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Effects of cryopreservation on the survival rate of the seven-band grouper (*Epinephelus septemfasciatus*) embryos

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ABSTRACT

The effects of cryopreservation and the vitrification solution on the embryo hatchability of the seven-band grouper *Epinephelus septemfasciatus* were evaluated in this study. Six small molecule cryoprotectants (PG, MeOH, Gly, DMF, DMSO and EG) and four macromolecular cryoprotectants (glucose, fructose, sucrose and trehalose) were used to determine the embryo toxicity levels. Results showed that the embryo survival rate was higher when the PM (24% PG + 16% MeOH):Gly ratios were 3:1 and 4:1. Further experiments showed that the embryo survival rates in PMG3S (35% PMG3 + 5% sucrose) and PMG3T (35% PMG3 + 5% trehalose) were relatively higher, which are 29.24 ± 10.81% and 27.01 ± 3.39%, respectively. When treated with PMG3S and PMG3T by using 5-step method, embryos at somite stage and tail-bud stage shrank in the first 6 min and gradually recovered in volume to the original. This indicated the successful permeation of the vitrification solutions into cells. Then, embryos at the embryoid body formation stage, the somite stage and the tail-bud stage were cryopreserved with PMG3S and PMG3T. In total, 82 floating embryos were obtained, 14 of which developed further, with 8 embryos at the tail-bud stage developing to the heartbeat stage, 4 embryos at the body formation stage development to the somite stage, and 2 embryos at the somite stage hatched to larval fish.

1. Introduction

The seven-band grouper, *Epinephelus septemfasciatus* (Perciformes: Serranidae: Epinephelinae), is a protogynous hermaphrodite species. In China, the seven-band grouper is mostly distributed in the South China Sea and East China Sea [1]. Among the 30 grouper species in China's coastal waters, the seven-band grouper inhabits the highest latitude and is the only species found along the coast of the Yellow Sea [2]. In the 1960's there were only a few reports published related to the seven-band grouper [3]. And, the studies on the seven-band grouper have been increasing in recent years, being focused on the reproductive biology [4], early development [5], artificial reproduction [6], viral nervous necrosis (VNN) [7], muscle composition analysis [8], population genetic diversity [9] and chromosome karyotype analysis [10]. In addition, methods for the cryopreservation of the sperm of the seven-band grouper have been developed [11,12]. Due to the high market value of wild-caught fish [4], the seven-band grouper is considered as a potential candidate for aquaculture and sea ranching in Japan [4,13]. As viral nervous necrosis of the grouper is one of the most serious diseases in aquaculture worldwide [14], it affects grouper aquaculture a lot. Therefore, the storage of gametes and embryos can facilitate genetic and breeding studies as well as enhance seed production [15], [16].

Successful embryo cryopreservation and transplantation studies have already been published in cows [17], goats [18], equines [19] and mice [20]. However, there has not been a successful method developed for the cryopreservation of fish embryos [21], for fish embryos are more sensitive to cryoprotectants due to their bigger size and higher amounts of yolk and water. Vitrification cryopreservation is a technology that the embryo's metabolic activities are

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stopped at temperature of −196 °C and then restored after thawing by using special protective measures and a cooling process. In this process, solution is transformed into a stable amorphous glass, and maintained in a solidified form by using ultrarapid cooling, which prevents ice crystal formation and chilling injury [22]. As an important method for germplasm resource preservation, vitrification cryopreservation has been modestly successful for embryo cryopreservation and transplantation in mammals. In the present study, we aim to explore the vitrification method for cryopreservation of the seven-band grouper embryos, which would provide technological approaches for fish germplasm preservation.

2. Materials and methods

2.1. Embryo collection

Eggs and sperm were obtained from the seven-band grouper (*E. septemfasciatus*) females and males (3.4–4.8 kg body weight, 30 females and 20 males) from Laizhou Mingbo Aquatic Products Limited Company (China) (lat: 37° 7′N, long: 119° 56′E). The sperm were collected from the genital pore by using a 5 mL straw with a gentle abdominal massage. The eggs were collected in 2 L plastic cubes by using the same method.

Fertilization was performed by using 10 μL of sperm for each 1 mL of eggs. The sperm was spread over the eggs for 5 min, and the sperm motility was activated with 1 mL of seawater. After 10 min activation, more seawater was added to the eggs (20 times larger than its initial volume). The fertilized eggs were washed and incubated in a incubator at 21–23 °C. Next, as the embryonic cell differentiation occurred, normal eggs and embryos floated at the surface, whereas abnormal eggs or embryos did not. These abnormalities often appeared as embryonic body malformation, increasing body pigmentation, or a white appearance of the yolk. Wherein, the malformation took the form of scoliosis of the embryo's body, in which it formed a semicircle shape: the tail got shorter and reversed [23,24]. Pigmentation referred to the color of the whole embryo, in which the pigmentation of the tail of the embryonic body increased compared with the normal embryonic body. Well-developed embryos were picked up as they float on the surface, and then incubated in a constant temperature incubator at 22 °C. Embryos at the embryoid body formation stage (10 h post-fertilization, hpf), the somite stage (30 hpf), and the tail-bud stage (36 hpf) were collected for toxicity test and vitrification cryopreservation test.

2.2. Cryoprotectants

The diluent BS2 [25] was prepared by using NaCl 24.72 g/L, $CaCl_2 \cdot 2H_2O$ 1.46 g/L (Tianjin Regent Chemicals Co., Ltd., Shanghai China), KCl 0.865 g/L, $MgCl_2 \cdot 6H_2O$ 4.86 g/L and $NaHCO_3$ 0.19 g/L (Sinopharm Chemical Reagent Co., Ltd., Shanghai China). Distilled water was used in the diluent preparation.

