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Effects of Slow Cooling Methods and Vitrification Methods on Red Seabream (*Pagrus major*) Embryos

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1. Introduction

Fish gametes cryopreservation would benefit global germplasm shipping and supply, aquaculture, aquatic resources conservation and scientific research [1]. In fish sperm cryopreservation, more than 200 species have been successfully cryopreserved [2]. However, the cryopreservation of fish embryo has not been successful due to its complex multi-compartmental system, large content of water, high sensitivity to chilling, large amount of egg-yolk and low membrane permeability [3,4]. In recent years, many researches have been carried out on cryopreservation protocols [5] and mechanism of cryoinjuries in fish embryos [6].

Conventional slow cooling and vitrification are commonly used methods for long term storage of mammalian embryos. Conventional slow cooling method has been widely used in various species, but it suffers from several limitations such as chilling injury, ice formation damage, expensive equipment and tedious cooling protocols [7]. Vitrification, a solidification of a liquid without crystallization, seemed to be a promising approach. It is an extreme increase of viscosity and requires either rapid cooling rates or high concentration cryoprotectants [8], and can greatly simplify the process of cooling, avoids physical damage, and lessens the chilling injury to embryo [7]. However, the embryo cryopreserved by vitrification may still be injured by toxicity and osmotic effects of cryoprotectants [9].

Despite the fact that cryopreservation of embryos for some fish species have been attempted, successful results were not achieved [10]. Slow-cooling method had been approved to be not suitable for cryopreservation of zebrafish embryos, starfish oocytes and *Xenopus laevis* oocytes [11,12]. Therefore, some researchers suggested that vitrification would be a good option for its absence from ice crystal formation. In olive flounder, some researchers reported survival embryos were obtained after vitrification [13], however, it could not be replicated by others

[14]. In spite of this, as a promising method, attempts have been made on various fish species by vitrification.

Red seabream (*Pagrus major*) is one of the most important cultured fish species in China, Japan and South Korea. We have carried out some researches on red seabream embryos in cryopreservation protocols [15] and mechanism of cryoinjuries [6]. Potential protocols of slowing cooling and vitrification (sorts of cryoprotectant, programs of cooling and thawing) have been screened [15]. However, little was known upon the morphological changes issue during cooling and thawing process by the two methods. The objective of the present study was to investigate the effects of two conventional slow cooling methods (S1 and S2) and two vitrification methods (V1 and V2) on red seabream embryos. The main topics are as follows: 1) the effect of different cryoprotectant solutions on hatching rate of red seabream embryos; 2) the morphological changes during exposure to different cryoprotectant solutions; 3) and the changes of embryos during cooling and thawing process under cryomicroscope.

2. Materials and methods

2.1. Fish breeding and embryo collection

Sexually mature red seabream (8 female, 12 male; body weight, 3–4 kg) were maintained in a 12 m³ concrete rearing pond (temperature: 16–18 °C) with filtered seawater changed two times a day and pumped air supply. The photoperiod was fixed at L: D=16 h: 8 h. They were fed twice a day with cooked meat of mussel. Naturally fertilized embryos were collected each morning before feeding and then incubated in filtered seawater with pumped air supply at 18 ± 1 °C in a small plastic barrel. Embryos developed to heart-beat stage (heart rate: 60–90 beats/min (fig.1); approximately 36 h after fertilization) were used for experiments. The developmental stages of the embryo were determined morphologically using a light microscope (Nikon-YS100).

