical analyses.

RESULTS

Cryopreservation of Blastomeres

Cryopreservation reduced the viability of the blastomeres (Figure 2). The addition of DMSO to MEM solution improved the post-thaw viability of blastomeres ($P < 0.0001$). Blastomere cryopreservation without DMSO resulted in a post-thaw viability of 1.8 ± 0.8%. The DMSO additions at 5, 10, 15, and 20% improved the post-thaw viability to 31.1 ± 13.7%, 44.1 ± 11.5%, 32.0 ± 5.8, and 36.9 ± 12.9%, respectively. These results are lower when compared to

control unfrozen samples (95.1 ± 1.5%, $P < 0.0001$). Based on these results, DMSO concentration at 10% was selected for further cryopreservation experiments.

Glycerol supplementation significantly decreased ($P < 0.0001$) the post-thaw viability (Figure 3). In the absence of glycerol, post-thaw viability was 34.4 ± 12.5%. Addition of glycerol at 1, 2, and 4% to the cryo-solution reduced post-thaw viability to 26.5 ± 5.5%, 11.7 ± 4.6%, and 4.7 ± 1.2%, respectively. By comparison to fresh controls, cryopreservation reduced ($P < 0.0001$) viability.

The addition of external cryoprotectants did not significantly improve post-thaw viability when compared to control (cryopreserved with 10% DMSO) (Figure 4). Post-thaw viability after addition of external cryoprotectants ranged from 60.9 ± 2.3% (sucrose at 1%) to 67.0 ± 2.1% (FBS at 10%). These data did not significantly differ from control (65.1 ± 0.6%, $P < 0.0531$), in which the blastomeres were cryopreserved without any external cryoprotectants. However, post-thaw samples presented decreased viability when compared with control (fresh), in which the viability was 96.8 ± 0.8%.

Production of Germ-Line Chimeras

Transplantation of blastomeres immediately after thawing gave very low survival rates, and most of the embryos died before gastrulation (Table 1). The number of germ-line chimeras also was very low (3 of 264 or 1.1%). Intact and dechorionated groups produced hatching rates about 70%, in which ~3% were abnormal.

Incubation of cryopreserved blastomeres for 16 h at 20°C prior to transplantation improved hatching rates (~90%) and the percentage of chimeras (16%; Table 2). Intact and

223 dechorionated treatments gave hatching rates around 80% with 5% abnormal embryos.
224 Chimerism was confirmed in vivo by observation of host and donor PGC (Figure 5). Some donor
225 PGC exhibited different migration routes than host PGC (Figures 5C and 5E). However, at
226 hatching stages, donor and host PGC were localized to the genital area. Donor PGC migration
227 was confirmed by histological detection of biotin-streptavidin-horseradish peroxidase at 4 d post
228 transplantation (Figures 5G and 5H).
229
230 **DISCUSSION**
231
232 In previous studies with teleosts, blastomeres were cryopreserved using both slow- and
233 rapid-freezing protocols. Using the slow cooling, the post-thaw viability was 54% for *Carassius*
234 *auratus* (Kusuda et al., 2004), 59.3% for *Oncorhynchus keta* (Kusuda et al., 2002), 19.9% for
235 *Sillago japonica*, 67.4% for *Odontesthes bonariensis*, 34.1% for *Oryzias latipes* (Strussmann et
236 al., 1999), 70.2 to 84.8% for *Danio rerio* (Harvey, 1983; Lin et al., 2009); 96% for *Cyprinus*
237 *carpio* (Calvi and Maisse, 1999), and 36 to 95% for *Oncorhynchus mykiss* (Nilson and Cloud,
238 1993; Calvi and Maisse, 1998; Kobayashi et al., 2003). The rapid-cooling method gave viability
239 above 90% in the zebrafish (Cardona-Costa et al., 2009). Despite many protocols on blastomere
240 cryopreservation mentioned above, very few attempts successfully produced germ-line chimeras
241 using the cryopreserved samples. Cardona-Costa et al. (2009) failed to produce germ-line
242 chimeras, although the viability of post-thaw blastomeres was above 90%. In *C. auratus*, Kusuda
243 et al. (2002) used a similar procedure to the present study (transplantation of blastomeres
244 immediately after thawing) but obtained a very low percentage of germ-line chimeras (~3%). An
245 effective procedure was reported in zebrafish by Higaki et al. (2010), who succeeded in

246 production of germ-line chimeras using PGCs isolated from vitrified embryos (~8%). Kobayashi
247 et al. (2007) produced germ-line chimeras from cryopreserved PGC and 5.6 to 12.1% of the
248 transplanted fish successfully produced donor gametes in *Oncorhynchus mykiss*.