In the present study, 1, 2 - propylene glycol (PG), ethylene glycol (EG), dimethyl sulfoxide (DMSO), methanol (MeOH), glycerol (Gly), sucrose (S) (Sinopharm Chemical Reagent Co., Ltd., Shanghai China), dimethyl formamide (DMF), fructose (F), trehalose (T) (Amresco Co., Ltd., USA) and D-glucose (G) (Beijing Solarbio Science and Technology Co., Ltd., Beijing China) were used as the experimental cryoprotectants. All the cryoprotectants were combined with BS2 for the preparation of the experimental vitrification solutions (Table 1).

2.3. Toxicity tests of individual permeable cryoprotectants

Several vitrification solutions were prepared by using the BS2 diluent (v/v) to the final concentration of 40% PG, MeOH, Gly, DMF, DMSO and EG, respectively (Table 1). Embryos (100–150 fertilized eggs) at the somite stage were placed in the 40% vitrification solutions and equilibrated for 30 min at room temperature according to a five-step method [26] (Table 2). Briefly, embryos were first incubated in 1/4 diluted vitrification solution for 6 min, and then in 1/3, 1/2 and 2/3 diluted vitrification solutions for 6 min each. After equilibrium with undiluted vitrification solution for 6 min, the embryos were washed for 15 min in 0.125 M sucrose eluent and then carefully rinsed with seawater to remove any residual sucrose eluent. Finally, the embryos were incubated with seawater in petri dishes in a constant temperature incubator (22 °C), and the hatchability of each group was recorded. Each treatment was conducted in three replicates (n = 3). The water in the petri dishes was changed on a regular basis during the incubation period.

2.4. Combination of two types of small molecule cryoprotectants

By using BS2 as the diluent, PG was combined with five solutions in a 3:2 ratio (v/v) and then prepared as 40% cryoprotectant solutions (Table 1). The five solutions included MeOH, Gly, DMF, DMSO, and EG, with which the cryoprotectants were referred to as PM, PGly, PDF, PDO and PE, correspondingly. About 100 embryos were treated with the five cryoprotectants following the five-step equilibrium method. Then the treatment group was eluted in 0.125 M sucrose and transferred in seawater for incubation. Meanwhile, the same amount of fertilized eggs were collected without any treatment, used as the control. The test demonstrated that PM were less toxic, thus, PM was selected for further study. 40%, 35% and 30% PM solutions were prepared by combining PG and BS2 in different proportions for cryoprotectants preparation, respectively (Table 1). As described above, embryos were equilibrated, eluted and incubated, and the hatchability of each group was recorded (n = 3).

2.5. Combination of three types of small molecule cryoprotectants

Based on the BS2 diluent, four different 35% cryoprotectant solutions (PMG1, PMG2, PMG3 and PMG4) were prepared with PM (PG:MeOH = 3:2) and Gly at 1:1, 2:1, 3:1 and 4:1 ratios (Table 1). Using the methods described previously, the somite embryos were equilibrated, eluted and incubated before the hatchability of the groups was determined (n = 3). In addition, the method of treatment was the same as above, as was the establishment of a control group.

2.6. Combination of small molecule cryoprotectants with macromolecular cryoprotectants

Using the BS2 diluent, PMG3 was combined with four different macromolecular cryoprotectants (v/v), D-glucose (G), sucrose (S), fructose (F) and trehalose (T), and prepared as 40% cryoprotectant solutions, which were referred to as PMG3G, PMG3S, PMG3F and PMG3T in this study (Table 1). Using the methods as previously described, the somite stage embryos were equilibrated, eluted and incubated, and the hatchability of each group was recorded (n = 3). In addition, the method of treatment was the same as above, as was the establishment of a control group.

2.7. The effects of two vitrification solutions (PMG3S and PMG3T) on embryonic volume

200 embryos, either at somite stage or at tail bud stage, were used to test how vitrification solutions affected embryonic volume. After the removal of seawater, the embryos were treated with

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Table 1
Composition and vitrification ratio of all of the experimental vitrification solutions (n = 5).

Vitrification solution	Composition (%)	Vitrification ratio (%)		Thawing temperature/°C
		Cryopreservation	Thawing	
40% PG	40% PG + 60% BS2	86.67 ± 5.77	56.67 ± 5.77	37
40% MeOH	40% MeOH + 60% BS2	76.67 ± 5.77	13.33 ± 5.77	37
40% Gly	40% Gly + 60% BS2	53.33 ± 5.77	10.00 ± 8.66	37
40% DMF	40% DMF + 60% BS2	53.33 ± 11.54	9.00 ± 1.73	37
40% DMSO	40% DMSO + 60% BS2	36.67 ± 5.77	4.00 ± 1.73	37
40% EG	40% EG + 60% BS2	93.33 ± 5.77	40.00 ± 0.00	37
40% PM	24% PG + 16% MeOH + 60% BS2	76.67 ± 5.77	33.33 ± 5.77	37
40% PGly	24% PG + 16% Gly + 60% BS2	56.67 ± 11.55	16.67 ± 5.77	37
40% PDF	24% PG + 16% DMF + 60% BS2	53.33 ± 5.77	18.33 ± 5.77	37
40% PDO	24% PG + 16% DMSO + 60% BS2	36.67 ± 5.77	6.67 ± 2.89	37
40% PE	24% PG + 16% EG + 60% BS2	73.33 ± 11.54	43.33 ± 5.77	37
35% PM	21% PG + 14% MeOH + 65% BS2	53.33 ± 5.77	26.67 ± 11.55	37
30% PM	18% PG + 12% MeOH + 70% BS2	40.00 ± 0.00	9.00 ± 1.72	37
PMG1	10.5% PG + 7% MeOH + 17.5% Gly + 65% BS2	33.33 ± 5.77	4.67 ± 4.62	37
PMG2	13.998% PG + 9.332% MeOH + 11.67% Gly + 65% BS2	40.00 ± 0.00	4.00 ± 1.73	37
PMG3	15.75%P G + 10.5% MeOH + 8.75% Gly + 65% BS2	60.00 ± 0.00	30.00 ± 0.00	37
PMG4	16.8% PG + 11.2% MeOH + 7% Gly + 65% BS2	80.00 ± 0.00	4.00 ± 1.73	37
PMG3G	15.75% PG + 10.5% MeOH + 8.75% Gly + 5% Glucose + 60% BS2	43.33 ± 5.77	3.00 ± 1.73	37
PMG3S	15.75% PG + 10.5% MeOH + 8.75% Gly + 5% Surcose + 60% BS2	56.67 ± 11.55	41.67 ± 2.89	37
PMG3F	15.75% PG + 10.5% MeOH + 8.75% Gly + 5% Fructose + 60% BS2	70.00 ± 17.32	6.67 ± 5.77	37
PMG3T	15.75% PG + 10.5% MeOH + 8.75% Gly + 5% Trehalose + 60% BS2	66.67 ± 11.55	36.67 ± 5.77	37