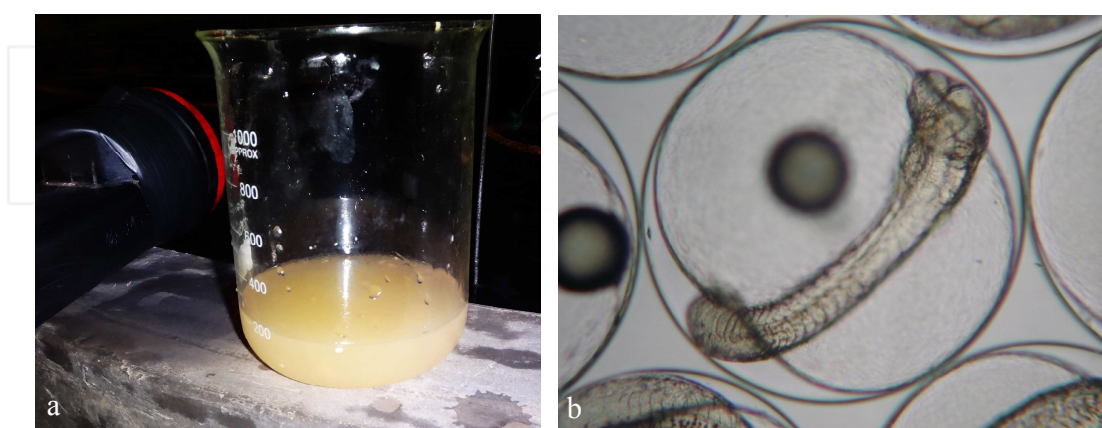


Figure 1. The collected fresh embryos of red seabream. Naturally fertilized embryos were collected each morning before feeding and then incubated in filtered seawater with pumped air supply at 18 ± 1 °C in a small plastic barrel. Embryos developed to heart-beat stage were used for experiments. a. The collected fertilized embryos. b. The heart-beat stage embryo.

2.2. Chemicals and solutions

Dimethyl sulfoxide (DMSO) was purchased from Sigma-Aldrich, methanol (MeOH), 1,2-propylene glycol (PG) and the other chemicals were purchased from Beijing Chemical Agents Ltd. All the chemicals were analytical grade. The cryoprotectant solutions used in the following experiments were made with Hank's solution [16] (8 g/L NaCl, 0.4 g/L KCl, 0.14 g/L CaCl₂, 0.1 g/L MgSO₄ 7H₂O, 0.1 g/L MgCl₂ 6H₂O, 0.06 g/L Na₂HPO₄ 12H₂O, 1g/L glucose, 0.35 g/L NaHCO₃) as the extender. The concentration of cryoprotectant was expressed as the percentage of volume (v/v).

2.3. Effect of cryoprotectant solutions on the embryos

For each exposure test, approximately 50 embryos were exposed to 10 mL cryoprotectant solutions with various concentrations for different exposure time (Tab 1) at room temperature (18 ± 1 °C). Three replicates were taken for each experiment group.

| | Cryoprotectants | extender | Exposure time(min) | | |
|------|-----------------|-----------------|--------------------|----|----|
| S1* | 5%DMSO+5%PG | Hank's solution | 10 | 30 | 60 |
| S2* | 8%MeOH | | | | |
| V1** | 40%DMSO | | 5 | 10 | 15 |
| V2** | 40%PG | | | | |

* For conventional slow cooling methods

** For vitrification methods

Table 1. Cryoprotectant concentrations and treatment time used in cryopreservation of red seabream.

After immersion the embryos were first removed from the cryoprotectant solution using a nylon mesh, and carefully washed three times with fresh seawater. Then the embryos were transferred to a 100 mL beaker containing 80 mL fresh seawater for incubation with a change of seawater an hour later. Control groups were incubated in the filtered seawater at room temperature. The toxicity of cryoprotectant was assessed by the hatching rate which was calculated as the percentage of hatched larvae (48 h after fertilization) in relation to the total number of each group.

2.4. Morphological changes during exposure to cryoprotectant solutions

One embryo with seawater was loaded on a concave slide under a light microscope (Nikon-YS100). We removed the seawater with filter paper and added 100 µl cryoprotectant solution. Morphological changes were observed by taking pictures using a digital camera (Nikon CoolPix 4500) under microscope with 40× magnification and the interval was about 1 min. 5 embryos were observed for each cryoprotectant solution.

