

Effect of Cryoprotectants on the Cryopreservation of Manila Clam, *Ruditapes philippinarum* Embryo

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바지락 발생배의 냉동보존에 관한 보존액의 효과

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Abstract The possibility and effectiveness of cryopreservation was determined to assess survival rates and improve stock management of thawed embryos of Manila clam, *Ruditapes philippinarum*. The ideal freezing rates were designed and tested to allow cryoprotectants to equilibrate across the membrane during freezing. Survival rates ranging from 0 to 64.3% were obtained using a stepwise freezing protocol compared with 82.3% control rates. Embryos of *Ruditapes philippinarum* were equilibrated in 2 CPAs plus sea water for 10 min at 25°C and then cooled at -1°C/min from 20°C to -12°C. Straws containing more than 100 embryos were held at 12°C for 5 min allowing equilibration after seeding and slowly cooled at 2°C /min. to -35°C for 30 min for equilibration before quenching in liquid nitrogen. Dimethyl sulfoxide (DMSO) is the best cryoprotectant indicated for embryos of *R. philippinarum* with a survival rate of $64.3 \pm 3.28\%$ in the presence of 2.0 M DMSO.

Key Words : Cryopreservation, Embryo, Cryoprotectant, Liquid Nitrogen, *Ruditapes philippinarum*

요약 바지락 발생배의 냉동보존을 위한 동해방지제의 효과를 규명하기 위하여 4종의 보존액, dimethyl sulfoxide, ethylene glycol, glycerol 및 1,2-propanediol을 사용하여 발생배의 생존율을 조사한 결과로, 생존율의 범위는 0-64.3%였고, 대조구의 생존율은 82.3%를 보였다. 발생배의 냉동보존을 위한 프로그래 동결 과정은 최초 25°C에서 10분 동안 유지하다가 자동 냉동보존 프로그램 장치에 의하여 -12°C까지 분당 -1°C씩 조정하였으며 -35°C까지는 분당 -2°C씩 하강시켜 액체 질소통에 스트로우를 넣어 해동 후 관찰한 결과, 바지락 생존율은 DMSO(dimethyl sulfoxide) 2.0M 실험구에서 64.3%로 가장 좋은 결과를 보였다.

주제어 : 냉동보존, 발생배, 동해방지제, 액체질소, 바지락

1. Introduction

Advances in cryopreservation of gametes and embryos in aquatic organisms are modest compared with progress in terrestrial animals and plants. Cryopreservation has been successfully investigated in oyster's sperm and D-shaped

larvae include fish sperm and shrimp (Nauplius) larvae[1-4]. Cryopreservation of late embryos and early larvae was achieved in the oysters and hard clams[5]. However, Tiersh and Green[6] reported that cryopreservation technology is a potential tool for preservation of genetic material. Liu and Terrence[7] also reported

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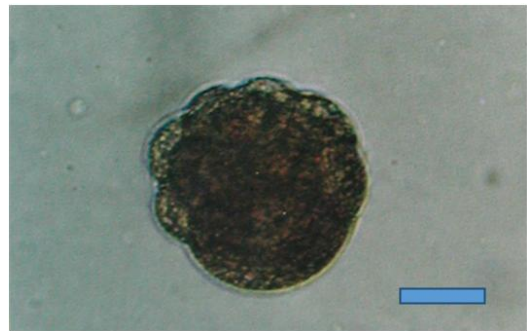
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earlier that cryopreservation of milt from several aquatic species has facilitated hybridization, gynogenesis as well as domestication and conservation of important traits in commercial aquaculture. In the recent past, a number of researchers carried out artificial insemination studies in various shrimp species and reported varying degrees of success[8-11]. Cryopreservation of shellfish embryos compared to fish embryos is easy because of their smaller size, which enables effective permeability of water and cryoprotectants, low yolk content and holoblastic cleavage of the developing embryos[12]. Among shellfish, cryopreservation studies involving embryos and larvae have been carried out mainly in pacific oysters because of their high commercial value [13,14]. Cryopreservation of finfish ova and embryos has not been successful until now. Unlike shellfish eggs and embryos, finfish ova and embryos are large, contain a large amount of yolk and are covered with a relatively thick chorion. These large and dense specimens do not show uniform penetration of conventional cryoprotectants and cooling during the freezing process. The potential role of cryopreservation in the artificial propagation of many aquatic animals is similar to that of breeding of terrestrial plants and animals. Furthermore, cryopreservation plays a significant role in aquatic biodiversity, eco-toxicology, and environmental conservation[15]. Currently, cryopreservation techniques in domestic animals are well known and are applied commercially. In contrast to research and advances in the cryopreservation of sperm and embryos of domestic animals and humans, few methodologies are available for the application of cryopreservation in aquatic organisms except fish sperm[16]. Cryopreservation is considered as an effective strategy to save endangered species by facilitating the storage of their

