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The Effects of Cryopreservation on Fine Structures of Pearl Oyster (*Pinctada fucata martensii*) Larvae

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냉동보존이 진주조개(*Pinctada fucata martensii*) 유생의 미세구조에 미치는 영향

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ABSTRACT : The freezing susceptibilities of two larval stages (D-shaped and umbo) of the pearl oyster (*Pinctada fucata martensii*) were evaluated by the electron microscopy (light, transmission electron and scanning electron). The morphological shapes were examined from each pre-frozen or frozen-thawed stage of the cryopreserved larvae in liquid nitrogen by using the cryoprotectant, dimethyl sulfoxide (Me_2SO) mixed with sucrose. Although a portion of the shell was damaged, the hinge and prodissoconch were intact and clearly visible after preservation in liquid nitrogen. In addition, the cytoplasm of the frozen-thawed larvae maintained the normal organelle integrities, e.g., endoplasmic reticula, lipid droplets, mitochondria, nucleus and microvilli. However, some of the frozen-thawed larvae showed irregularly arranged cilia, rough shell surfaces and round-lumped cilium heads. These results indicate that *P. fucata martensii* larvae are susceptible to freezing, at least at those two critical developmental stages (D-shaped and umbo), and suggest a new industrial investigation including reduction method of cell injury for preserving microbial starter cultures need to be developed.

Key words : Pearl oyster, *Pinctada fucata martensii*, Larvae, Cryopreservation, Fine structure.

요 약 : 냉동보존이 진주조개(*Pincata fucata martensii*) 유생의 형태 및 구조에 미치는 영향을 알아보기 위하여 냉동전후 D형 및 각정기 유생을 광학 및 전자현미경으로 조사하였다. 동해방지제는 0.2 M sucrose를 첨가한 2.0 M Me_2SO를 사용하였다. 냉동후 유생은 일부 패각이 손상되긴 했지만 hinge와 prodissoconch가 뚜렷하게 나타났으며, 소포체, 지질 과립, 미토콘드리아, 핵 등을 포함한 세포내 소기관들이 고르게 분포되어 있었다. 또한, 섬모가 규칙적으로 배열되어 있었고, 섬모 아래 미토콘드리아와 지질과립이 위치해 있는 것이 관찰되었으나, 일부 해동된 유생에서 섬모의 불규칙적인 배열과 섬모환이 둥글게 뭉쳐져 있는 모습이 관찰되었다. 이러한 결과는 진주조개의 D형 유생과 각정기 유생이 냉동에 쉽게 영향을 받을 수 있다는 것을 보여준다. 따라서 냉동보존 시 세포의 손상을 감소시킬 수 있는 연구가 이루어져야 할 것으로 사료된다.

INTRODUCTION

Cells, in general, are highly susceptible to severe damage during freezing and thawing. To circumvent intracellular injury of aquacultured or food industry species,

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various studies have examined different cryopreservation methods. These studies have investigated parameters such as optimal developmental stage, extender composition, cryoprotectant composition and cooling and thawing rates in bivalve embryos and larvae (Chao *et al*., 1997; Naidenko, 1997; Chang *et al*., 1999; Choi & Chang, 1999, 2003; Chao & Liao, 2001; Paniagua-Chavez & Tiersch, 2001). Most of these studies, however, determined larval survival rates, rather than non-fatal morphological alterations. In general, larval development in mollusc could be influenced by many factors including states of nurtrition and hatch rate. Studies on bivalve species have focused on the internal or external systems of fresh larvae (Chang *et al*., 1989; Cragg, 1989).

Thus, the effects of cryopreservation on the fine structures of bivalve larvae remain poorly understood, and few studies have described the feature of embryo or larva according to pre/post freezing. Therefore, this study was conducted to evaluate the effects of pre-freezing and frozen-thawing on the pearl oyster, *Pinctada fucata martensii* larvae.