Note: PG: 1,2-propylene glycol; MeOH: methyl alcohol; Gly: glycerin; DMF: dimethyl formamide; DMSO: dimethyl sulfoxide; EG: ethylene glycol. All vitrification solutions and combinations of cryoprotectants were prepared according to the volume ratio.

Table 2
Five-step equilibrium method used before the cryopreservation of embryos.

Vitrification solution	Equilibrium Time/min
1/4 Dilution	6
1/3 Dilution	6
1/2 Dilution	6
2/3 Dilution	6
Undiluted	6

PMG3S and PMG3T, respectively, according to the five-step method (Table 2). The images of 30 embryos were captured between each step on BDS300 inverted microscope (Chongqing Optec Instrument Co.,Ltd,China) using software (Scopephoto 3.0) for image recording and their diameters were measured by using Adobe Photoshop CS6. Embryos without any treatment were served as control. The embryonic volume was calculated by using the formula $V = 4/3\pi R3$ (V, embryonic volume; R, embryonic radius). The relative volume was obtained with the normalization of the embryonic volume in control group and the penetration of the vitrification solutions to the embryos were compared after each step during the treatment.

2.8. Embryo vitrification cryopreservation test

100–150 embryos at the embryoid body formation stage, the somite stage and the tail-bud stage were collected. After treated with the vitrification solutions PMG3S and PMG3T according to the five-step method (Table 2), embryos were sucked into straws along with the vitrification solution whose total volume was up to 2/3 straws (250 μL). Then both ends of the straw were sealed by using an alcohol lamp, and it was immediately immersed in an 800 mL vacuum cup filled with liquid nitrogen (LN) for at least 30 min. After cryopreservation, the straws were thawed using a 37 °C water bath for 30–50s. The thawed embryos were transferred to petri dishes containing the 0.125 M sucrose eluent and incubated at 22 °C for 15 min. Next, the sucrose eluent was carefully removed and the embryos were carefully washed 2–3 times in seawater (22 °C) to remove any residual sucrose eluent. Finally, 10–15 mL seawater was added to the petri dishes, and embryos were placed in a constant temperature incubator for cultivation. The hatchability of each group was recorded (n = 3). Meanwhile, the same amount of fertilized eggs without any treatment were collected as the control group and directly incubated for cultivation and measurement of hatchability.

2.9. Statistical analyses

The results were analyzed using a one-way analysis of variance (ANOVA). The results are shown as the mean ± standard deviation (SD). Differences between the treatments were detected by using the Student-Newman-Keuls (SNK) test in the SPSS software. The significance level was set at $P < 0.05$.

3. Results

3.1. Effect of individual small molecule cryoprotectants on embryo hatchability

Seven-band grouper embryos at the somite stage were treated with 40% solutions of PG, MeOH, Gly, DMF, DMSO and EG, and the hatchabilities of the embryos from the six vitrification solutions were 15.96 ± 1.72%, 5.95 ± 0.45%, 3.62 ± 0.33%, 5.26 ± 0.94%, 4.03 ± 1.17% and 0.00 ± 0.00%, respectively. As can be seen, the 40% PG treatment resulted in the greatest hatchability. There were significant differences between the treatment groups and the control (83.81 ± 3.23%) ($P < 0.05$) (Fig. 1).

3.2. Effect of combination of two types of small molecule cryoprotectants on embryo hatchability

PG was combined with MeOH, Gly, DMF, DMSO and EG to generate 40% cryoprotectant solutions, which were referred to as PM, PGly, PDF, PDO and PE, and the embryos were treated with these different solutions. The results showed that the hatchability rates of the treatment groups for the PM, PGly, PDF, PDO and PE vitrification solutions were 33.80 ± 8.27%, 10.72 ± 0.77%, 26.15 ± 4.39%, 15.03 ± 2.01% and 2.07 ± 1.90%, respectively. It can be seen that 40% PM resulted in the greatest hatchability and 40% PE in

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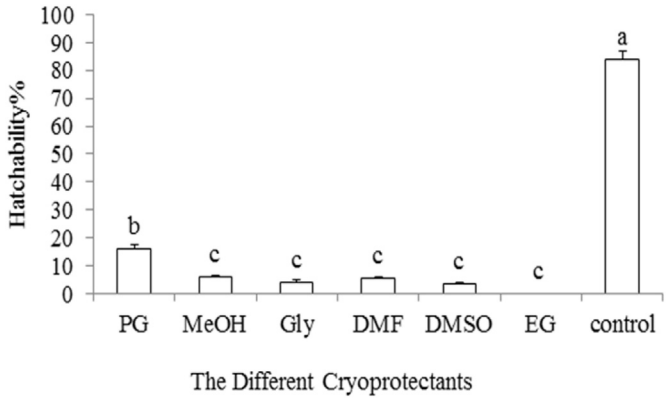

Fig. 1. Hatchability of embryos (somite stage) treated with individual small molecule cryoprotectants. Values are presented as means ± S.D. (n = 3) and a, b, and c indicate significant differences among the treatments, as determined by one-way ANOVA followed by Student-Newman-Keuls test ($P < 0.05$). PG: 1,2-propylene glycol; MeOH: methanol; Gly: glycerol; DMF: dimethyl formamide; DMSO: dimethyl sulfoxide; EG: ethylene glycol; Control: control group (fresh embryos).