2.5. Changes of embryos during cooling and thawing process

The embryos were immersed in the four cryoprotectant solutions for different time (Tab 2), respectively. After immersion, the embryos suspended in 20 μ l of cryoprotectant solution were loaded into a small quartz holding vessel and placed onto a Linkam Cryostage (Linkam-THMS600, UK). The embryos were cooled with different methods (Tab 2). After thawing, the embryos were transferred to a 100 mL beaker containing 80 mL filtered seawater for incubation. Each experiment was repeated three times. The morphological changes during the cooling-thawing process were recorded using a microscope (Olympus BX-51, Japan) with a video attachment and monitor (Nikon-E200, Japan). In addition, two temperature values, T_{EIF} and T_{IF} were recorded. They were identified as the temperature when a flash appears in the field of view and the temperature when the embryo suddenly blackens, respectively. The temperatures reported were obtained by the Linkam cryostage thermocouple.

| Cryoprotectants | | Cooling methods | Thawing methods |
|-----------------|------------------------------------|--|--|
| S1 | 5%DMSO+5%PG Immersed for 50 min | 0°C for 1 min, to -15.5°C with -3.5 °C /min, to -30°C with 3 °C /min, then to -150°C with -130°C /min and for 1 min; | to -80°C with 30°C/min, to 20°C with 130°C/min |
| S2 | 8% Methanol Immersed for 30 min | 0°C for 1 min, to -20°C with -2°C /min and for 5 min, to -60°C with -2°C/min and for 5 min, then to -150°C with -10°C/min and for 1 min; | to -70°C with 30°C/min, to 20°C with 130°C/min |
| V1 | 40%DMSO Immersed for 5 min | cooling to -150°C with -130°C /min and for 1 min; | to 20°C with 130°C/min |
| V2 | 40%PG Immersed for 10 min | cooling to -150°C with -130°C /min and for 1 min; | to 20°C with 130°C/min |

Table 2. Cooling methods used in cryopreservation of red seabream.

After thawing, the morphological changes of embryos were observed and recorded by the video attachment and monitor under the microscope. The proportion of morphologically intact embryos was calculated as the percentage of embryos with normal morphology in relation to the total number of each group.

2.6. Statistical analysis

Percentage data were normalized through arcsine transformation and analyzed by one-way ANOVA with SPSS software (SPSS Inc., USA). The results were expressed as means \pm SD. The significant differences between treatments in the different experiments were detected using an SNK (Student Newman Keuls) statistical test ($P < 0.05$). The percentages of embryos with intact morphology were arithmetical means.

3. Results

3.1. Effect of cryoprotectant solutions on the hatching rate of red seabream embryos

The hatching rates of embryos treated with cryoprotectant solutions are shown in Table 3. After exposure to S1 and S2 solutions, the hatching rates of embryos showed no significant decrease compared to control except for S2 solution with 60 min exposure. However, the hatching rates of embryos exposed to V1 and V2 solutions decreased sharply, only the embryos immersed in V2 solution for 5 min and 10 min had higher hatching rates (>80%). So in the later cooling experiment, we choose 60 min, 30 min, 5 min and 10 min as exposure time for S1, S2, V1 and V2, respectively.

| | Cryoprotectants | Exposure time | Hatching rate (%) |
|---------|-----------------|---------------|---|
| Control | — | — | 99.67±0.82 ^a |
| S1 | 5%DMSO+5%PG | 10 min | 98.00±2.00 ^a |
| | | 30 min | 100.00±0.00 ^a |
| | | 60 min | 100.00±0.00 ^a |
| S2 | 8%MeOH | 10 min | 98.67±2.31 ^a |
| | | 30 min | 96.67±2.31 ^a 65.67±5.13 ^{bc} |
| | | 60 min | |
| V1 | 40%DMSO | 5 min | 59.33±14.19 ^c |
| | | 10 min | 15.33±5.03 ^d |
| | | 15 min | 15.00±15.52 ^d |
| V2 | 40%PG | 5 min | 96.67±5.77 ^a |
| | | 10 min | 81.33±22.74 ^{ab} |
| | | 15 min | 65.00±27.73 ^{bc} |

Values with different letters are significantly different ($P<0.05$) (means ± SD)

Table 3. Hatching rates of embryos treated with the four selected solutions

3.2. Morphological changes during exposure to cryoprotectant solutions

The morphological changes of the embryos in S1 and V1 are shown in Figure 2. The morphological changes in S2 and V2 were similar with those in S1 and V1, respectively. No obvious change was found after immersed in S solutions (after exposure for 30 min (Fig. 2-S-2)). But there was an obvious dark strip around the yolk sac in V groups as soon as embryo was exposed in the cryoprotectants (Fig. 2-V-2), and the dark strip became narrower until disappeared gradually at the time of 10 min (V1, Fig. 2-V-4)/13 min (V2). At the end of immersion, the

embryos presented different extent of degeneration (Fig. 2-S-6), and the degeneration in S2 was more serious (Fig. 2-S-7).