gametes in a gene bank because of several benefits. Stocks can be protected from being totally eliminated due to sudden disease outbreaks, natural disasters, or accidents such as oil spills[17]. *R. philippinarum* is an economically important shellfish cultured in Asia for its nutritious components, especially for boiled soup material. The demand for high-quality larvae is increasing due to excessive consumption of natural resources and expansion of artificial breeding programs. Therefore, the present study investigated the effect of cryoprotectants (DMSO : dimethyl sulfoxide, EG : ethylene glycol, Gly : glycerol PD : 1,2-propanediol) on cryopreservation of embryos derived from Manila clam, *R. philippinarum*.

2. Materials and methods



Scale bar : 20 μ m.

Fig. 1. Embryo of *Ruditapes philippinarum* used in the experiment.

Cryoprotectants such as dimethyl sulfoxide, ethylene glycol, glycerol and 1,2-propanediol were used in different concentrations ranging from 1.0 M to 3.0 M as shown in Table 1. As you can see in Fig. 1, embryos of *R. philippinarum* were diluted with filtered sea water and then mixed with cryoprotectants of different concentrations. After equilibration for 20 min. under room temperature, samples of embryo were loaded into 0.5 mL straws (200 embryos per straw) and then transferred to a

programmable freezer (Kryosave Integra, Rovers Polska from UK) for cryopreservation. Two-step freezing processes were performed: the samples were first cooled at $-1^{\circ}\text{C}/\text{min.}$ from 20°C to -12°C , holding for 10 min. and then cooled to -35°C at $-2^{\circ}\text{C}/\text{min.}$ holding for 30 min. Immediately the straws were plunged into liquid nitrogen (-196°C). The straws were thawed in a water bath at 25°C directly after recovery from liquid nitrogen. The embryos were placed in 50 mL beakers and mixed with seawater. The survival rate was estimated by counting the number of live and dead larvae under a microscope (CX 41RF, OLYMPUS, PHILIPPINES, $\times 100$). After storage for one day in liquid nitrogen, embryos were thawed and then cultured in seawater after the removal of cryoprotectants. Time intervals of 1, 3, 6, 9 and 12 days were set after thawing. Embryo survival in each of the four groups was estimated at the five concentrations. Untreated embryos were also cultured to measure the survival rate at different times as a control group.

Table 1. Experimental protocol according to concentrations of various cryoprotectants

Cryoprotectant	Con. (M)	Constituent (mL)
DMSO	1.0	DMSO 7.73/SW 100
	1.5	DMSO 10.81/SW 100
	2.0	DMSO 14.30/SW 100
	2.5	DMSO 17.10/SW 100
	3.0	DMSO 22.30/SW 100
EG	1.0	EG 5.48/SW 100
	1.5	EG 8.23/SW 100
	2.0	EG 10.97/SW 100
	2.5	EG 12.71/SW 100
	3.0	EG 16.30/SW 100
Gly	1.0	Gly 7.45/SW 100
	1.5	Gly 11.22/SW 100
	2.0	Gly 14.89/SW 100
	2.5	Gly 18.67/SW 100
	3.0	Gly 21.25/SW 100
PD	1.0	PD 7.42/SW 100
	1.5	PD 11.20/SW 100
	2.0	PD 14.85/SW 100
	2.5	PD 18.62/SW 100
	3.0	PD 21.30/SW 100

(Con : concentration, DMSO : dimethyl sulfoxide, EG : ethylene glycol, Gly : glycerol, PD : 1,2 - propanediol, SW : sea water).

To analyze the survival rate following treatment at each concentration and solution, we used SAS statistical software (SAS 9.4, SAS institute, Cary, NC, USA) to perform analysis of variance. When there was a significant difference at $P < 0.05$, the mean separation was conducted using Tukey's "honestly significant difference" test to compare the means among the treatments. Tukey-adjusted pairwise comparison was used to determine the survival rate under five different concentrations and four different solutions.