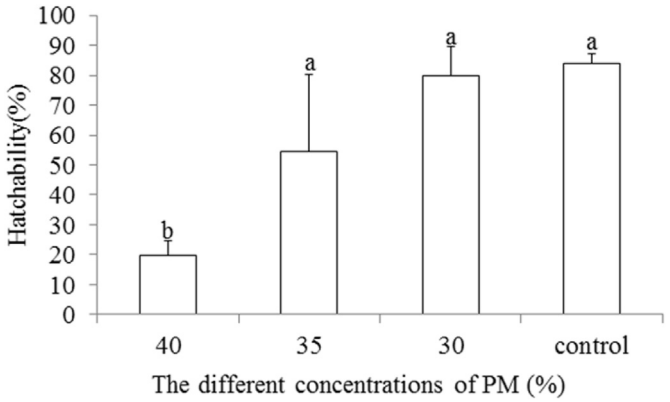

Fig. 3. Hatchability of embryos (somite stage) treated with different concentrations of PM. Values are presented as means ± S.D. (n = 3). a and b indicate significant differences among the treatments, as determined by one-way ANOVA followed by Student-Newman-Keuls test ($P < 0.05$).

the lowest. Furthermore, there were significant differences between the results for the control group and each experimental group ($P < 0.05$) (Fig. 2).

To determine the optimal PM concentration, the embryos of the treatment groups were treated with 40%, 35%, and 30% PM vitrification solutions. The results showed that there was a significant difference between the 40% PM treatment and the control group ($P < 0.05$), and that there were no significant differences between the others and the control group ($P > 0.05$) (Fig. 3).

3.3. Effect of combination of three types of small molecule cryoprotectants on embryo hatchability

PM (PG:MeOH = 3:2) and Gly were used in the ratios of 1:1, 2:1, 3:1 and 4:1 to prepare four different 35% vitrification solutions, which were referred to as PMG1, PMG2, PMG3 and PMG4 in this study. The treatment groups had the above vitrification solutions applied, and the results showed that the hatchabilities of the embryos treated with PMG3 and PMG4 were 50.10 ± 34.47% and 23.37 ± 3.69%, respectively. There were significant differences between these results and the result for the control group ($P < 0.05$). The hatchabilities of the embryos treated with PMG1 and PMG2 were 0.00 ± 0.00% and 8.98 ± 3.82%, respectively, and there were significant differences between these results and the result for the control group ($P < 0.05$) (Fig. 4).

3.4. Effect of the combination of small molecule cryoprotectants with macromolecular cryoprotectants on embryo hatchability

The embryos in the treatment groups were treated with the vitrification solutions, as presented above, and then cryopreserved. The hatchabilities of the embryos that were treated with PMG3G, PMG3S, PMG3T, and PMG3F were 24.38 ± 10.76, 29.24 ± 10.81, 27.01 ± 3.39 and 20.43 ± 8.97%, respectively, and there were significant differences between these results and the result for the control group (Fig. 5) ($P < 0.05$). Embryos at three different development stage (embryoid body formation stage, somite stage and tail-bud stage) were treated with PMG3S and PMGST vitrification solutions and cryopreserved. The results indicated that the hatchability of the embryos at the three

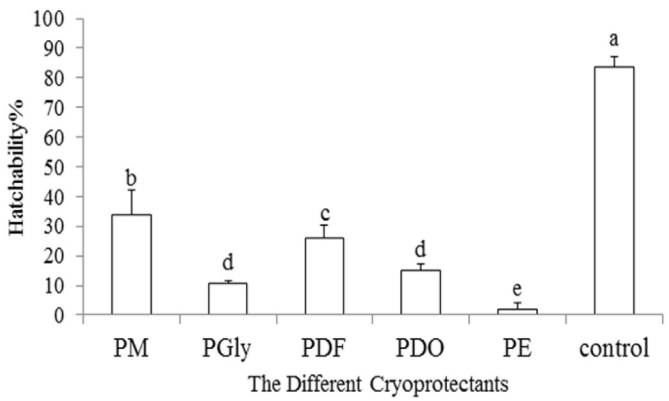

Fig. 2. Hatchability of embryos (somite stage) treated with five mixed cryoprotectants: PM, PGly, PDF, PDO and PE. Values are presented as means ± S.D. (n = 3) and a, b, c, d, and e indicate significant differences among the treatments, as determined by one-way ANOVA followed by Student-Newman-Keuls test ($P < 0.05$). PM: 24% PG + 16% MeOH + 60% BS2; PGly: 24% PG + 16% Gly + 60% BS2; PDF: 24% PG + 16% DMF + 60% BS2; PDO: 24% PG + 16% DMSO + 60% BS2; PE: 24% PG + 16% EG + 60% BS2; Control: control group (fresh embryo). PG: 1,2-propylene glycol; MeOH: methanol; Gly: glycerol; DMF: dimethyl formamide; DMSO: dimethyl sulfoxide; EG: ethylene glycol.

