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CRYOPRESERVATION OF HEART CELLS FROM THE EASTERN OYSTER

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SUMMARY

Conditions were developed to cryopreserve cells from pronase-dissociated atria and ventricles of eastern oysters (*Crassostrea virginica*). The effect of three concentrations (5, 10, 15%) of the cryoprotectants (dimethyl sulfoxide, glycerol, and propylene glycol), three thawing temperatures (25, 45, 75° C), and three cooling rates (slow, medium, fast) were compared. Cells were frozen at −80° C and plunged in liquid nitrogen. Thawed cells were seeded in 96-well plates and primary cultures were evaluated after 3 d by measuring the metabolic activity using a tetrazolium compound, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, and by comparing the relative spreading of cells between treatments. The best conditions for freezing and thawing of cells for each cryoprotectant were selected and a final study was performed to compare cryoprotectants. For this final study, we measured the number of cells and their viability 3 d after thawing, in addition to determining cell metabolic activity and cell spreading. Primary cultures of cells frozen without cryoprotectant and of nonfrozen cells were used as controls in all studies. Atrial cells were best cryopreserved with glycerol at a concentration of 10%, a medium cooling rate, and thawing at 45° C. After thawing, atrial cells showed 53 ± 5% of the metabolic activity, 84 ± 5% of the number, and 92 ± 2% of the viability of nonfrozen cells. For ventricular cells, 10% glycerol, a medium cooling rate, and thawing at 25° C yielded the best results. The thawed ventricular cells showed 83 ± 5% of the metabolic activity, 91 ± 5% of the number, and 96 ± 2% of the viability of nonfrozen cells.

Key words: Crassostrea virginica; bivalve mollusc; somatic cells; primary cultures.

INTRODUCTION

Primary cell cultures of oysters and other bivalve molluscs are being increasingly manipulated in various fields because of the absence of cell lines (Boulo et al., 1996; Birmelin et al., 1999; Canesi et al., 1999; Pazos and Mathieu, 1999; Berthelin et al., 2000). A potential problem of using primary cultures of oysters for long-term comparative studies is the variation in cell activities and viability which can arise from using animals at different times of the yr, of different ages, and from different locations. Oysters are osmoconformers and poikilotherms, and their physiology and biochemistry are influenced by environmental conditions (Shumway, 1996). Seasonal metabolic changes in oysters in relation to their reproduction have been studied extensively and can be expected to influence the activities of isolated cells in culture (Gabbott, 1983; Thompson et al., 1996). An additional problem encountered in culturing oyster cells is contamination by bacteria, protozoa, or fungi because oysters are filter feeders. Moreover, the likelihood of contamination of primary cultures increases in summer with the increase in microbial densities in seawater. Cryopreservation can be used to alleviate these problems by enabling the storage of a large number of oyster cells at ultralow temperatures at selected times of the yr. Cryopreservation can therefore provide a reliable and uniform source of oyster cells for conducting in vitro studies (Rinkevich, 1999). The usefulness of this methodology has recently been demonstrated by Pomponi et al. (1997), using batches of cryopreserved sponge cells to routinely establish primary cultures for experimentation.

Techniques have been developed to cryopreserve spermatozoa, embryos, and larvae of a number of bivalve molluscs with varying degrees of success (Chao et al., 1997; Naidenko, 1997; Paniagua-Chavez et al., 1998; Lin et al., 1999; Gwo, 2000). Interest in this technology arose from its potential use in bivalve aquaculture for extending seed production in hatcheries outside of the natural breeding season and for facilitating the development and preservation of valuable strains of bivalve molluscs. In contrast, only a few studies have reported the cryopreservation of somatic tissues or cells of bivalve molluscs. Odintsova and Tsal (1995) cryopreserved dissociated cells from mantle and gill tissues of the north Pacific mussel, *Mytilus trossulus*, and from the digestive gland and embryonic tissues of the Japanese scallop, *Mizuhopecten yessoensis*. Cells dissociated from the heart of the great scallop, *Pecten maximus*, were cryopreserved by Le Marrec-Croq et al. (1998). Horita et al. (1999) demonstrated that pearl oyster mantles cryopreserved for 14 mo could be thawed and grafted into recipient pearl oysters, resulting in the production of pearls. Effective cryopreservation conditions in each of these studies varied with the species, developmental stage, and cell type.