249 In our study, transplantation procedures played an important role in embryo survival as
250 well as for successful formation of chimeras from cryopreserved blastomeres. The
251 transplantation using blastomeres immediately post-thaw resulted in very low hatching rates and
252 most embryos died shortly after injection. In such procedure, the volume of the transplanted cells
253 was not the main cause of embryo mortality because germ-line chimeras were successfully
254 produced in the same species using similar transplanted volume from donor blastoderms
255 (Fujimoto et al., 2010b). However, in such method only the blastomeres were transplanted,
256 differing from our procedure in which some volume of solution was also injected and may affect
257 the embryo development. Another possible problem regards to the toxic residues from the cryo-
258 solution, including DMSO and sodium citrate, and the presence of dead cells in post-thaw
259 samples that may also disturb the development of transplanted embryos. Optimization of the
260 cryopreservation methods examined viability of post-thaw blastomeres and did not test whether
261 these surviving cells could develop in the host embryos. Later improvement in our procedure for
262 cryopreservation (packaging, cooling protocols, cryoprotectants, and thawing conditions) and
263 post-thaw management (centrifugeing, injection, and elimination of dead cells and toxic
264 components) may be used to increase the production of germ-line chimeras by such method. The
265 post-thaw management including washing and the usage of DNase used in this study is
266 recommended because it improves the survival rate of the transplanted fish (unpublished data)
267 but more adjustments may be necessary for successful production of germ-line chimeras.

Incubation of cryopreserved blastomeres prior to injection into the host allowed the blastomeres to reach the adequate period for expression of GFP-*nos1* mRNA in some of them. Transplantation of the GFP-positive blastomeres successfully produced 16% of germ-line chimeras. Such transplantation is interesting because the post-thaw recovery process probably eliminates, or dilutes, toxic components in the cryo-solution allowing for selection of viable, developing PGC.

In the case of fresh zebrafish (*Danio rerio*) samples from which PGC were isolated for transplantation, demonstrated that the yield of germ-line chimeras was around 5.7 to 32 %. (Kawakami et al., 2010) This range is similar to that observed in the present study (16.5%). The previous and present results showed that cryopreserved blastomeres could differentiate into PGC and they did not lose their migration ability to the genital ridges. It is possible that changes in post-thaw blastomere incubation periods may promote PGC differentiation and further improve our results.

A single PGC from the *Danio albolineatus* was successfully transplanted into *Danio rerio* and the host species produced heterospecific gametes (Saito et al., 2008). As discussed by many authors (Yoshizaki et al. 2003; Yamaha et al., 2003; Saito et al., 2008), successful production of chimeras may be facilitated when using closely-related species as hosts. In our case, in order to establish a protocol of production of germ-line chimeras using cryopreserved blastomeres, we performed intra-specific transplantation and the PGC were able to migrate and colonize to the genital ridges. This result suggested that our procedure may be effective for gamete production from donor PGC derived from cryopreserved blastomeres of loach.

In conclusion, we defined an effective protocol for cryopreservation of loach blastomeres. We also developed an improved method to produce germ-line chimeras from the cryopreserved

291 blastomeres integrating short-term culture and transplantation of differentiated PGC. These
292 results are applicable for cryobanking and restoration of genetic resources from cryopreserved
293 blastomeres.
294
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375 Tables
376
377 Table 1. Development of loach chimeric embryos by transplantation of cryopreserved blastomeres immediately after thawing. Number
378 within parenthesis indicates the number of embryos with primordial germ cells in the genital area.
379