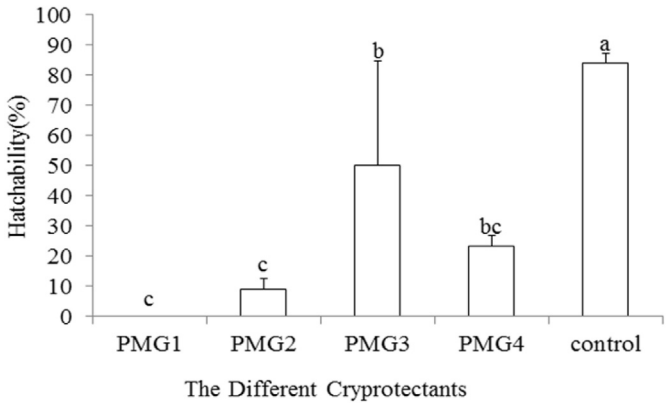

Fig. 4. Hatchability of embryos (somite stage) treated with different concentrations of PMG1-4. Values are presented as means ± S.D. (n = 3) and a, b, and c indicate significant differences among the treatments, as determined by one-way ANOVA followed by Student-Newman-Keuls test ($P < 0.05$). PMG1: 10.5% PG + 7% MeOH + 17.5% Gly + 65% BS2; PMG2: 13.998% PG + 9.332% MeOH + 11.67% Gly + 65% BS2; PMG3: 15.75%P G + 10.5% MeOH + 8.75% Gly + 65% BS2; PMG4: 16.8% PG + 11.2% MeOH + 7% Gly + 65% BS2. Control: control group (fresh embryo); PG: 1, 2- propylene glycol; MeOH: methanol; Gly: glycerol.

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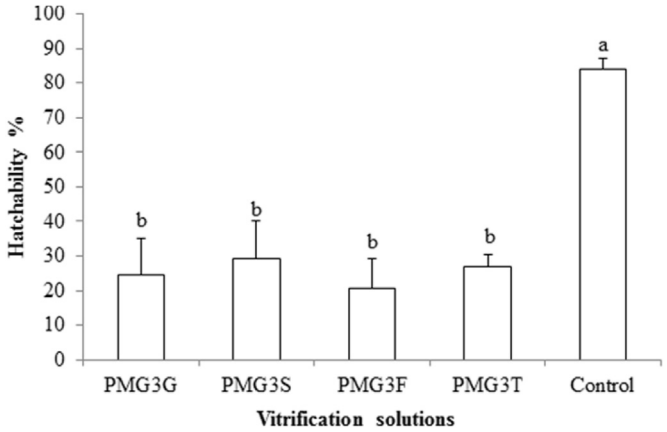

Fig. 5. Hatchability of embryos (somite stage) treated with three mixed small molecule cryoprotectants in combination with non-permeable cryoprotectants. Values are presented as means ± S.D. (n = 3). a and b indicate significant differences among the treatments, as determined by one-way ANOVA followed by Student-Newman-Keuls test ($P < 0.05$). The hatchability rates of PMG3S and PMG3T were relatively higher, but not significantly different than the other treatment groups. For all of the treatment groups, their values were significantly different than the control group ($P < 0.05$). PMG3G: 15.75% PG + 10.5% MeOH + 8.75% Gly + 5% glucose + 60% BS2; PMG3S: 15.75% PG + 10.5% MeOH + 8.75% Gly + 5% surcose + 60% BS2; PMG3F: 15.75% PG + 10.5% MeOH + 8.75% Gly + 5% fructose + 60% BS2; PMG3T: 15.75% PG + 10.5% MeOH + 8.75% Gly + 5% trehalose + 60% BS2; Control: control group (fresh embryo). PG: 1,2-propylene glycol; MeOH: methanol; Gly: glycerol.

developmental stages treated with the PMG3S and PMGST vitrification solutions but not cryopreserved were 60.11 ± 29.55, 21.26 ± 4.59 and 29.24 ± 10.81% and 64.93 ± 20.65, 21.11 ± 13.43 and 27.01 ± 3.40%, respectively (Fig. 6; A and B). There were significant differences in the hatchabilities between all the treatment groups and the control ($P < 0.05$).

3.5. The variation of embryonic volume with the treatment of two vitrification solutions

Fig. 7A showed the volume of embryos at somite stage changed with the treatment of two vitrification solutions from 0 to 30 min. The original embryonic volume was set as the control, which was 1 ± 0.003. After treated with PMG3S and PMG3T for 6 min, embryos shrank and the volume reduced significantly by 0.934 ± 0.002 and 0.946 ± 0.002, respectively ($p < 0.05$). Embryos started to recover after 12 min, whose volume was 0.967 ± 0.002 (PMG3S treated) and 0.979 ± 0.002 (PMG3T treated). The embryonic volume increased significantly by 1.029 ± 0.003, 1.051 ± 0.003, and 1.062 ± 0.003 as much as the original ($p < 0.05$) when the embryos were treated with PMG3S for 18min, 24 min, and 30 min. Similarly, the embryonic volume were significantly larger than the original by 1.048 ± 0.002, 1.051 ± 0.002, and 1.057 ± 0.004 folds ($p < 0.05$) when the embryos were treated with PMG3T on the same time scale. All these results showed the same variation trend of embryos at somite stage with the treatment of PMG3S and PMG3T that embryos shrank most in 6 min, gradually recovered after 12 min, and regained the original volume and significantly expanded in 18–30 min ($p < 0.05$).

Fig. 7B showed the volume of embryos at tail-bud stage changed with the treatment of two vitrification solutions from 0 to 30 min. The original embryonic volume was set as the control, which was 1 ± 0.003. After treated with PMG3S and PMG3T for 6 min, embryos shrank most, whose volume significantly reduced by 0.925 ± 0.002 and 0.927 ± 0.002 folds, respectively ($p < 0.05$). The embryonic volume was 0.972 ± 0.001, 0.983 ± 0.001, 0.993 ± 0.001, 0.994 ± 0.004 folds as much as the original when the embryos were