Conditions to cryopreserve cells from dissociated oyster tissues

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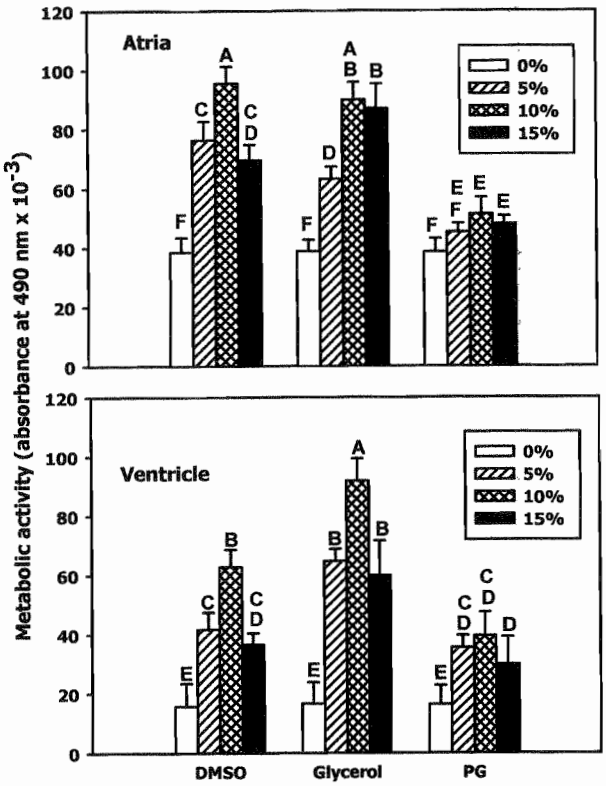

FIG. 1. Metabolic activity of thawed atrial and ventricular cells of the eastern oyster, *C. virginica*, exposed to different cryoprotectants and concentrations. Cells were cryopreserved with propylene glycol DMSO, glycerol, and propylene glycol PG at three concentrations, and thawed at 45° C. Metabolic activities of thawed cells were evaluated after 3 d of culture in three separate studies (N = 3). For each study, cells from 30 atria or 30 ventricles were used. *Bars* sharing *letters* were not significantly different ($P > 0.05$).

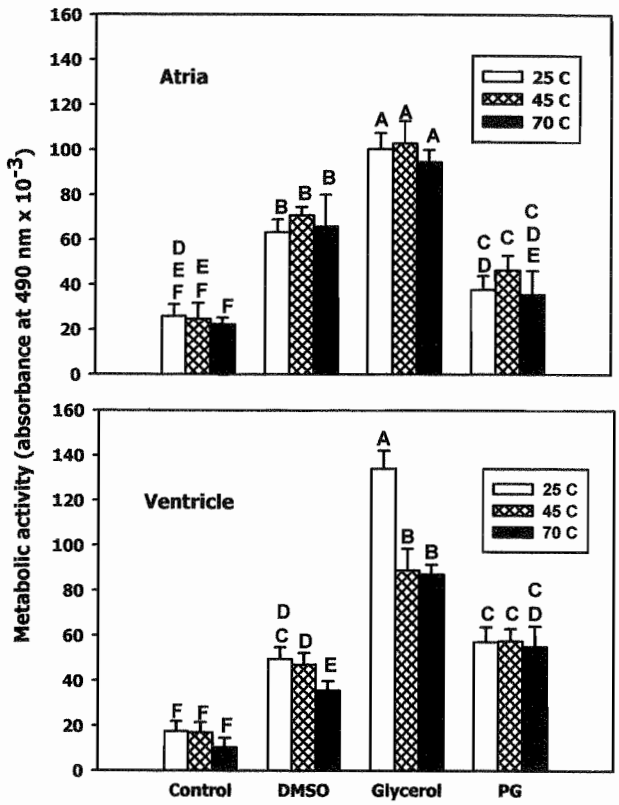

FIG. 2. Metabolic activity of atrial and ventricular cells of the eastern oyster, *C. virginica*, frozen with a 10% final concentration of glycerol, DMSO, or PG, and thawed at three temperatures. The control indicates cells that were frozen without cryoprotectant. Metabolic activities of cells were evaluated after 3 d of culture in three separate studies (N = 3). For each study, cells from 30 atria or 30 ventricles were used. *Bars* sharing *letters* were not significantly different ($P > 0.05$).