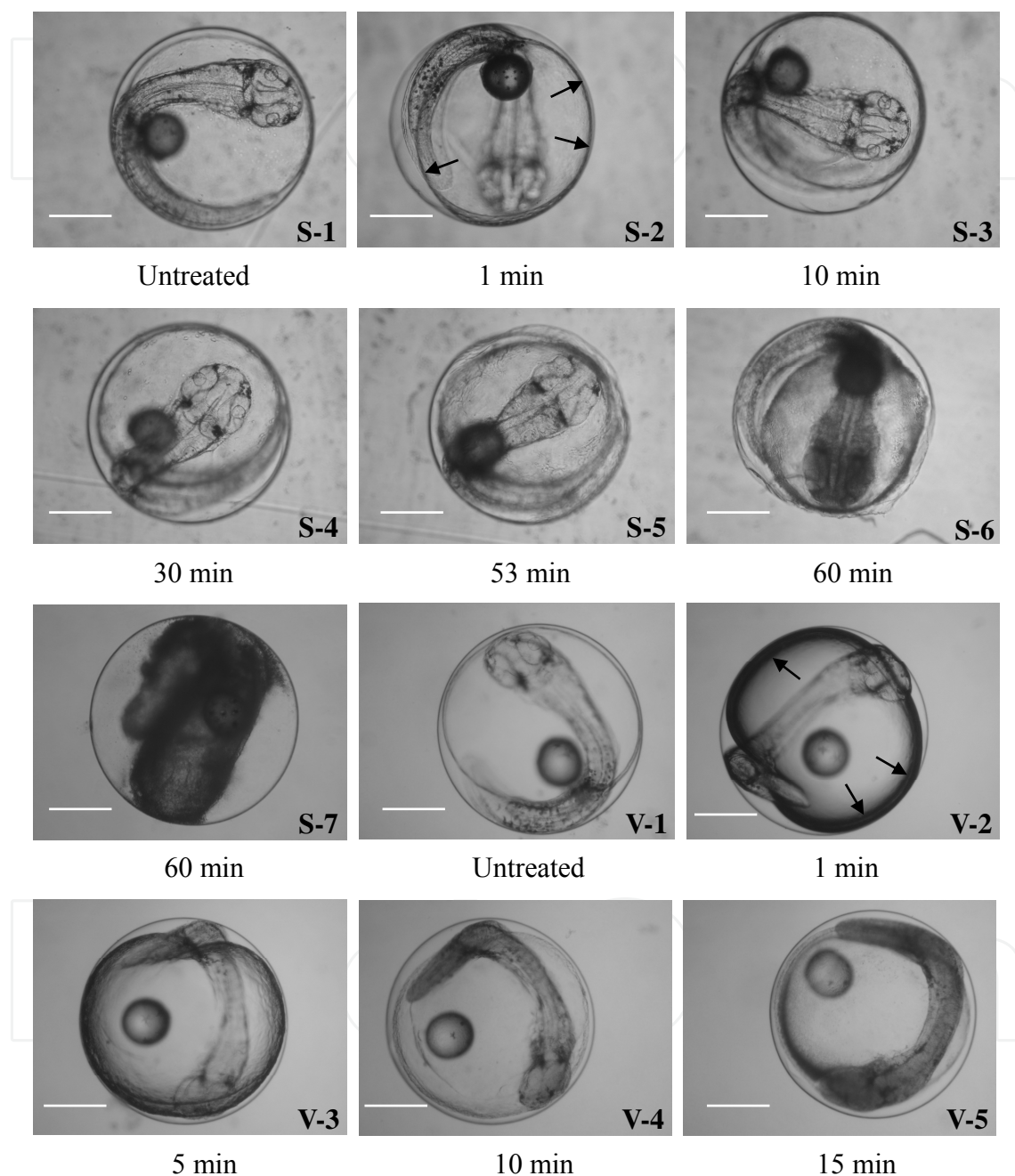


Figure 2. Representative morphological changes of embryo immersed in cryoprotectant solutions. This figure shows the morphological changes of red seabream embryo immersed in cryoprotectant solutions. (S-1) Untreated embryo for S1 group. (S-2~6) Morphological changes of embryo immersed in S1 for different times. (S-7) Degeneration of embryo immersed in S2 for 60 min. (V-1) Untreated embryo for V1 group. (V-2~5) Morphological changes of embryo immersed in V1 for different time. Scale bar=300.00 μm .