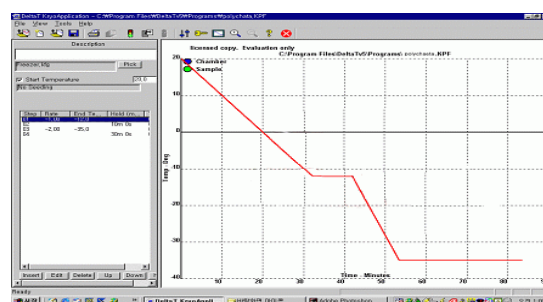
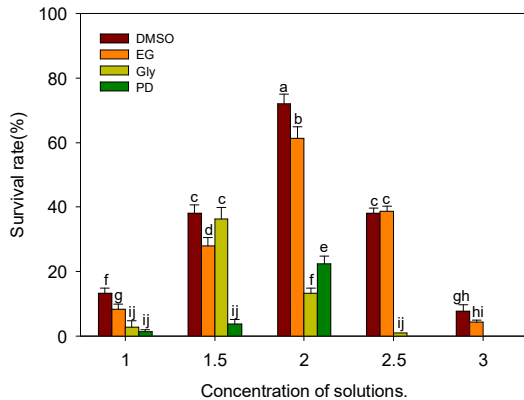


Fig. 2. Freezing protocol for various cryoprotectants according to elapsed time

3. Results

In this experiment, the effects of 4 cryoprotectants (CPAs) on the growth and survival rate of post-thawed embryos of *R. philippinarum* were assessed after rearing for 1 h daily in a nitrogen tank over 12 days. The results are shown in Figure 3. It is obvious that the DMSO was better than the other three cryoprotectants. The maximum survival rate of the embryo (82.3%) was obtained with 2.0 M DMSO. DMSO was the best of the four cryoprotectants and PD was the worst, while EG and Gly were intermediate. The effect of the cryoprotectants varied with the concentration. The survival of embryo after thawing varied with time after exposure to different cryoprotectants. The survival rate definitely decreased with lapse

of time even in the control group. The DMSO group yielded the best results at a concentration less than 2.0 M. DMSO treatment increased the percentage of survival rate progressively as the cryoprotectant concentration was increased from 1.0 M to 2.0 M, but dramatically decreased from 2.5 M to 3.0 M as shown Fig. 3 and Fig. 4. In case of treatment with 1.0 M DMSO, on day one, the survival rate was $15.3 \pm 2.1\%$, while on day 3, it was $11.6 \pm 1.2\%$. The survival rate on day 9 was $8.3 \pm 0.4\%$, but surprisingly all embryos were dead. The survival rate under 1.5 M DMSO was $40.3 \pm 3.7\%$ on day 1, $26.7 \pm 2.7\%$ on day 6, $19.3 \pm 0.9\%$ on day 9, and $14.3 \pm 0.9\%$ on day 12. However, treatment with 2.0 M DMSO yielded the highest value, $64.3 \pm 3.2\%$. In case of 2.5 M DMSO, a survival rate of $45.0 \pm 3.6\%$ to $22.6 \pm 1.9\%$ was observed on day 6. However, all embryos were dead after 9 days as shown in Fig. 4.



concentrations (DMSO : dimethyl sulfoxide, Gly : glycerol, PD : 1,2-propanediol, EG : ethylene glycol). Different letters on the bars indicate significant difference ($p < 0.05$).

Fig. 3. Survival rates of *Ruditapes philippinarum* embryos at various cryoprotectant

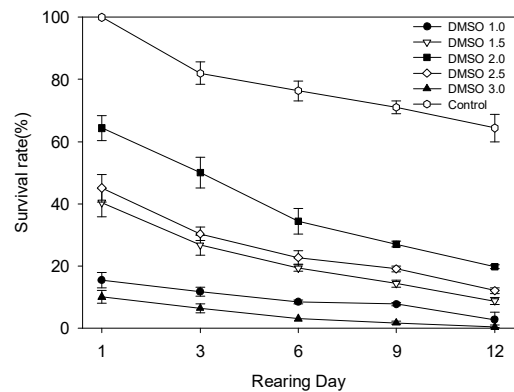


Fig. 4. Survival rates of *Ruditapes philippinarum* embryos according to elapsed time after thawing with DMSO (dimethyl sulfoxide)