have not been reported. Our goal was to develop conditions to cryopreserve cells from eastern oyster atria and ventricles. Heart cells were selected because they have most often been used to establish primary cultures for in vitro studies and for attempts to develop an oyster cell line (Li et al., 1966; Brewster and Nicholson, 1979; Hetrick et al., 1981; Boulo et al., 1996; Chen and Wen, 1999). During the cooling and thawing phases of cryopreservation, major stresses, such as the physical and chemical effects of ice crystals and osmotic shock can damage cells. These effects can be reduced by controlling the cooling rate, thawing rate, and by addition of chemicals such as dimethyl sulfoxide (DMSO) (Lovelock and Bishop, 1959) and glycerol (Leung, 1991) as cryoprotectants to modify intracellular and intercellular environments. In the present study, we compared for the first time the effects of three cryoprotectants (DMSO, glycerol, and propylene glycol), cryoprotectant concentration (5, 10, 15%), thawing temperature (25, 45, 75° C), and cooling rate (slow, medium, fast) on the cryopreservation of oyster cells. The best conditions for freezing and thawing oyster cells for each cryoprotectant were selected and a final study was performed to determine optimal conditions for cryopreservation of oyster heart cells.

MATERIALS AND METHODS

Oyster depuration. Oysters were collected at Grand Isle, Louisiana (29°40′00″N, 90°02′30″W), scrubbed with a 5% bleach solution, and rinsed thoroughly in tap water. They were maintained in a recirculating system containing ultraviolet-light treated artificial seawater (Instant Ocean, Aquarium Systems, Mentor, OH) at 15 ppt and 25° C. Oysters were kept in the tank for at least 1 wk without being fed, in order to reduce microbial contaminants.

Tissue dissection and decontamination. Ventricles or atria of 30 oysters were dissected, pooled, and washed three times with 30 ml of sterile 0.2-μm–filtered artificial seawater (ASW) at 15 ppt. All chemicals were of reagent grade and were purchased from Sigma Chemical Co. (St. Louis, MO), unless otherwise indicated. Each tissue type was transferred to 50-ml tubes containing 10 ml of decontamination solution (penicillin 500.000 U/L, streptomycin 500 mg/L, gentamycin 250 mg/L, kanamycin 500 mg/L, neomycin 250 mg/L, chloramphenicol 25 mg/L, polymyxin B 250 mg/L, erythromycin 200 mg/L, amphotericin B 2.8 mg/L), and the tubes were incubated for 30 min at room temperature on a rocking platform (3 cycles/min) to reduce microbial contamination (Stephens and Hetrick, 1979; La Peyre et al., 1993). Decontaminated tissues were centrifuged at 200 × *g* for 3 min and the supernatants were discarded. The tissues were weighed and minced into 1–2-mm^3 pieces to facilitate dissociation.

Tissue dissociation. The tissues were dissociated in 50-ml beakers with 45 U/ml of pronase (Calbiochem, La Jolla, CA) in 30 ml of saline I (NaCl 11.611 g/L, KCl 0.310 g/L, $NaHCO_3$ 0.350 g/L, $CaCl_2$ 0.480 g/L, $MgSO_4$ 1.456 g/L, $MgCl_2{\cdot}6H_2O$ 2.182 g/L). The beakers were placed on a stir plate (Model Nuova II, Thermolyne Corporation, Dubuque, IA) and octagonal stir bars (8-mm diameter × 12-mm long) were used to agitate the solution at 700 revolutions per min for 60 min. Any undissociated tissue was separated from the cells in 50-ml conical tubes by centrifugation (Model TJ-6 centrifuge, Beckman, Palo Alto, CA) at 20 × *g* for 3 min. The supernatants containing dissociated cells were transferred to 50-ml conical tubes, and the undisso-