MATERIALS AND METHODS

1. Freezing and Thawing Procedures

A modified version of the method (Choi & Chang, 2003) was employed for cryopreservation of larvae. The late D-shaped (3 days after fertilization, 83.8±4.6 μm of shell length (SL) and 72.6±5.1 μm of shell height (SH)) and umbo stage (7 days after fertilization, 112.3±8.7 of SL and 103.5±9.6 μm of SH) larvae were stabilized at room temperature in a freezing medium composed of 0.2 M sucrose and 2.0 M dimethyl sulfoxide (Me_2SO) for 15 min, and were transferred into 0.5 ml straws (FHK, Japan). The straws were immediately placed in a programmed freezer (Samwon Freezing Engineering Co., Korea) at 0℃. The temperature was dropped subsequently at a rate of 1℃/min from 0℃ to −12℃. After seeding began at −12℃, the larvae were kept at −12℃, for 10 min. The straws were then cooled at a rate of 1℃/min from −12℃ to −35℃, at which point they were quickly immersed in liquid nitrogen and held constantly for 1 h. To thaw the larvae, the straws were immersed in a water bath at 25℃ for 10 sec. After thawing, the larvae were transferred to a glass tube, and fresh artificial seawater (ASW) was gradually added to avoid osmotic shock. ASW contained 2.7 g NaCl, 0.07 g KCl, 0.05 g $NaHCO_3$, 0.12 g $CaCl_2$, 0.46 g $MgCl_2$ in 100 ml distilled water. The larvae were fed mixed diets of *Isochrysis galbana*, *Chaetoceros calcitrans* and *Pavlova lutheri* (these three microalgae were provided by South Sea Mariculture Research Center, NFRDI, Korea) during experimental period .

2. Examination of the External Larval Structures

To observe the shapes of the D-shaped and umbo stage larvae, pre-frozen and frozen-thawed larvae were prepared, and samples were placed into a hole slide to be observed by light microscope (BX 504F, Olympus, Japan). The scanning electron microscope (SEM; DMS 940-A, Carl Zeiss, Germany) analyses methods were applied to investigate the external fine structures of the larvae. The larvae were pre-fixed for 2 h at 4℃ in 2.5% glutaraldehyde solution buffered with 0.1 M phosphate buffer solution (PBS, pH 7.2). Subsequently, the samples were dehydrated in a series of ethanol (50% to 100%). The samples were treated with a gold palladium coating (15 to 20 mm) and were observed with the SEM.

3. Examination of the Internal Larval Structures

To observe the inner structures of the D-shaped and umbo stage larvae, we performed microscopy with a transmission electron microscope (TEM; JEM 1200 E-ⅩII, 60-80 Kv, JEOL, Tokyo, Japan). The samples were sectioned to 70 nm with toluidine blue stain (Fluka, Swiss), placed onto a hole slide and observed under the light microscope. To investigate the inner fine larval structures, scanning electronic samples were produced. The samples were pre-fixed in the same manner as the transmission

electronic samples, in a 2.5% glutaraldehyde solution buffered by 0.1 M PBS, pH 7.2 for 2 hours at 4℃. After washing with PBS for 10 minutes, the samples were post-fixed in 1% osmium tetroxide (OsO_4) for 2 hours at 4℃. The samples were washed again with PBS, serially dehydrated in a series of ethanol and embedded in epon 812. We cut 0.5 μm sections with an ultramicrotome (LKB, Nova, Sweden) and then stained them with toluidine blue to determine an investigation region. After that ultra-thin sections of 70 nm thicknesses were cut with a diamond knife, and the sections were stained with uranyl acetate and lead citrate solutions and examined by TEM.

4. Statistical Analysis

Survival rates of larvae in cryopreservation experiments were expressed as the means (S.E of 50 samples. The significance of differences between mean survival rates for each factor were tested by Duncan's multiple range post-hoc test. Differences with a probability value (*P*) of 0.05 or less were considered significant.

RESULTS AND DISCUSSION

1. Examination of the External Larval Structures

The pre-frozen larvae in two different developmental stages were transparent with conspicuous granules, and the cilia of velum moved actively (data not shown). Data from the SEM for the shell surface of D-shaped larvae revealed that the prodissoconch I and II were clearly visible and with growth rings (Fig. 1A). The provincular structure was simple. The prodissoconch I shell of the pearl oyster bore the same punctate stellate pattern as described by Ansell (1961). Carriker & Palmer (1979) and Waller (1981) described the punctuate regions of *Crassostrea virginica* and *Ostrea edulis*, respectively. The punctate region in *C. virginica* is roughly 30 μm in diameter, and the region in *O. edulis* is ovoid, approximately 30×50 μm. However, in the pre-frozen or frozen-thawed *P. fucata martensii* larva, the punctate pattern covered an area of approximately 59×77 μm (n=68).