As showing in Fig. 5, the survival rate in the presence of 1.0 M EG was $9.6 \pm 0.4\%$ on day 1, and $6.0 \pm 0.8\%$ on day 3. However, all *R. philippinarum* embryos died after 6 days. The survival rate increased gradually at concentrations higher than 1.5 M to $26.7 \pm 0.9\%$ on day 1, $11.3 \pm 0.9\%$ on day 6, and $6.0 \pm 0.8\%$ on day 9. However, all *R. philippinarum* embryos died after 9 days as shown in Fig. 5. An EG concentration of 2.0 M yielded the highest survival rate of $50.0 \pm 2.1\%$, which declined to $19.3 \pm 1.2\%$ on day 9, and $12.3 \pm 2.1\%$ by day 12. The survival rate at a concentration of 3.0 M was $5.3 \pm 0.5\%$, $1.3 \pm 1.2\%$ on day 6, but all *R. philippinarum* embryos died after 6 days. The survival rate in the presence of 1.0 M glycerol was $5.0 \pm 0.8\%$ on day 1, but all embryos were dead eventually as shown in Fig. 6. In case of 1.5 M, it was $30.6 \pm 0.9\%$ on day 1, $17.0 \pm 0.8\%$ on day 6 and $11.0 \pm 1.4\%$ on day 9, which eventually declined to $7.0 \pm 1.4\%$ on day 12. However, the survival rate at 2.0 M was $15.0 \pm 1.6\%$, $8.3 \pm 1.2\%$ on day 6. However, all *R. philippinarum* embryos died subsequently. No surviving *R. philippinarum* embryo was observed at 3.0 M. The survival rate on day 1 was $2.3 \pm 0.4\%$ (1.0 M) and $5.0 \pm 0.8\%$ (1.5 M), but all embryos died subsequently as shown in Fig. 7. In the case of

2.0 M, the highest survival rate of $16.3 \pm 1.2\%$ was observed, which declined to $4.3 \pm 1.2\%$ on day 9 and $2.0 \pm 0.8\%$ on day 12. There was no survivor left at 2.5 M and 3.0 M.

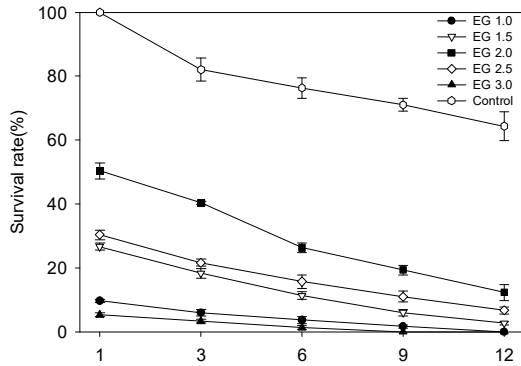


Fig. 5. Survival rates of *R. philippinarum* embryos according to elapsed time after thawed with ethylene glycol

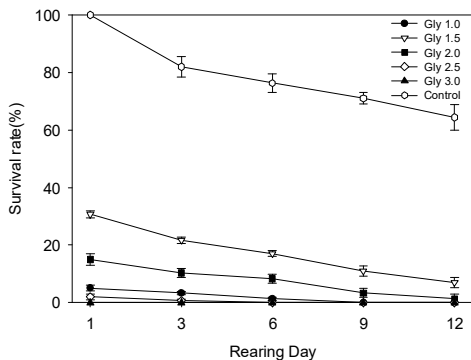


Fig. 6. Survival rates of *R. philippinarum* embryos according to elapsed time after thawing with glycerol

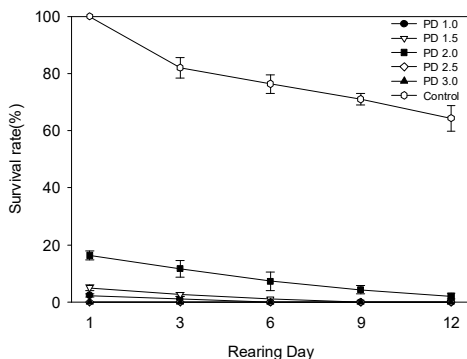


Fig. 7. Survival rates of *R. philippinarum* embryos according to elapsed time after thawing with 1,2-propanediol

4. Discussion

In order to ensure good freezing tolerance and survival rates during cryopreservation, it is essential that only the best *R. philippinarum* embryos are used. Other studies[18] corroborate the findings of the present one in that embryos of poor quality are of little value in assessing the effect of various parameters. The use of good quality embryos should improve the prospects of obtaining higher survival rates using stepwise freezing protocols and vitrification protocols. Asahina and Takahashi[19] reported that late developing embryos of sea urchin survive at least for a short period of time at -196°C in the presence of cryoprotectants like DMSO and ethylene glycol. The viability and growth of shellfish and oyster eggs cryopreserved[14] at -196°C and thawed after 30-60 min were compared with that of non-frozen control embryos derived from the same parents. Growth of *Manila clam* larvae was monitored for 35 days and the shell length, which was high at 84%, was not significantly different compared to that of the control. In an early trial, frozen pacific oyster larvae were reared successfully to settlement, but survival was reported to be poor. However, after modification of the freezing process, the survival of larvae was substantially elevated[20].