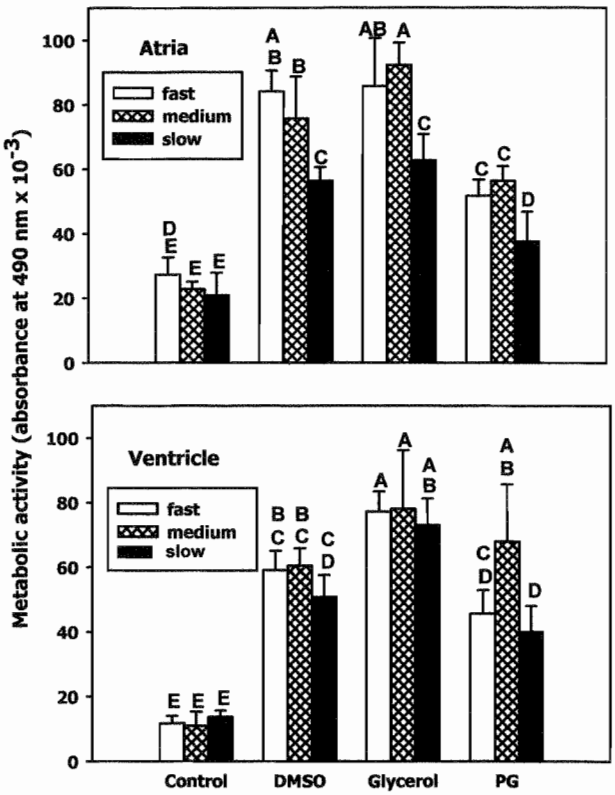

FIG. 3. Metabolic activity of atrial and ventricular cells of the eastern oyster, *C. virginica*, frozen with a 10% final concentration of glycerol, DMSO, or PG at three cooling rates. The control indicates cells that were frozen without cryoprotectant. Atrial cells were thawed at 45° C and ventricle cells were thawed at 25° C. Metabolic activities of cells were evaluated after 3 d of culture in three separate studies (N = 3). For each study, cells from 30 atria or 30 ventricles were used. *Bars* sharing *letters* were not significantly different ($P > 0.05$).

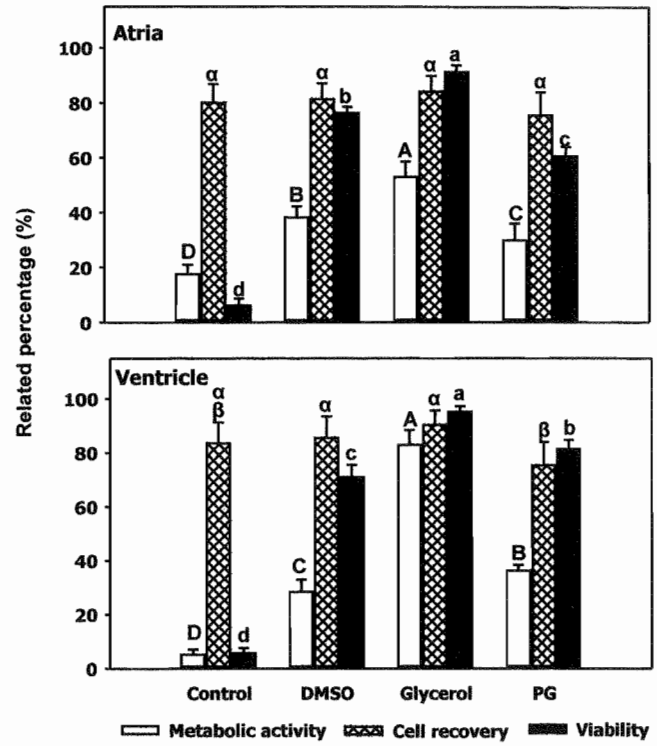

FIG. 4. Percentages of metabolic activity, cell recovery rate, and viability of atria and ventricular cells of the eastern oyster, *C. virginica*, after thawing. Cells were frozen and thawed at experimentally determined optimal conditions. For atrial cells: 10% final concentration of glycerol, DMSO, or PG, cooling at a medium rate, and thawing at 45° C. For ventricular cells: 10% final concentration of each cryoprotectant, cooling at a medium rate, and thawing at 25° C. The control indicates cells that were frozen without cryoprotectants. These percentages were compared to those of untreated cells that received no cryoprotectant and were not frozen. Results for each parameter measured were analyzed separately. *Bars* sharing *letters* were not significantly different ($P > 0.05$) with *uppercase letters* for metabolic activity, *Greek letters* for cell recovery, and *lowercase letters* for viability.

ciated tissue pellets were discarded. To remove pronase, the cells were pelleted by centrifugation at 200 × *g* for 10 min. The supernatants were discarded and the cells were resuspended in 30 ml of saline II (NaCl 16.600 g/L, KCl 0.595 g/L, $NaHCO_3$ 0.666 g/L, glucose 0.555 g/L, galactose0.111 g/L, trehalose 0.111 g/L). This washing was repeated twice. After the last centrifugation, the cell pellets were resuspended in 10 ml of cooling medium composed of JL-OPRD-4 medium (Buchanan et al., 1999) without antibiotics and with 20% (v/v) fetal bovine serum. Cell numbers were counted with a hemocytometer, and the cell density was adjusted to 5 × 10^6 cells/ml with the cooling medium. Viability of the dissociated cells, as determined by the trypan blue–exclusion test, was always 90% or greater.