The bilobe configuration of the outer velum margin was reported among *C. virginica* larvae (Elston, 1980). The cilia on the velum rim were lucid and the apical tuft was visible through the shell (Fig. 1B). The velum cilia provide the means of bivalve propulsion. The contribution of the various bands of cilia to this propulsion action can be best understood by considering the relative sizes of the cilia, their arrangement, and the directions in which they beat (Cragg, 1989). These cilia might play a sensory role though no sensory receptors have been detected in the region of the inner ciliary ring in *P. maximus* (Cragg, 1989). Mackie *et al*. (1976) described the velum function, anatomy and physiology in gastropod veligers, while Strathmann and Leise (1979) investigated the beating of the velar cilia of the pacific oyster, *Crassostrea gigas*.

In the frozen-thawed larvae, the hinge and prodissoconches

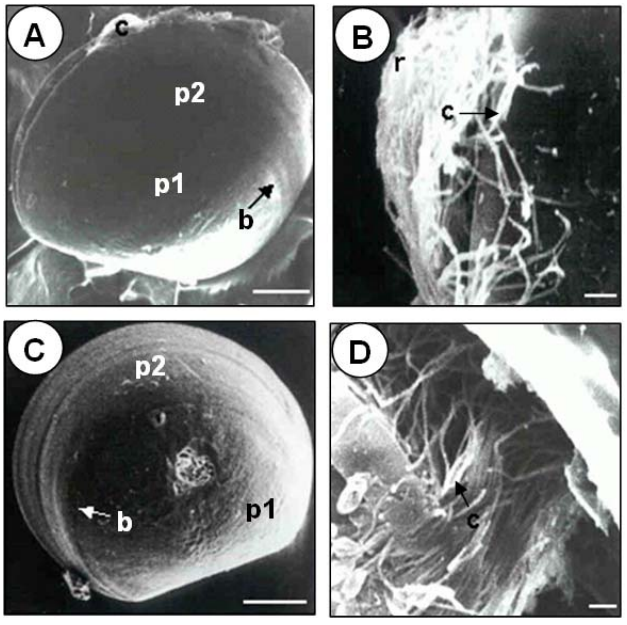

Fig. 1. SEM of external surface of D-shaped larvae of pearl oyster, *Pinctada fucata martensii*. A and B: view of pre-frozen larvae. C and D: view of frozen-thawed larvae. b: prodissoconch Ⅰ/Ⅱ boundary, c: cilia, p1: prodissoconch I shell, p2: prodissoconch Ⅱ shell, r: rim of velum. A and C scale bar = 20 μm. B and D scale bar = 2 μm.

of the D-shaped larvae were intact and clearly visible in spite of freezing (Fig. 1C). However, some of the D-shaped larvae removed possessed cilia on the velum, irregularly arranged cilia and a round-lumped cilium head. The edge of the velum sported injured cilia (Fig. 1D). Similar result was observed in the umbo stage larvae (data not shown).

2. Examination of the Internal Larval Structures

In the pre-frozen larvae, all components of cellular organelles as well as every vital organs were well-developed in two different stages. Cilia on the outer margin of the velum arranged in a low and a great number of cilia in the umbo stage larvae appeared more than the D-shaped larvae (Figs. 2A and 3A). The rootlets of the cilia were surrounded by numerous mitochondria (Figs. 2A and 3B). These mitochondria and rootlets are distinctly larger than their counterparts in adjoining cells. The rootlets extend deep into the cytoplasm to the level of the nucleus.

As a velum is an organ of locomotion and is located in the margin of a body, it could be extremely affected with freezing. After thawing, intracellular structures including mitochondria, nucleus, lipid droplets, yolk granules and endoplasmic reticulum were partially altered in some of the frozen-thawed cells (Figs. 2B and 3C-D) but shell was not severely damaged. The frozen-thawed larvae had an irregular shaped nucleus with few heterochromatin and mitochondria with more electron-dense. Especially, the damaged extents were increased as larval development proceeded (Figs. 2B and 3C-D). In addition, the margin of organs including the region of the rootlets of cilia was not remained intact. The appearance of numerous vacuoles was featured in the frozen-thawed cell (Fig. 3D).

