Effect of vitrification solutions on survival rate of cryopreserved Epinephelus moara embryos

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ABSTRACT

Embryo cryopreservation is important for long-term preservation of germplasm and assisted reproduction. However, it is still very difficult to obtain viable embryos from cryopreserved fish embryos. In this study, embryos of Epinephelus moara were used to investigate the effects of various cryopreservation methods. Embryos in stages 10 pairs somite (10S), 18 pairs somite (18S), 22 pairs somite (22S), tail-bud (TB), embryo twitching (ET) and pre-hatch (PH) were treated with five-step equilibrium penetration in 40% PMG3T vitrification solution, which contained 15.75% 1,2-propylene glycol, 10.50% Methanol, 8.75% Glycerol and 5.00% Trehalose. We found that 18S, 22S, TB and ET stage embryos had higher survival rates and were more tolerant to the vitrification solution. Five-step equilibrium treatments on the embryos at the tail-bud stage were performed using two vitrification solutions: 40% PMG3T and 40% PMG3S, which consisted of 15.75% 1,2-propylene glycol, 10.50% Methanol, 8.75% Glycerol and 5.00% Sucrose. The embryonic survival rate under PMG3S treatment (63.36%) was significantly higher than PMG3T treatment (43.93%) (P < 0.05). PMG3S and PMG3T with concentrations of 35%, 40% and 45% were tested on tail-bud stage embryos. Higher concentration of the vitrification solution led to significantly lower embryonic survival rate (P < 0.05). The survival rate was 36.79 – 72.05% in PMG3S, and 37.11 – 55.18% in PMG3T, and there were non-significant differences in embryonic development and malformation rates among the groups treated with different concentrations. The embryonic normal development rates in PMG3S and PMG3T were 21.27% and 11.04%, and the malformation rates were 36.13% and 31.04%, respectively. The optimum treatment condition was 40 min using 40% PMG3S on embryos at the tail-bud stage. Both PMG3S and PMG3T were used for cryopreserving embryos at 16 pairs somite, tail-bud and ET stage in liquid nitrogen, where we obtained 190 surviving embryos, and 44 fishes underwent normal development and hatched. The survival rate of cryopreserved embryos was 5.15%, the normal development rate was 1.31%, and the malformation rate was 3.66%. We found that PMG3S and PMG3T were effective for cryopreservation of Epinephelus moara embryos. The results provide a foundation for further explorations of fish embryo cryopreservation techniques.

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The cryopreservation of embryos can preserve the genetic information of species, and is crucial for long-term preservation of germplasm. The concept of cryopreservation of animal embryos and gametes was proposed by Chang in 1948 to preserve rabbit oocytes, fertilized eggs and embryos [1]. Following the original description, cryopreservation of embryos of human [2], mouse [3], cow [4], horse [5], and sheep [6] were conducted. At present, the survival rate of animal blastocysts after cryopreservation can reach up to 52 – 83.7% [5, 6]. Fish embryos were subjected to cryopreservation slightly later than mammals, with Blaxter’s work to preserve fish gametes in 1953 [7]. Although it has been more than 60 years,
the cryopreservation of fish embryos is still an intricate problem in cryobiology. Fish eggs have large yolk content, double membranous structure, large perivitelline space, abundant water content, and high amount of fat droplets, limiting the application of cryopreservation techniques for fish embryos.

In freshwater fishes, three surviving embryos of Cyprinus carpio were obtained using the slow-freezing method [8], four embryos of Misgurnus anguillicaudatus at blastopore closed period were obtained through the vitrification cryopreservation method, which developed to the tail-bud stage [9]. In marine fishes, Tian et al. used vitrification cryopreservation methods for the embryos of Lateolabrax japonicus [10], Paralichthys olivaceus [11,12], Scorpaenichthys maximus [13], Epinephelus septemfasciatus [14] and Epinephelus moara [15], obtaining a total of 350 surviving embryos at the somite, tail-bud and heart beating stages, and 261 juveniles that hatched. The embryos of Persian sturgeon (Acipenser persicus) were obtained using the slow-freezing method [8], four embryos of Epinephelus moara (1.5. The effects of vitrification on the embryonic survival rate of Epinephelus moara [15], and obtained surviving embryos and fishes after freezing, providing a basis for further studies on the cryopreservation of Epinephelus moara embryos.