Study 1: Effects of cryoprotectant concentrations. The effects of DMSO, glycerol, and propylene glycol (PG) at, respectively, 5, 10, and 15% final concentrations on the metabolic activity and spreading of atrial and ventricular cells after cryopreservation were evaluated. All procedures before cooling at −80° C were performed at 25° C, unless otherwise indicated. This study was repeated three times. Each time, 30 atria or 30 ventricles were pooled from individual oysters. Metabolic activities of cells frozen with cryoprotectants were compared to the metabolic activities of cells frozen in cooling medium without cryoprotectant. Spreading of cells frozen with cryoprotectants was compared to spreading of cells that were not frozen.

To add cryoprotectants and cool the cells, an equal volume of atrial or ventricular cells at 5 × 10^6 cells/ml was mixed with cooling medium containing a 2× final concentration of the cryoprotectant. The mixture was distributed into 2-ml cryovials (Sarstedt, Leicester, UK) in 700-μl aliquots for cooling. Cryovials containing atrial cells were packed into a polystyrene foam box with outer dimensions of 21 × 19 × 7 cm and 0.7-cm thickness and were wrapped with two layers of cotton (80 g/layer). Cryovials containing ventricle cells were packed into an identical box without additional wrapping. Atrial and ventricular cells were equilibrated at 25° C for 20 min, placed in and held at −80° C for 16 h, and plunged into liquid nitrogen (−196° C) for storage.

After 3 d of frozen storage, cells in individual cryovials were thawed in a 45° C water bath with shaking until all ice crystals had melted (~1 min). The thawed cell suspension was gently transferred to a 15-ml conical tube. The cryoprotectant was removed by adding the culture medium drop-by-drop over a 20-min period until the cooling medium was diluted 10-fold. The cells were then pelleted by centrifugation at 200 × *g* at 4° C for 10 min. The supernatants were discarded, and the cells resuspended in culture medium were seeded in 96-well plates at a volume of 100 μl/well (Falcon Plastics, Becton Dickinson Inc., Franklin Lakes, NJ).

To evaluate the metabolic activity and extent of spreading, thawed cells were cultured for 3 d at 25° C in 96-well plates. The metabolic activity of the cells in a well was determined by measuring the reduction of the tetrazolium compound 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) in the presence of phenazine methosulfate (PMS), to a soluble formazan dye by measuring absorbance at 490 nm (A_{490}) (Cell Titer 96™ Aqueous Cell Proliferation Assay, Promega, Madison, WI). Past studies have demonstrated the validity of this assay for use with oyster cells (Buchanan et al., 1999). Reagents were mixed according to the manufacturer's instructions, and 20 μl of the MTS/PMS solution was added to each well. Assay plates were incubated for 4 h at 25° C in the dark, and absorbances were read at 490 nm with a microplate reader (Dynatech, Chantilly, VA). The results of this assay as reported in the present study give an estimate of the overall metabolic activity for a well. These values reflect