3.3. Changes of embryos during cooling and thawing process

The representative changes of embryos during cooling and thawing process under cryomicroscope are shown in Figure 3 and Figure 4. Intra-cellular ice formation occurred in both group (Fig. 3-S-4,5; Fig. 4-V-2,3) when the embryos suddenly blacken. According to the extent of blackening, we separated the intra-cellular ice formation temperatures (T_{IF}) into two categories, high temperature blackeners (T_{IF1}) and low temperature blackeners (T_{IF2}). The values of T_{EIF} and T_{IF} in the four groups are shown in Table 4.

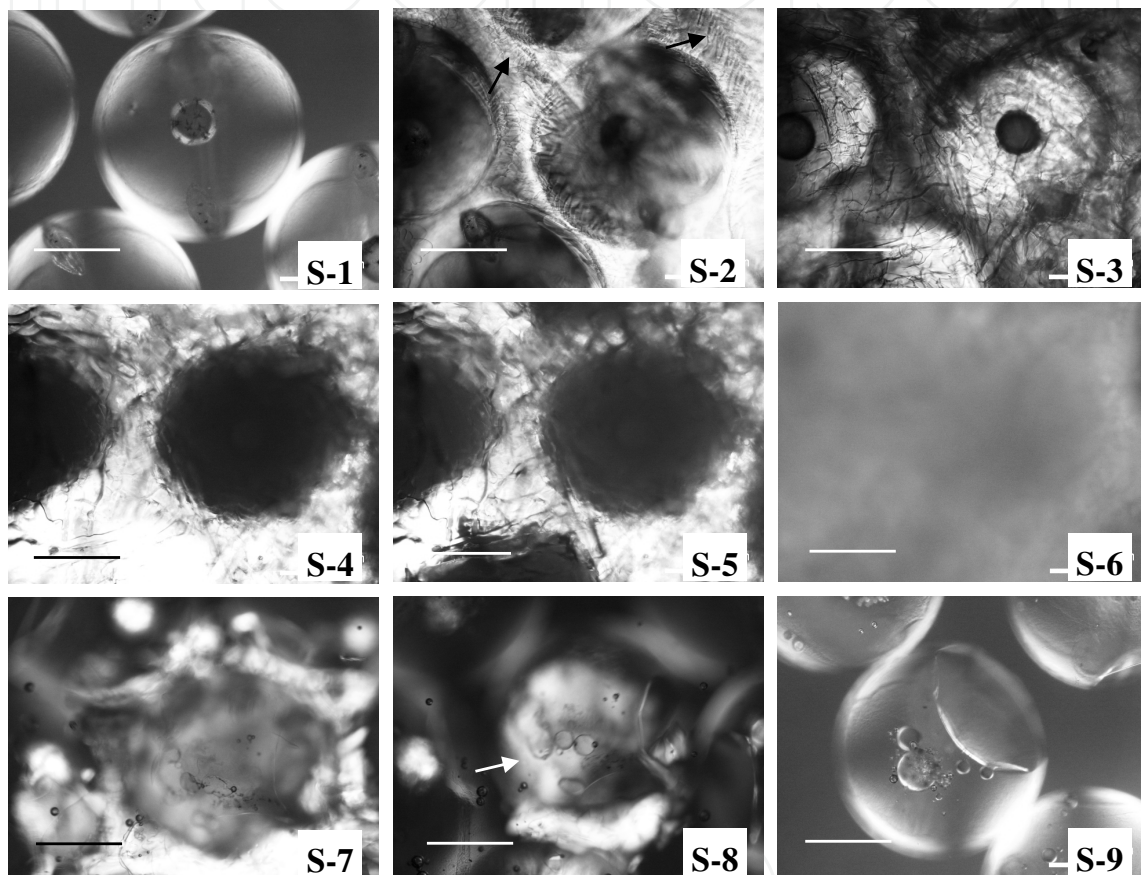


Figure 3. The representative changes of embryos during cooling and thawing in conventional slow cooling groups. This figure shows the representative changes of embryos during cooling and thawing in conventional slow cooling groups. (S-1) Embryos before cooling. (S-2) Extra-cellular ice formation: a flash appears and dendritic spears of ice (arrow) project in the field of view. (S-3) Extra-cellular ice formation. (S-4) Intra-cellular ice formation in partly embryos. (S-5) Intra-cellular ice formation in all embryos (S-6) Completely frozen embryos. (S-7) Embryo with extra-cellular ice thawing. (S-8) Embryo with intra-cellular ice thawing. (S-9) Completely thawed embryos. Scale bar=400.00 μm .

Both T_{EIFS} and T_{IFS} in S groups were significantly higher than those in V groups. In V groups, T_{IF} was higher than T_{EIF} , indicating intra-cellular ice formed earlier than extra-cellular ice (Fig. 4-V-2), opposite to S groups (Fig. 3-S-2). And the intra-cellular ice firstly formed in the yolk and developing embryo (Fig. 4-V-3), then the perivitelline space (Fig. 4-V-4). Besides, dendritic ice crystals were observed in S groups (Fig. 3-S-2, arrow), and no big ice crystals were observed in V groups.

When the embryos cooled to -150°C , the phenomena was similar in S groups and V1, the whole field of view was obscured by the extra-cellular ice (Fig.3-S-6, Fig.4-V-5). In V2, the embryos were obscure, while the solutions outside the embryos were transparent, just like solid glass (Fig.4-V-9). The transparent spaces among embryos were obscured during the thawing process (Fig.4-V-10).