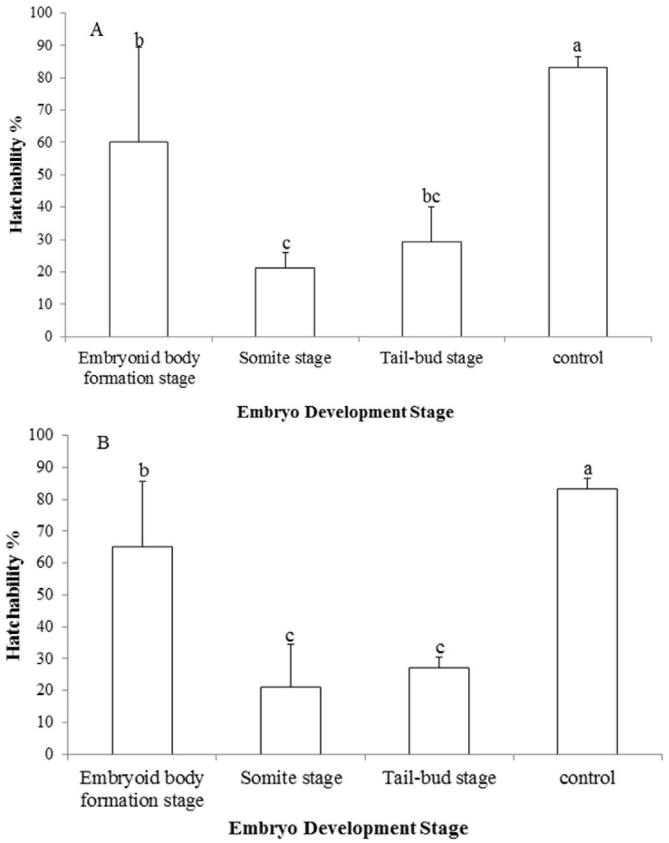

Fig. 6. Hatchability of embryos at three different stages (embryoid body formation stage, somite stage and tail-bud stage) treated with of PMG3S (A) and PMG3T (B) vitrification solutions. Values are presented as means ± S.D. (n = 3) and a, b, and c indicate significant differences among the treatments, as determined by one-way ANOVA followed by Student-Newman-Keuls test ($P < 0.05$). PMG3S: 15.75% PG + 10.5% MeOH + 8.75% Gly + 5% surcose + 60% BS2; PMG3T: 15.75% PG + 10.5% MeOH + 8.75% Gly + 5% trehalose + 60% BS2; Control: control group (fresh embryo). PG: 1,2-propylene glycol; MeOH: methanol; Gly: glycerol.

treated with PMG3S for 12 min, 18min, 24 min, and 30 min. There is no significant difference between the embryonic volume in 24min or 30 min and the control ($p > 0.05$). When the embryos were treated with PMG3S for 12 min, 18min, 24 min, and 30 min, the embryonic volume was 0.961 ± 0.002, 0.972 ± 0.002, 0.992 ± 0.002, 0.998 ± 0.002 folds as much as the original. No significant difference was shown between the embryonic volume in 24 min or 30 min and the control ($p > 0.05$). All these variation trends were similar with those of the embryos at somite stage treated with PMG3S and PMG3T; however, after the treatment by using five-step method, the embryonic volume has no significant difference with the control ($p > 0.05$).

3.6. Embryo vitrification cryopreservation

Using all the above vitrification solutions, the cryopreservation test was performed, and the results indicated that the chorion almost shrunk and ruptured, the cell contents spilled, the embryonic body structure was not clear (some even beginning to dissolve), and the pigmentation of the embryonic body increased to appear white in most cryopreserved embryos. The results (Table 3) showed that some embryos survived after cryopreservation when using the vitrifcation solutions PMG3S and PMG3T. In 78 embryos at the somite stage cryopreserved with PMG3T, 6 floating embryos were obtained after thawing. Some showed no difference with

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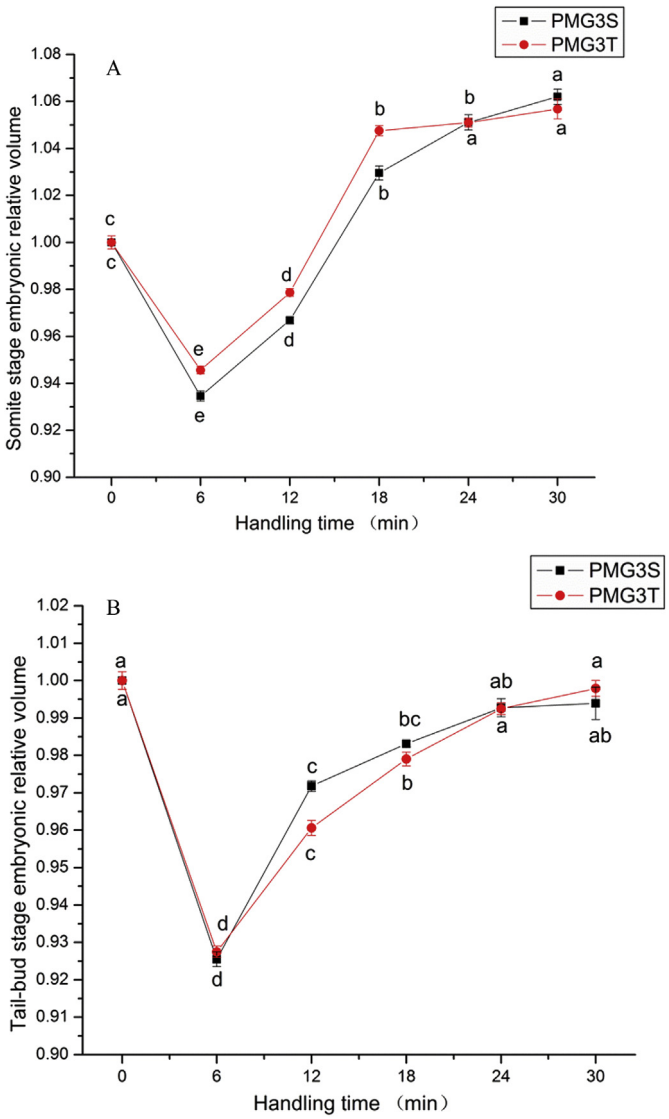