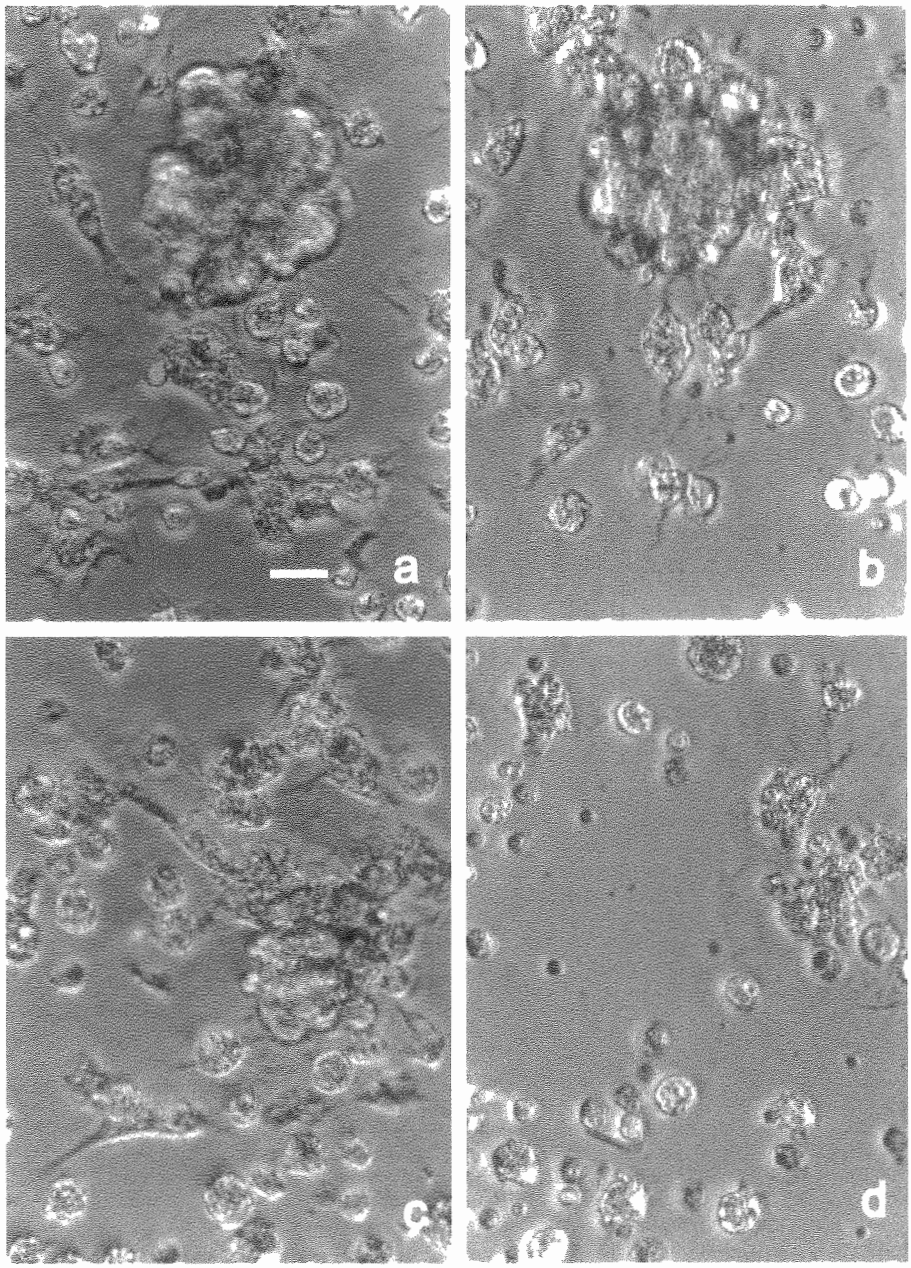

FIG. 5. Spreading of atrial cells of the eastern oyster, *C. virginica*, after thawing and 3 d of culture. Cells were cryopreserved at optimal conditions (10% cryoprotectant, cooling at a medium rate, and thawing at 45° C). (*a*) Control cells not treated with cryoprotectant or frozen; (*b*) cells treated with 10% DMSO; (*c*) cells treated with 10% glycerol, and (*d*) cells treated with 10% PG. *Bar*, 20 μm.

the number of cells in a well (i.e., the number surviving thawing) and the metabolic activity of these cells.

The extent of cell spreading was ranked by use of a phase-contrast inverted microscope (Diaphot-TMD, Nikon, Garden City, NY) at 400× magnification. Cells that were flattened and extended were counted as "spread." The ratio of the number of spread cells to the total cell number (from at least 300 cells) was calculated as the percentage of cell spreading. The extent of cell spreading in control wells containing cells that were not frozen was ranked as the highest (100%) and was designated "++++." By comparison with this control, the extent of cell spreading of other treatments were ranked as "+" (≤25%), "++" (>25% but ≤50%), "+++" (>50% but ≤75%), or "++++" (>75%). The concentration for each cryoprotectant that yielded the highest metabolic activity and level of cell spreading was selected for subsequent studies.

Study 2: Effects of thawing temperature. Cryopreservation conditions and evaluation criteria were the same as in Study 1, except that thawing temperatures of 25, 45, and 70° C were tested. Samples were thawed in a water bath at the appropriate temperature with shaking until all ice crystals melted. This study was repeated three times. Each time 30 atria or 30 ventricles were pooled from individual oysters. The thawing temperature for each cryoprotectant that yielded the highest metabolic activity and level of cell spreading was selected for subsequent studies.