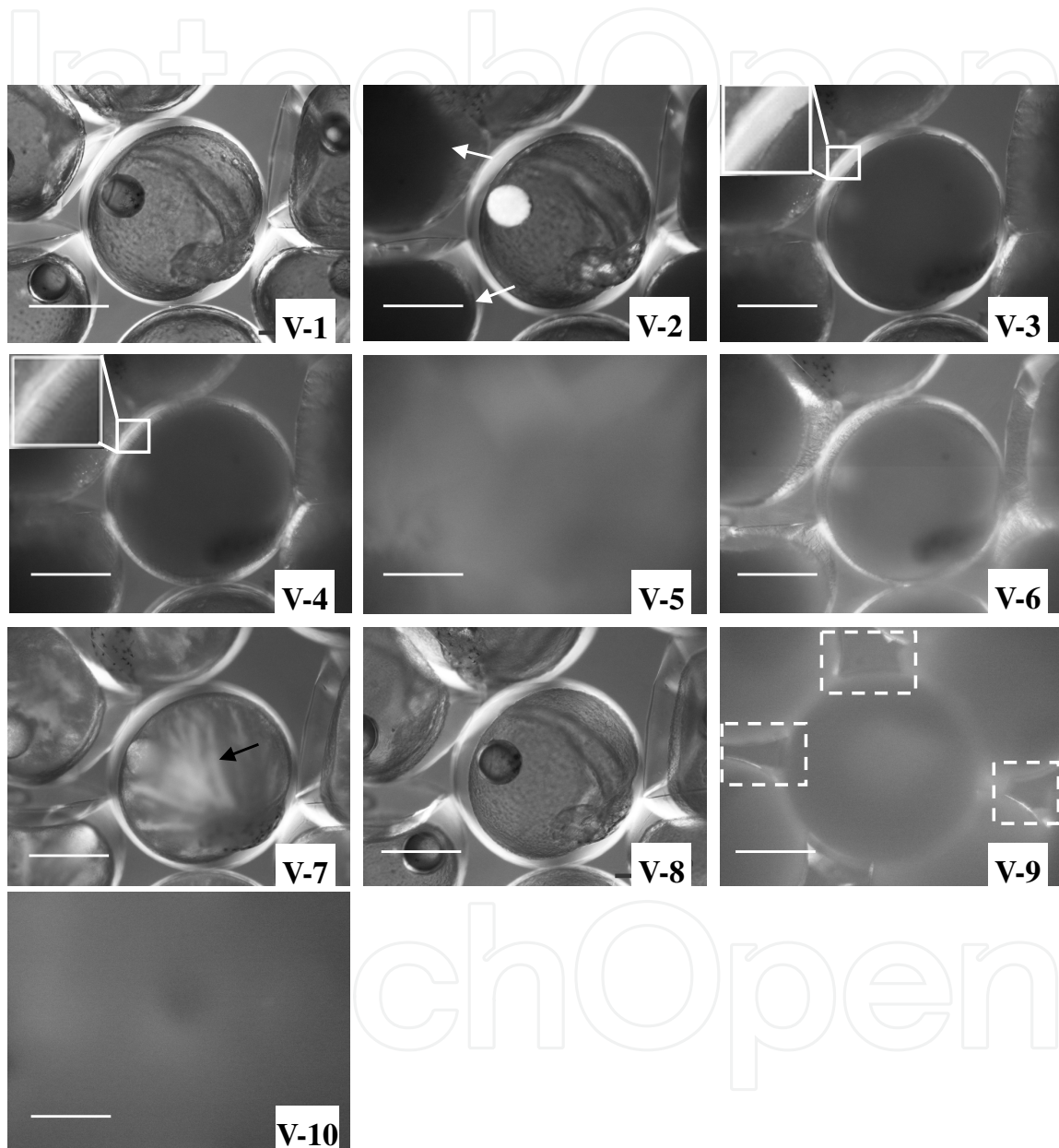


Figure 4. The representative changes of embryos during cooling and thawing in vitrification groups. This figure shows the representative changes of embryos during cooling and thawing in vitrification groups. (V-1) Embryos before cooling. (V-2) Intra-cellular ice formation in partly embryos. (V-3,4) Intra-cellular ice formation: the intra-cellular ice firstly forms in the yolk and developing embryo (arrow), then the perivitelline space (arrow). (V-5) Completely frozen embryos in V1 group. (V-6) Embryo with extra-cellular ice thawing. (V-7) Embryo with intra-cellular ice thawing. (V-8) Completely thawed embryos. (V-9) Vitrification in the solutions outside embryos in V2 group. (V-10) Devitrification during the thawing process in V2 group. Scale bar=400.00 μm .

During the thawing process, the ice of the outside solution thawed firstly and then thawed the ice inside the embryo with the embryo brightening (Fig. 3-S-8, Fig. 4-V-7) in all four groups. After thawing, the percentage of embryos with intact morphology in the four groups were S1, 10.26%; S2, 8.19%; V1, 55.26%; and V2, 70.00%, respectively. In total, the percentage of embryos with intact morphology in V groups (62.82%) was significantly higher than that in S groups (9.21%).