Treatment	n	Unfertilized	Blastula	Gastrula	10 somite	Hatched embryos	
						Normal	Abnormal
Intact control	1,112	25.9 ± 11.3%	74.1 ± 11.3%	72.2 ± 12.9%	70.1 ± 14.0%	67.6 ± 13.2%	3.1 ± 1.4
Dechorionated	386	28.3 ± 11.6%	71.7 ± 11.6%	71.2 ± 12.3%	69.7 ± 14.2%	64.6 ± 12.0%	2.9 ± 1.2
Chimera	264	-	100.0 ± 0.0%	14.9 ± 5.9%	4.5 ± 3.5% (4)	1.4 ± 1.9% (1)	1.7 ± 1.4% (2)

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381

382 Table 2. Development of loach chimeric embryos by transplantation of green fluorescence protein-positive blastomeres. Number
383 within parenthesis indicates the number of embryos with primordial germ cells in the genital ridge.
384

Treatment	n	Unfertilized	Blastula	Gastrula	10 somite	Hatched embryos	
						Normal	Abnormal
Intact control	653	9.8 ± 8.5%	90.2 ± 8.5%	87.8 ± 8.1 %	86.6 ± 8.9 %	81.71 ± 13.1%	4.86 ± 6.2%
Dechorionated	472	9.7 ± 7.9%	90.3 ± 7.9 %	89.3 ± 9.3 %	84.3 ± 15.5 %	79.2 ± 15.4 %	5.15 ± 3.5%
Chimera	206	-	100.0 ± 0.0 %	92.1 ± 7.4 %	90.1 ± 10.9 %	80.2 ± 13.6% (28)	9.5 ± 2.5% (5)

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Figure captions:

Figure 1. Apparatus used to collect cryopreserved blastomeres, prepared using an oil microinjector (Celltram Vario, Eppendorf, Hamburg, Germany) and a 1-mL syringe barrel. The pipette holder was attached to a micromanipulator (M-152, Narishige, Tokyo, Japan). Blastomere suspension was centrifuged in a 0.5-mL microtube, and the cells were collected from the bottom using an inverted capillary micropipette connected to the apparatus. Arrows indicates the flow of oil pumping for cell collection.

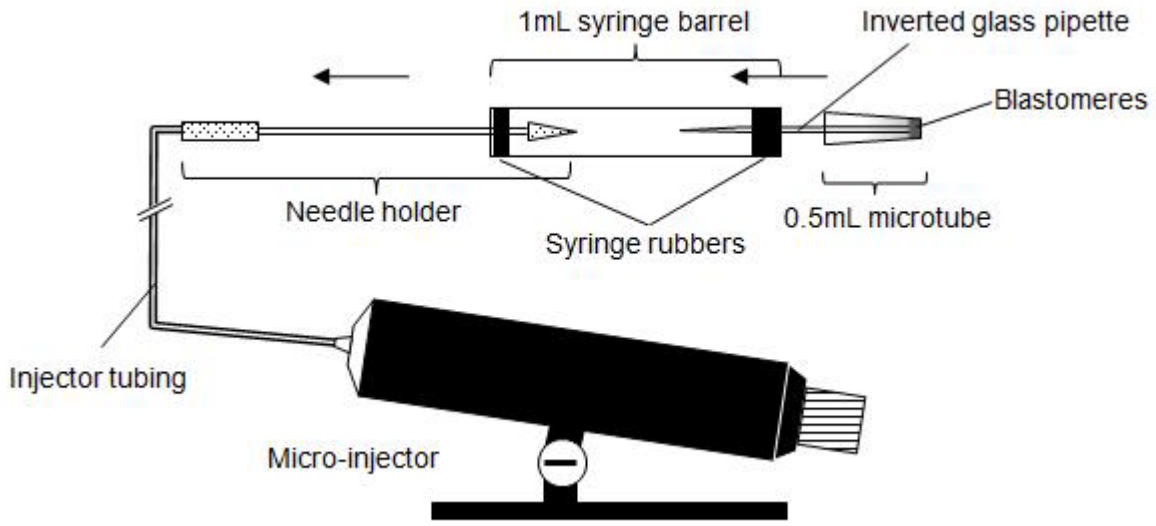
Figure 2. Viability of cryopreserved blastomeres using cryo-solutions containing increasing concentrations of dimethyl sulfoxide in Eagle's minimum essential medium. Percentage of live and dead cells was assessed by propidium iodide fluorescent staining. Columns without a common letter differ as determined by the Tukey test ($P < 0.05$).