In the present study, we explored the appropriate development stages for embryonic cryopreservation of Epinephelus moara, as well as the optimal concentration, type and penetration time of vitrification solutions. Our results provide theoretical and technical basis for the improvement of vitrification cryopreservation technology in Epinephelus moara embryos. Furthermore, the results can be used for the establishment of embryo bank in this species.

1. Materials and methods

1.1. Preparation for artificial reproduction and embryos of Epinephelus moara

The experimental fishes were provided by LaiZhou MingBo Aquatic Co., Ltd. (Laizhou, Shandong province). April to May of each year, when Epinephelus moara were sexually mature, female that weighed 5–10 kg and 40–60 cm long were selected as parents. 200–300 IU/kg human chorionic gonadotropin (HCG) and 5 µg/kg ovulation-stimulating hormone analogue (LHRH-A3) were applied through intramuscular injection to induce spawning [14,15]. At 48 h after inducing spawning, eggs were collected through artificial extrusion of the abdomen, and 1 ml cryopreserved sperm of Epinephelus moara was used for fertilization of 200 ml eggs [15]. The fertilized eggs were rinsed using seawater with 35% salinity. Fertilized eggs that floated were isolated and put in the 500 L net cage containing seawater at 24 °C, 30% salinity and dissolved oxygen of 6–10 mg/L to allow for hatching. Embryos were selected at 10 pairs somite (10S, 20 h after fertilization), 16 pairs somite (16S, 21 h after fertilization), 18 pairs somite (18S, 22 h after fertilization), 22 pairs somite (22S, 23 h after fertilization), tail-bud (TB, 25 h after fertilization), embryo twitching (ET, 27 h after fertilization) and pre-hatch (PH, 29 h after fertilization) for osmotic treatments using vitrification solutions and for cryopreservation experiments.

1.2. The preparation of vitrification solution

The diluent BS2 [15] (24.72 g/L NaCl, 1.46 g/L CaCl2·2H2O, 0.865 g/L KCl, 4.86 g/L MgCl2·6H2O and 0.19 g/L NaHCO3) was used to dilute vitrification solutions PMG3T and PMG3S to 35%, 40% and 45% concentrations (Table 1). The vitrification solutions were diluted in BS2 to the ratio of 1:4, 1:3, 1:2 and 2:3. In addition, 0.125 mol/L sucrose was added to BS2 in order to prepare the embryonic eluent [14,15].

1.3. The adaptation of embryos at different stages to vitrification solutions

Epinephelus moara embryos developed to 10S, 18S, 22S, TB, ET and PH stages, and three duplicate samples with each an average of 500 embryos were taken at each stage (n = 3). Embryos were treated with a gradient liquid of 40% PMG3T for the “five-step” osmotic treatment [14]. They were treated in 1/4, 1/3, 1/2, 2/3 and 1 × PMG3T for 7 min each, and then 0.125 mol/L sucrose was used to elute for 10 min. During elution, seawater at 24 °C was gradually added to wash embryos, then embryos were cultured in seawater and observed under a microscope at pre-hatching stages. In addition, the survival, normal development and malformation rates of embryos were measured as the percentages of developed embryos, normally developed embryos and abnormally developed embryos against the total ones, respectively.

1.4. The comparison between two vitrification solutions, PMG3S and PMG3T

When the embryos developed to the tail bud stage, 5–6 duplicate samples each with average 250 embryos were taken for treatment. Two vitrification solutions of 40% PMG3S and PMG3T were applied for the “five-step” osmotic treatment, then 0.125 mol/L sucrose was applied for elution. The control group was not treated with vitrification solution. Embryos were cultured in 24 °C seawater, and survival, normal development and malformation rates of embryos were calculated at pre-hatching stages. The morphology of the embryos and hatchlings were observed under the microscope before and after treatment with vitrification solution.

1.5. The effects of vitrification solution concentration on embryos

When the embryos developed to the tail bud stage, 35%, 40% and 45% PMG3S and PMG3T solutions were applied using the “five-step” method. PMG3S and PMG3T were diluted with BS2 to prepare 1/4, 1/3, 1/2, 2/3 and 1 times gradient liquids. Osmotic treatments of embryos were conducted gradually in the gradient liquids, and for 7 min in each gradient. 0.125 mol/L sucrose was applied to elute samples. The control group contained embryos at the tail bud stage without any treatment. Embryos were cultured in 24 °C seawater, and survival, normal development and malformation rates of embryos were measured at pre-hatching stages. There were an average of 400 embryos in each treated sample with 3–7 repetitions.

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