| | Cryoprotectants | T_{EIF} (°C) | T_{IIF1} (°C) | T_{IIF2} (°C) | $T_{EIF} - T_{IIF2}$ (°C) |
|----|-----------------|--------------------------|--------------------------|--------------------------|---------------------------|
| S1 | 5%DMSO+5%PG | -5.00±0.17 ^a | -17.74±0.59 ^a | -21.40±0.31 ^a | 16.40±0.47 ^a |
| S2 | 8%MeOH | -4.93±0.35 ^a | -16.34±0.08 ^a | -17.51±0.48 ^b | 12.58±0.74 ^b |
| V1 | 40%DMSO | -70.30±2.72 ^b | -56.83±1.48 ^b | -64.33±0.51 ^c | -5.97±2.21 ^c |
| V2 | 40%PG | — | -46.23±0.31 ^c | -53.70±1.00 ^d | — |

Values with different letters within the same row are significantly different ($P < 0.05$) (means ± SD)

Table 4. The values of T_{EIF} and T_{IIF}

4. Discussion

4.1. Morphological changes during exposure to cryoprotectant solutions

Fish embryo is composed of three membrane-limited compartments: a large yolk surrounded by the yolk syncytial layer, a developing embryo limited by its own cell plasma membranes, and perivitelline space surrounded by chorion [17]. The different layers and membranes represent permeability barriers hindering the movement of water and cryoprotectants, and consequently, making the balance of cryoprotectant in the whole embryo difficult [18].