Figure 3. Viability of cryopreserved blastomeres using cryo-solutions containing 10% dimethyl sulfoxide in Eagle's minimum essential medium and increasing glycerol additions. Percentage of live and dead cells was assessed by propidium iodide fluorescent staining. Columns without a common letter differ as determined by the Tukey test ($P < 0.05$)..

Figure 4. Viability of cryopreserved blastomeres using cryo-solutions containing 10% dimethyl sulfoxide in Eagle's minimum essential medium and various external cryoprotectants and concentrations. Control fresh refers to non-cryopreserved blastomeres and in control the

408 blastomeres were cryopreserved without external cryoprotectant (10% dimethyl sulfoxide only).
409 Percentage of live and dead cells was assessed by propidium iodide fluorescent staining.
410 Columns without a common letter differ as determined by the Tukey test ($P < 0.05$)..
411
412 Figure 5. Embryos transplanted using cryopreserved, thawed and incubated blastomeres. Left
413 images show embryos at 12-somite stage (A, C, E). Right images show hatched larvae (B, D, and
414 F). Host (C and D) and donor primordial germ cells (PGC) (E and F) were labeled by injection of
415 DsRed and GFP-*nos*1 3'UTR mRNA, respectively, and observed by fluorescence. Chimeric
416 embryo observed under a normal light (A and B). Histological sections of transplanted PGC by
417 biotin-streptavidin-horseradish peroxidase complex and 3,3´-diaminobenzidine substrate and
418 then stained with hematoxylin and eosin (G and H). Arrows indicate PGC. No = notocord; p =
419 pronephric duct, and y = yolk.
420

421 Figure 1.



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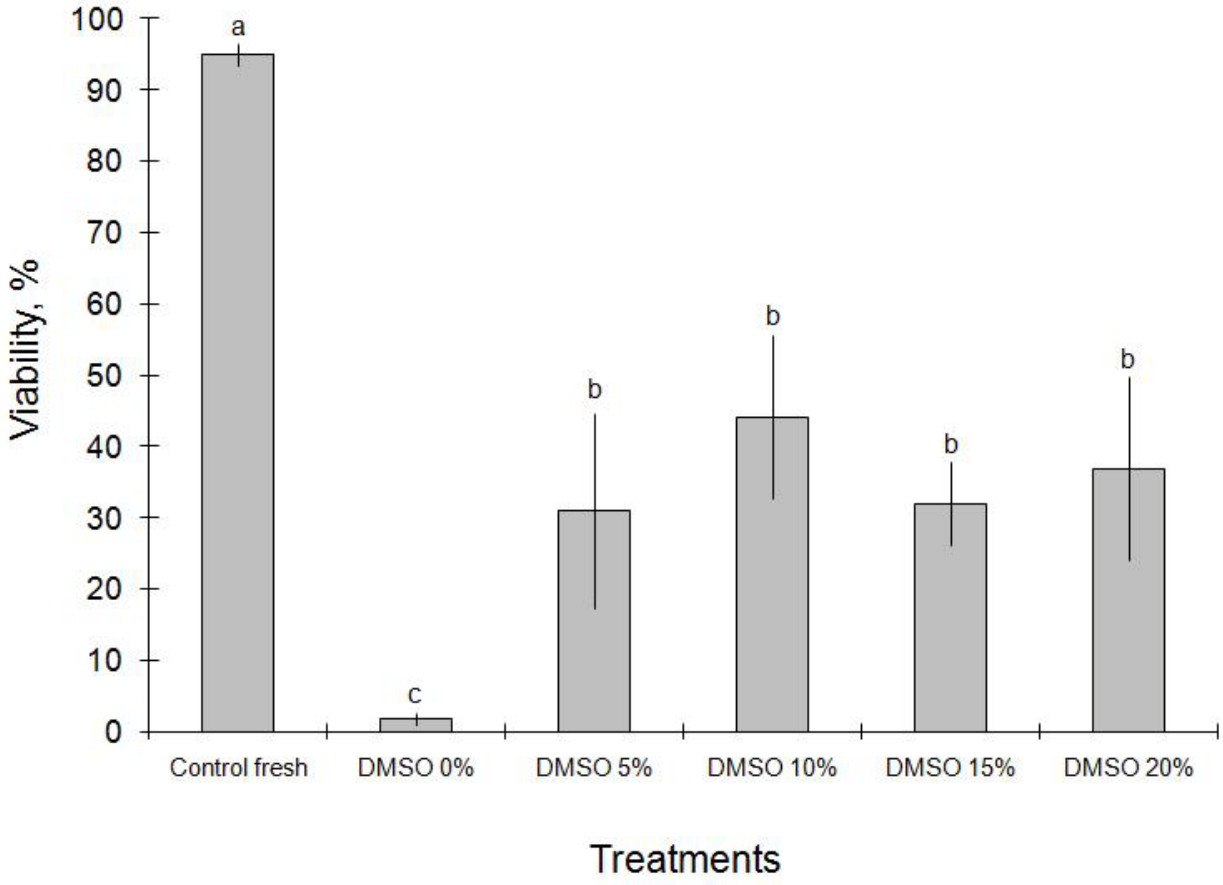
424 Figure 2.