Survival rates of the frozen-thawed D-shaped and umbo stage larvae were 87.3% and 17.8%, respectively (Table 1). However, when the frozen-thawed larvae were incubated in the fresh sea water for 3 days, the larvae did not grow to next developmental stage and failed to survive (data not showed). These data suggest that the histological observations by SEM and TEM may not be sufficient to find out the effects of cryopreservation on the larval survival.

It has been reported that embryonic tolerance of biochemical and osmotic damage from cryoprotectant and

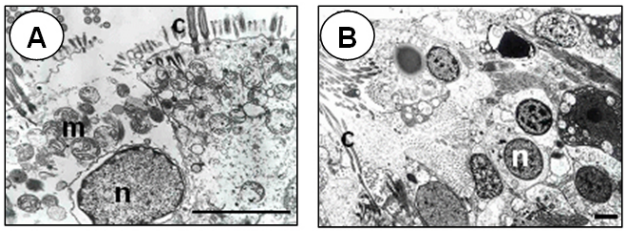

Fig. 2. TEM of internal structures of D-shaped larvae of pearl oyster, *Pinctada fucata martensii*. A: view of pre-frozen larva. B: view of frozen-thawed larva. c: cilia, m: mitochondrion, n: nucleus. Scale bar = 2 μm.

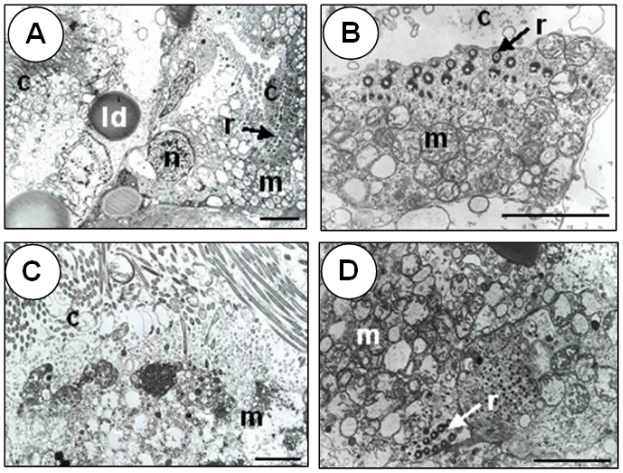

Fig. 3. Internal structures of umbo stage larvae of pearl oyster, *Pinctada fucata martensii*. A and B: view of internal structure of pre-frozen larva. C and D: view of internal structure of frozen-thawed larva. c: cilia, ld: lipid droplet, m: mitochondrion, n: nucleus, r: root of cilia. Scale bar = 2 μm.

Table 1. Survival rates of frozen-thawed pearl oyster (*P. fucata martensii*) larvae

Developmental stage	Survival rates (%, mean±S.E.)
D-shaped larva	87.3±9.7[a]
Umbo larva	17.8±6.3[b]

Values with different subscripts within a column differ (p<0.05).

freezing (Renard & Cochard, 1989; Toledo *et al*., 1991). Gwo (1995) suggested that the trochophore larva with lower lipids has the highest survival rates in the various developmental stages. In the previous study, we found that the survival rates of the D-shaped larva were higher than those of the trochophore, and the survival rates were different at various developmental stages in the pearl oyster (Choi & Chang, 2003).

Freezing is commonly used for preserving and storing microorganisms and for the production of concentrated starter cultures for the food industry (Calcott, 1986). Cryopreservation optimization requires further understanding of the biophysical responses in the target cells or tissues during freezing (Devireddy *et al*., 1998). These biophysical responses are linked directly to cell injury and survival.

In conclusion, freezing-thawing, as compared to pre-freezing, did somewhat disrupt the morphology or fine structures of the larvae in the pearl oyster. The frozen-thawed larvae showed irregularly arranged cilia, rough shell surfaces and round-lumped cilium heads. These results indicate that *P. fucata martensii* larvae are susceptible to freezing, at least at those two critical developmental stages (D-shaped and umbo stage larva), and suggest a new industrial investigation to reduce cell injuries for preserving microbial starter cultures need to be developed.

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