Fig. 7. The variation of embryonic volume with the treatment of two vitrification solutions PMG3S and PMG3T for 30 min at 22 °C. A presents the variation in the embryos at somite stage and B presents the variation in the embryos at tail-bud stage. Values are presented as means ± S.D. (n = 3) and a, b, and c indicate significant differences among the treatments, as determined by one-way ANOVA followed by Student-Newman-Keuls test ($P < 0.05$).

normally cultured embryos (Fig. 8I); while the other floating embryos stopped development before the tail-bud stage, in which malformations were observed, such as the increasing pigmentation (Fig. 8VI). With the 6 floating embryos, 2 developed to larval fish with normal heartbeats and then survived for 13 h. One was morphologically identical to the control during the development (Fig. 8II), while the other had a curved embryonic body and a pigmented tail (Fig. 8III). In 102 embryos at the tail-bud stage cryopreserved with PMG3T, 8 embryos floated, developing to the heartbeat stage and then survived for 11 h. In 247 embryos at the embryoid body formation stage cryopreserved with PMG3S, 68 floating embryos were observed, and only 4 floating embryos developed to the somite stage and then survived for 7 h (Fig. 9). The somite of the embryo was observed clearly under a microscope (Fig. 9 III and IV).

4. Discussion

Vitrification, an ice-free cryopreservation technology, is based on program freezing, which could improve the concentration and osmotic pressure of the cryoprotectant during the cryopreservation process. Since the first report of successful embryo cryopreservation was in mouse via vitrification cryopreservation [27], more studies have been focused on the development of vitrification technology. Vitrification solutions are the media used in embryo cryopreservation, and selecting an appropriate cryoprotectant is a key step in the cryopreservation process [28]. Moreover, cryoprotectants are always vitrified in high concentration, but meanwhile it may cause the damage in embryos. Thus, the toxicity of the cryoprotectant should be tested in advance and an appropriate concentration needs to be decided [29]. In the present study, seven-band grouper embryos were cryopreserved with six different types of cryoprotectants (PG, MeOH, Gly, DMF, DMSO and EG) that are small molecule cryoprotectants with low molecular weights. They ensure the timely dehydration of the cell and lower the freezing point of the solution as well, which allows embryos to dehydrate longer and therefore prevents the formation of ice crystals [30]. Our results show that the survival rate of the seven-band grouper embryos was the highest with 40% PG (15.96 ± 1.72%), as compared with the other cryoprotectants (Fig. 1). This result indicated that PG had a relatively lower toxicity level and a stronger ability for vitrification, which was consistent with the study by Hua et al. [31]. But when a vitrification solution contains only one single cryoprotectant, the vitrification degree is relative low if the concentration of the cryoprotectant is higher than 45% [32]. This situation has been improved by using the combination of two different cryoprotectants in a vitrification solution, so that the concentration of each cryoprotectant is reduced [32]. It was found the survival rate of embryos was higher when PG and MeOH (PM) were combined as one vitrification solution (33.80 ± 8.27%) (Fig. 2), as compared with those treated with PG alone. Similarly, Wang et al. [33] reported that the toxicity of the mixed PG and MeOH (PM) was relatively low during the cryopreservation of the Japanese flounder embryos by using a programmed freezing method. In addition, the tolerance to different cryoprotectants varies in different fish embryos [34]. Kasai et al. found that 97–98% mouse morule normally developed after cryopreservation by using EG as an intracellular fluid cryoprotectant and then thawing [35]. Zhu et al. [36] used EFS40 to cryopreserve expanded mouse blastocysts after pretreating them with 10% EG, and the development rate of the thawed

Table 3
Survival of seven-band grouper (*Epinephelus septemfasciatus*) embryos after vitrification.

Vitrification solution	Embryo stage frozen	Number of embryos	Number of floating embryos after thawing	Number of embryos that developed	Embryonic development stage	Survival time/h
PMG3T	Somite stage	78	6	2	Larval fish	13
PMG3T	Tail-bud stage	102	8	8	Heartbeat stage	11
PMG3S	Embryoid body formation stage	247	68	4	Somite stage	7
Total		472	82	14		

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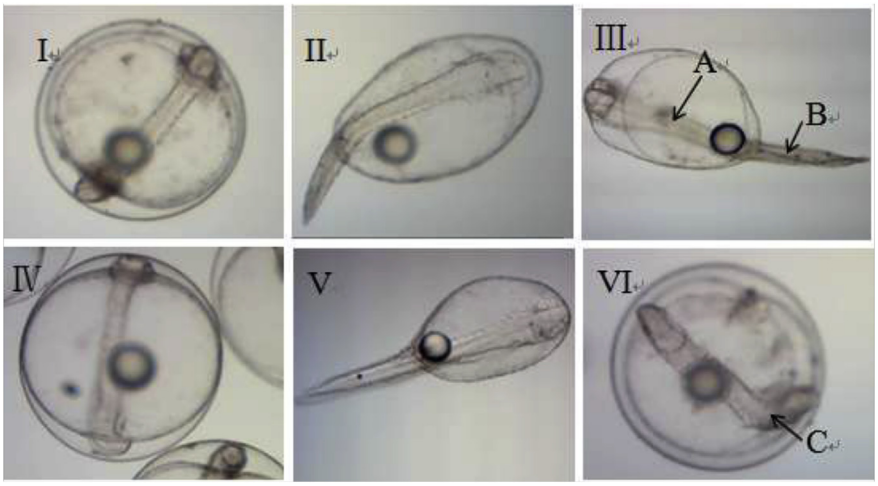

Fig. 8. Surviving embryos and larval fish cryopreserved with PMG3T vitrification solution and post-thawed. I: A frozen-thawed Embryo at the somite stage. Eyes and somite can be clearly observed, and there is no difference from normally cultured embryos (IV). II: Larval fish hatched from the cryopreserved embryos after developing for 13 h post-thawing. Embryonic body is straight, color of tail is normal and there are no differences with normally hatched larval fish (V). III: Hatched larval fish with slight malformations after cryopreservation, such as the curved embryonic body (A) and the pigmentation in the tail (B), which is clearly different from normal larval fish (V). IV: An embryo at the somite stage in normal culture. V: Normal larval after 13 h development. VI: A frozen-thawed embryo with a straight body and increasing pigmentation (C). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