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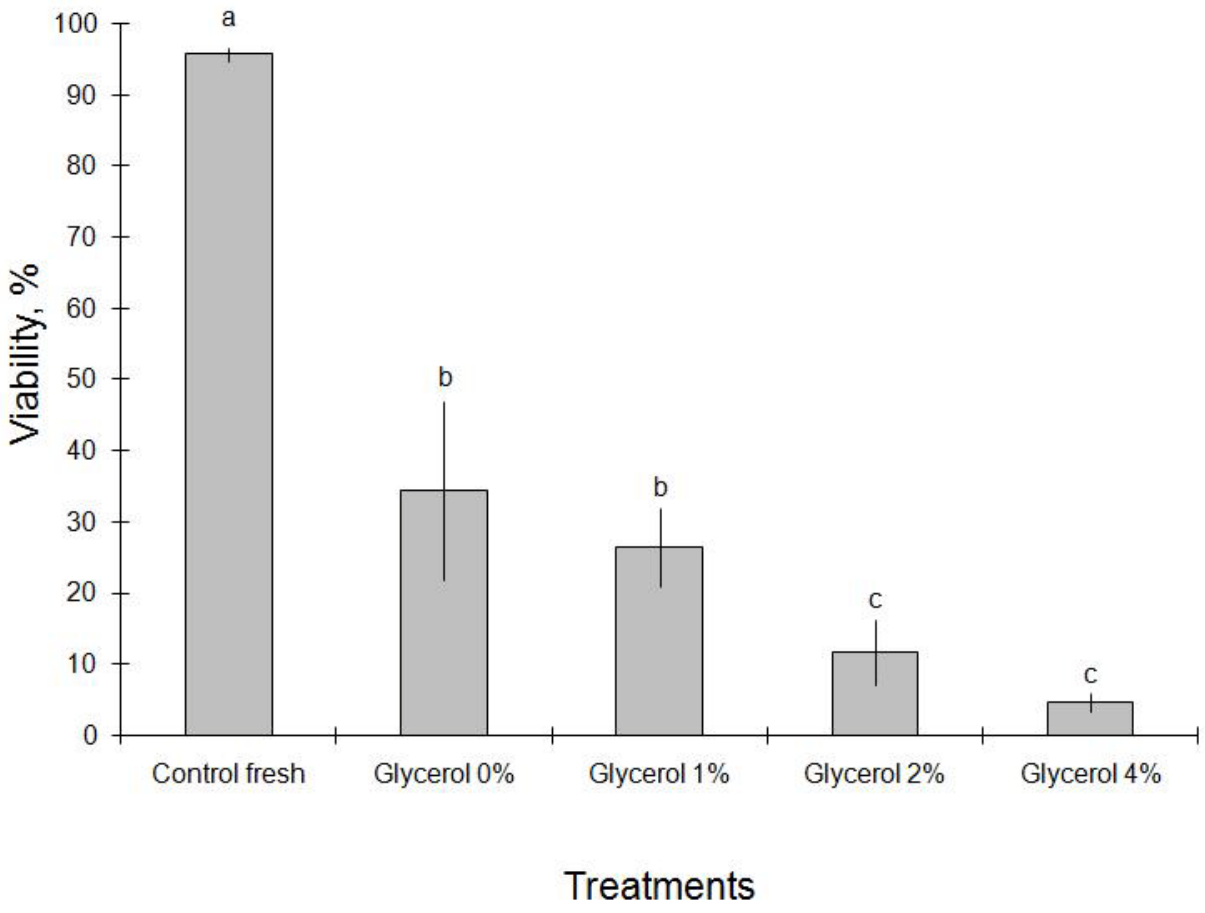
428 Figure 3.