Rana et al.[20] also reported poor cryopreservation of eggs and embryos of the oyster, *Crassostrea gigas*. However, varying degrees of success have been reported using early trocophore (7 h) larvae. In all the trials, DMSO concentrations of 1.0-1.5 M resulted in consistently better thaw ability of larvae that glycerol. At least, 40-50% of the larvae were recovered after thawing. The post-thaw viability was found to increase by 15% using larger embryos. As regards freezing rate, which is the most influential factor in this study, the

optimum freezing rate should allow sufficient time for extraction of cellular water, while minimizing the damage caused by dehydration or biotoxicity. Based on similar principles, protocols with optimum freezing rates were designed and tested to allow CPAs to equilibrate across the membrane during freezing. The freezing program for late larvae of hard clams was developed. The experience obtained with the oyster trials provided a valuable standard of reference. DMSO is a useful CPAs for the cryopreservation of fish sperm[6] and fertilized fish eggs[21]. However, no study reported the effect of DMSO and its optimal formula to meet the species-specific characteristics of hard clam embryos/larvae. Cryopreservation of spermatozoa has long been used in the breeding of many animal species and is now slowly spreading to aquaculture, for the countless advantages it offers[3]. During the cryopreservation of gametes, embryos and larvae of marine organisms, studies focused increasingly on shellfish besides fish. Studies investigating cryopreservation of oyster *Crassostrea gigas* embryos and larvae analyzed various parameters[22,23]. During cryopreservation, low-temperature dehydration is critical to prevent internal ice formation. Organisms are cooled by CPAs and ice formation in the external solute mixture during the cooling process. Progressive dehydration then occurs as the solute ice mixture is further cooled until a point is reached, where ice does not form internally or transfer to liquid nitrogen. Usually the presence of internal cryoprotectants can lead to vitrification, without formation of new ice subsequently[24].

Cryoprotectants are classified as permeating and non-permeating, according to their ability to pass through the cell membrane. Permeating cryoprotectants such as DMSO, EG, Gly and PD

used in this experiment usually reduce the ice point of the internal suspension. For the cryopreservation of spermatozoa of marine fish, DMSO generally yields the best results [13]. Similar results also were achieved in the current studies of cryopreservation involving *R. philippinarum* embryos. Other cryoprotectants not discussed here may show good cryoprotective effects in other organisms or cells. An optimal cryopreservation procedure exists for each cell or organism, and is defined by the cryoprotectant used, and the freezing and thawing protocol [25]. Slow freezing can prevent ice formation, while rapid thawing is necessary to avoid recrystallization. The two-step cooling method is a widely used protocol [26], in which the cells are initially cooled slowly to -40°C over a period and then immersed in liquid nitrogen. Cell dehydration occurs during the initial slow cooling to prevent intracellular freezing, and the slow cooling to -40°C at around -1°C per min facilitates successful cryopreservation regardless of cell type [27]. Cooling rate is the most critical factor when optimizing the cryopreservation protocol. The basic concept of cryopreservation is slow freezing and fast thawing. Rapid cooling can freeze the intracellular water mostly resulting in great damage. Conversely, a slow cooling rate results in significant toxic effect of cryoprotectants. Cooling rates selected in this experiment ($-1^{\circ}\text{C}/\text{min}$ and $-2^{\circ}\text{C}/\text{min}$) were based on this idea and on our previous successful experience. Diwan et al. [15] mentioned that eggs are fundamentally more difficult to freeze successfully than sperm, primarily because the large size of eggs interferes with the penetration of cryoprotectants and uniform cooling during the cryopreservation process. Consequently, eggs with a large yolk-sac tend to develop crystals, which damage the egg during

freezing. It has been also stated that the chromosomes in the egg are more vulnerable to damage those in sperm. Also, the loss of membrane integrity both in sperm and eggs is a critical risk factor for damage during the freeze/thaw process. Therefore, further efforts are strongly needed, particularly to improve this technology not only for cryopreservation of eggs, but also for embryos and larvae of economically important shellfish species.

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