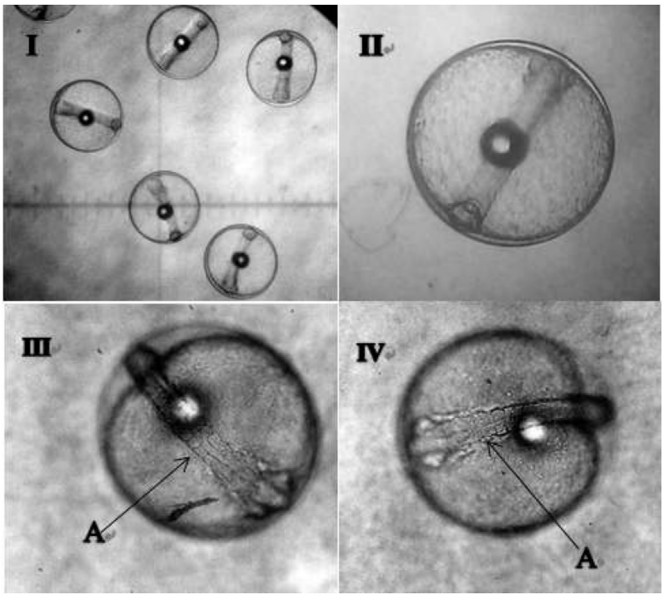

Fig. 9. Surviving embryos from cryopreservation with PMG3S vitrification solution and post-thaw. I and II: Surviving cryopreserved embryos at the embryoid body formation stage, in which somites have not formed. III and IV: Embryos develop to somite stage after 7 h post-thawing. A: Embryonic somite.

embryos was 94%. In this study, the toxicity of EG alone and the mixture of EG and PG was relative higher in the seven-band grouper embryos at the somite stage.

When the concentration of mixed cryoprotectants remains the same, several solutions combined in different ratios show different toxicity to embryos [37]. In this study, we tried four ratios in the combination of PM and Gly. The results showed that the hatchability of embryos were highest in the 3:1 (PM:Gly) ratio, which was significantly different from other ratios ($P < 0.05$, indicating that the toxicity of cryoprotectants in the 3:1 (PM:Gly) ratio was relatively lower to the seven-band grouper embryos.

Small molecule cryoprotectants at high concentrations are very toxic for embryos. Macromolecular cryoprotectants with low molecular weights, such as glucose, fructose, sucrose and trehalose, play an important role in embryo cryopreservation, protecting cells from damage. First, sugars help improve the osmotic pressure of the vitrification solution, under which cells can be fully dehydrated and their integrity can be well maintained. Second, less cryoprotectants is required with the addition of sugars, which may potentially minimize the toxicity of the solution to the embryo. Third, sugars serve as an osmotic pressure buffer, which decreases cell expansion rate and prevents cell from damage in osmosis process [30]. In this study, after vitrification cryopreservation with PMT3T (35%PMG3 + 5% trehalose), elution and incubation, 12 embryos at the somite stage recovered including 1 well developed to larval fish and another 4 embryos developed from the embryoid body formation stage to the somite stage. This indicated the potential protection of trehalose during cryopreservation.

Embryos will have some changes in volume when treated with cryoprotectant solutions. Pedro et al. [38] compared the permeability and volume variation in different developmental stages of mouse oocytes and embryos by using several cryoprotectants, such as propylene glycol, dimethyl sulfoxide, acetamide, and ethylene glycol. The results showed that oocytes/embryos became more permeable as the development proceeded, and the increase in the permeating rate varied among different cryoprotectants. However, the pattern of permeability to propylene glycol did not change during the development of oocytes/embryos. The study on the permeability of dechorionated zebrafish embryos to water and methanol showed that the water permeability remained stable while the methanol permeability decreased during embryo development [39]. Later, the hydraulic conductivity (Lp) and the solute (cryoprotectant) permeability (Ps) of different cryoprotectants were tested during the development of zebrafish oocyte development [40]. It was shown that stage III oocyte membrane permeability was lower than those successfully cryopreserved mammalian oocytes, but was higher than sea urchin eggs and other fish embryos. In this study, the embryonic variation at somite stage and tail-bud stage of the seven-band grouper were measured during the treatment of 2 mixed vitrification solutions, PMG3S and PMG3T. The variation trends were similar. When treated with 1/4 diluted vitrification solution for 6 min, Embryos shrank quickly with the diffusion of water out of cells and the reduction of cell

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volume. After treated with 1/3 diluted vitrification solution for another 6 min, they gradually recovered with the permeation of the solutions into cells. Embryos at somite stage expanded larger than the control when treated with 1/2 diluted vitrification solution in 18 min. While, embryos at tail-bud recovered in the same volume as the original after treated with 2/3 diluted vitrification solution in 24 min. This showed the different permeability of somite stage embryos and tail-bud stage embryos to the vitrification solutions. And during the treatment with the vitrification solutions, all the embryos shrank quickly first, and then gradually recovered, indicating that the solutions could permeate into cells and effectively protect embryos. The results obtained in this work provide some information for the studies on the permeability of embryos to mixed cryoprotectants, which would help the development of cryopreservation protocols for fish embryos.

In conclusion, surviving embryos were obtained in the present study when using vitrification cryopreservation with PMG3S and PMG3T solutions, which will provide a strong basis for subsequent studies. However, the number of surviving embryos is not sufficient, and further investigations and experiments are necessary to standardize the techniques so that the amount of surviving embryos is increased.

Conflict of interest

The authors have declared that no conflict of interests exist.

Acknowledgments

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