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431 Figure 4.

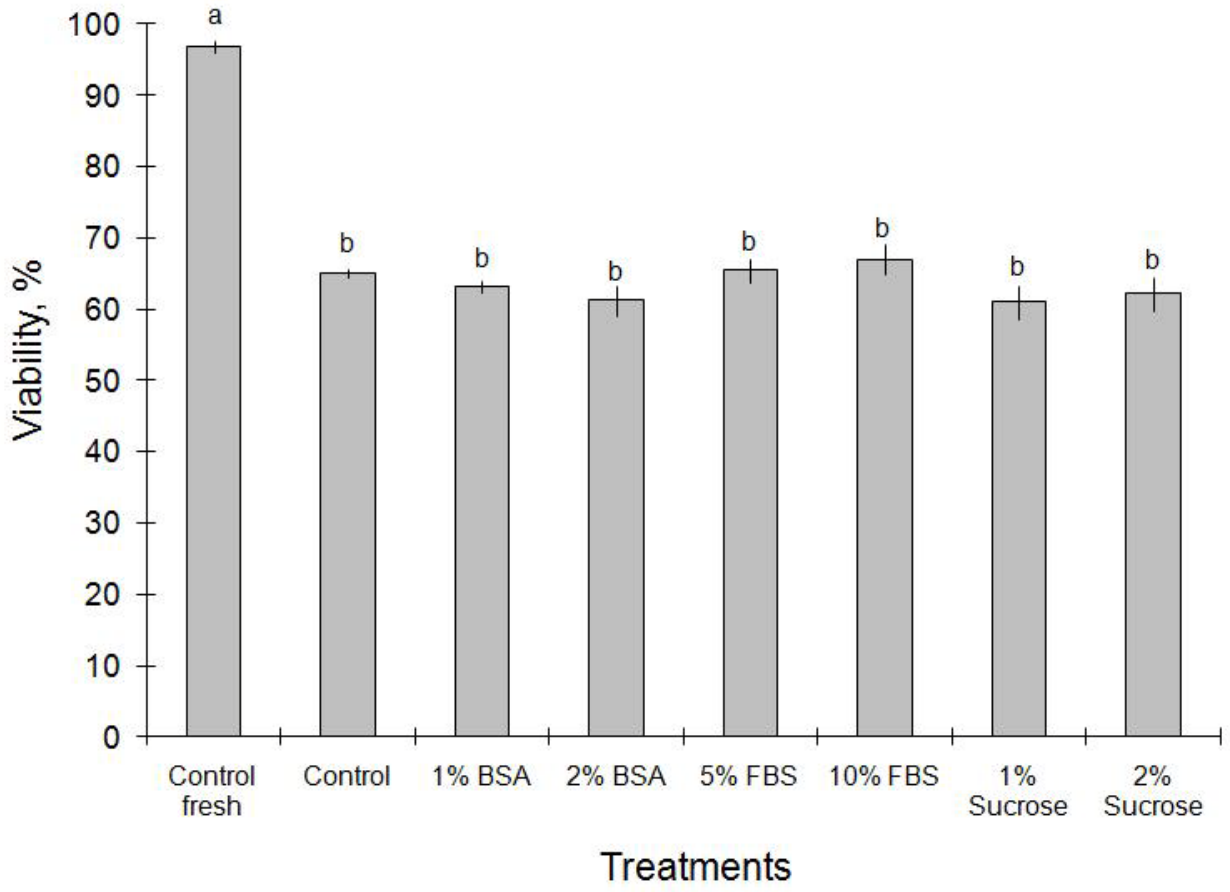