In the observation of morphological changes, we found a dark strip around the yolk sac after immersion in the V solutions. This may ascribe to the different refractive indexes between outer solution and perivitelline space. With the time going on, the strip became narrower and eventually disappeared. It indicated that the chorion of red seabream embryo was permeable to the cryoprotectant solutions. Similar results were found in studies on zebrafish embryos and medaka oocytes [18,19]. In V groups, the dark strip was obvious, while in S groups, the dark strip was not observed. This may be due to higher concentrations of V solutions than S solutions, which caused higher refractive index difference.

4.2. Changes of embryos during cooling and thawing process

In our cooling experiment, we have observed some different phenomena between the two methods. The first representative phenomenon in our experiment was the large dendritic ice crystals observed in S groups, but not in V groups. High cooling rate and cryoprotectant

concentration were reported to be prone to vitrify and avoid dendritic ice crystal formation [20], which may explain the phenomenon in this study.

Secondly, in our result, T_{IIF} s in S groups were significantly higher than those in V groups. This may be because the nucleation temperature of red seabream embryos is too high to achieve sufficient cell dehydration to avoid IIF [21]. T_{IIF} is a main factor many researchers focus on in the study of cryopreservation. Slow cooling usually causes dehydration and decreases T_{IIF} largely in mammal oocytes or embryos, and contributes to successful cryopreservation [22]. However, it was not the case in red seabream embryos, zebrafish embryos [23] and starfish oocytes [24] mainly due to the high water content and multi-membrane structures which inhibit sufficient dehydration of fish embryos.

Thirdly, intra-cellular ice formed earlier than extra-cellular ice in V groups, which is opposite to the phenomenon in S groups. And the intra-cellular ice in V groups firstly formed in the yolk and developing embryo, then in the perivitelline space. Based on the result of section 3.2 and previous research [19], the cryoprotectant could penetrate into the chorion but hardly penetrate into the yolk syncytial layer and the developing embryo. So the concentration of cryoprotectant outside the chorion was the highest, followed by the perivitelline space, and the yolk and developing embryo was the lowest. We speculated that the sequence of ice formation was determined by the different cryoprotectant concentration in the different parts.

At the point of $-150\text{ }^{\circ}\text{C}$, the phenomena in the four groups were different. In the group of S groups and V1, the whole field of view was obscured by the extra-cellular ice. However, in V2 group, the solutions outside embryos were transparent, like solid glass. We inferred that the outer solution vitrified. The difference of cryoprotectants caused the different phenomena between V1 and V2, because PG (used in V2) vitrifies more easily than DMSO [25]. The embryos in V group did not vitrify, which indicated that the cryoprotectant concentration of inner embryo was not high enough to form vitrification at this cooling rate. It indicated that limited or no cryoprotectant penetrated into the yolk and developing embryo. Yolk syncytial layer (YSL) was the major obstacle for the penetration of the cryoprotectant into the embryo. This was coincide with many researches [26].

In the thawing process, devitrification or/and recrystallization usually take place. Devitrification is the transition of glassy to crystalline state and recrystallization is the growth of existing ice crystals. In our experiment, the transparent space in V2 group was obscured during thawing, which indicated that devitrification had occurred in extra-cellular ice. Recrystallization had also occurred within the embryos in all four groups, for the embryos were all turned bright in the thawing process. Devitrification and recrystallization were thought to be the major reason for cellular damage associated with the thawing process of cryopreserved cells, for they could cause ice crystals formation and larger ice crystals [27]. To avoid devitrification and recrystallization, the specimens must be warming at higher rate than the critical warming rate [23]. However, there are no effective means to avoid them at the present time because of the technique limitation.

5. Conclusion

In the present study, we compared conventional slow cooling method and vitrification method in red seabream embryos cryopreservation. In V groups, the T_{EIFS} and T_{IFS} were significantly lower than those in S groups, and no big ice crystals formed. Besides, after thawing, the percentage of embryos with intact morphology in V groups was significantly higher than that in S groups. All the results indicated that vitrification method would be a good option for red seabream embryos cryopreservation, though the embryos were better tolerant of S solutions than V solutions. Therefore, further study is still required to optimize the cryopreservation protocol for reducing toxicity of cryopreservation as well as improving the internal cryoprotectant concentration in red seabream embryos.

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