Study 3: Effects of cooling rates. Cryopreservation conditions and evaluation criteria were the same as in Study 2, except for the cooling rates. Atrial cells were packed into a polystyrene foam box with four layers of cotton (slow cooling rate), two layers of cotton (medium cooling rate), or no cotton (fast cooling rate). Ventricle cells were packed into a polystyrene foam box (slow cooling rate), a freezer paper box (13 × 13 × 4.5 cm, Sarstedt, (Newton, NC, medium cooling rate), or were frozen without any box (fast cooling rate). This study was repeated three times. Each time, 30 atria or 30 ventricles were pooled from individual oysters. The cooling rate for each cryoprotectant that yielded the highest metabolic activity and level of cell spreading was selected for subsequent studies.

Study 4: Comparison of cryoprotectants. The best conditions (i.e., cryopro-

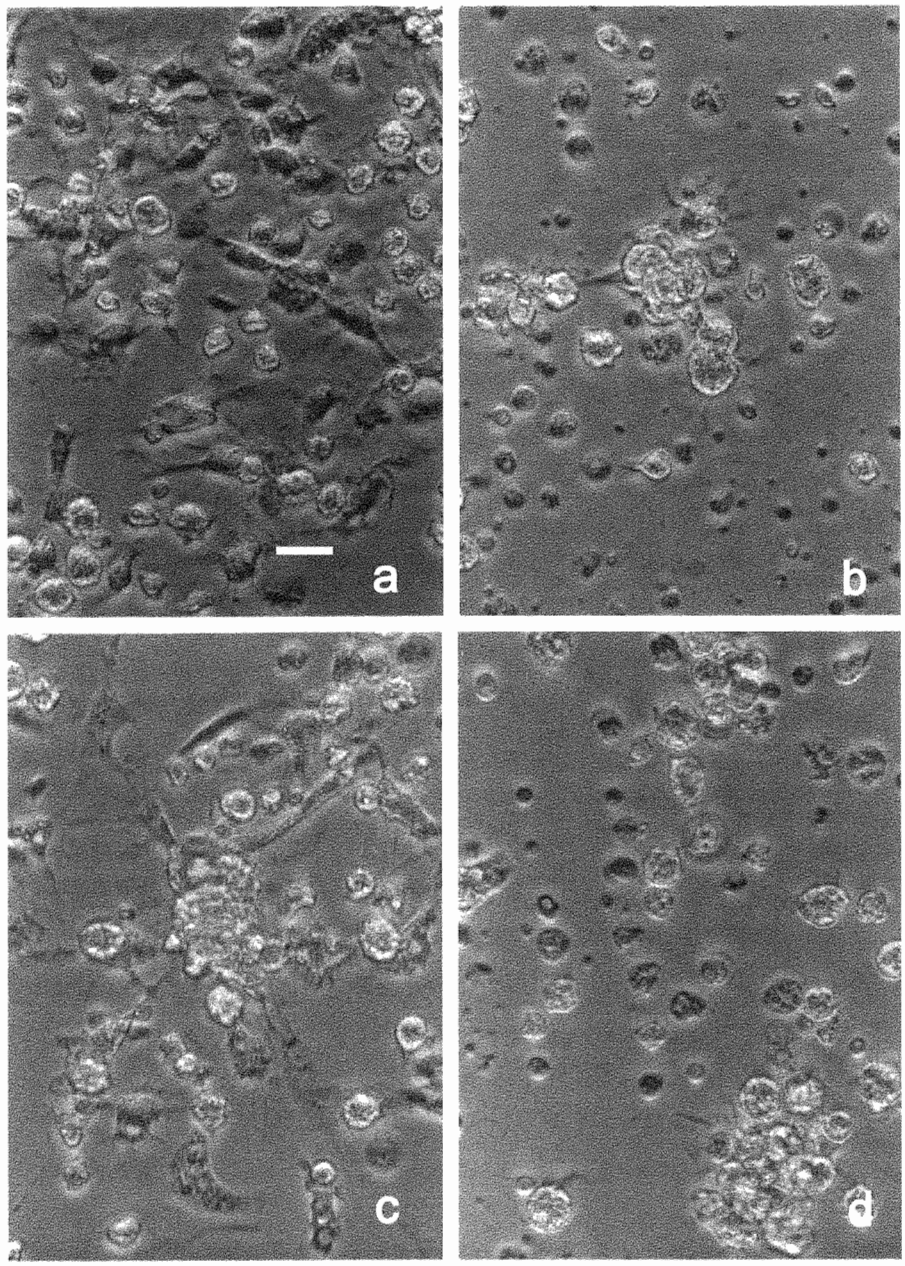

FIG. 6. Spreading of ventricular cells of the eastern oyster, *C. virginica*, after thawing and 3 d of culture. Cells were cryopreserved at optimal conditions (10% cryoprotectant, cooling at a medium rate, and thawing at 25° C). (*a*) Control cells not treated with cryoprotectant or frozen; (*b*) cells treated with 10% DMSO; (*c*) cells treated with 10% glycerol, and (*d*) cells treated with 10% PG. *Bar*, 20 μm.