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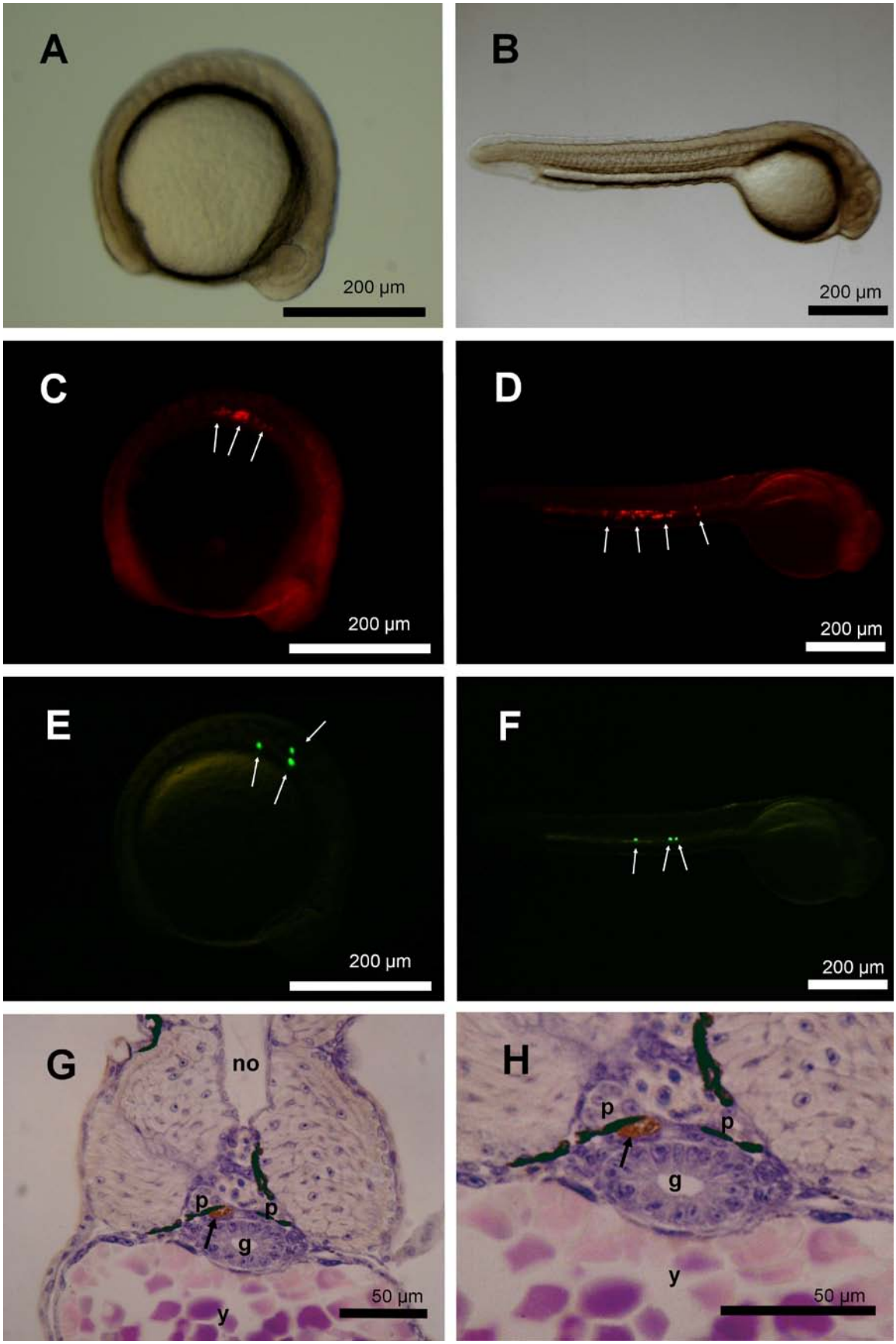
Fig. 5.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