tectant concentration, cooling rate, thawing temperature) for freezing and thawing of cells for each cryoprotectant were selected and Study 4 was conducted to identify the best cryoprotectant. For atrial cells, 10% of each cryoprotectant, a medium freezing rate, and thawing at 45° C were used. For ventricular cells, 10% of each cryoprotectant, a medium freezing rate, and thawing at 25° C were used. In addition to determining cell metabolic activity and extent of cell spreading, we also measured (1) the number of cells recovered from wells after detachment with trypsin with a hemocytometer, and (2) the viability of cells by using the LIVE/DEAD® viability/cytotoxicity kit L-3324 for animal cells from Molecular Probes Inc. (Eugene, OR). Results were compared with data obtained from primary cultures of cells frozen without the cryoprotectant. The percentages of metabolic activity, cell viability, and cell recovery were also calculated from data obtained with primary cultures of cells that were not frozen.

Statistical analysis. All studies were repeated three times. Each time, the cells used were obtained 30 atria or 30 ventricles pooled from individual oysters. The data for cell metabolic activity were then analyzed by a two-factor analysis of variance (ANOVA) in Study 1 (cryoprotectant concentrations and types), Study 2 (thawing temperature and cryoprotectant type), and Study 3 (cooling rate and cryoprotectant type). The data for cell metabolic activity, cell viability, and total cell number were analyzed by one-factor ANOVA in Study 4 (cryoprotectant type). Whenever a significant difference was found ($P < 0.05$), Tukey's multiple comparison of means was performed.

RESULTS

Study 1: Effect of cryoprotectant concentration. For atrial cells, the concentration and type of cryoprotectant significantly affected the metabolic activity of thawed cells (Fig. 1). Significant interaction was observed between concentration and type of cryoprotectant used ($P < 0.0001$). The metabolic activity of cells cryopreserved in 10% DMSO was significantly greater than in 5 or 15% DMSO. The met-

abolic activity of cells cryopreserved in 10 or 15% glycerol was significantly greater than in 5% glycerol. However, no significant difference was found in the metabolic activity of cells cryopreserved in 5, 10, and 15% PG, although the metabolic activity of the cells cryopreserved in 10% PG tended to be higher than in 5 and 15% PG. Overall, atrial cells cryopreserved in 10% DMSO or in 10 and 15% glycerol showed the greatest metabolic activity. The metabolic activity of cells frozen and thawed without cryoprotectant was significantly lower than that of cells cryopreserved with any cryprotectant except for PG at 5% (Fig. 1).

For ventricular cells, the concentration and type of cryoprotectant significantly affected the metabolic activity of thawed cells (Fig. 1). Significant interaction was observed between concentration and type of cryoprotectant ($P < 0.0001$). The metabolic activity of cells cryopreserved in 10% DMSO was significantly greater than in 5 or 15% DMSO. The metabolic activity of ventricular cells cryopreserved in 10% glycerol was significantly greater than in 5 or 15% glycerol. However, no significant difference was found in the metabolic activity of cells cryopreserved in 5, 10, and 15% PG, although the metabolic activity of the cells cryopreserved in 10% PG tended to be higher than in 5 and 15% PG. Overall, ventricular cells cryopreserved in 10% glycerol showed the greatest metabolic activity.

In the evaluation of cell spreading for each cryoprotectant treatment, atrial cells tended to form clumps in all cryoprotectant treatments. The ranked order of cell spreading for each cryoprotectant treatment was 10% glycerol (++++), 10% DMSO (+++), and 10% (++) PG. Atrial cells frozen without cryoprotectant showed the least cell spreading (+). For ventricular cells, less clumping was found in all cryoprotectant treatments. The ranked order of cell spreading for each cryoprotectant treatment was 10% glycerol (+++), 10% DMSO (++), and 10% PG (++). Ventricular cells frozen without cryoprotectant showed the least cell spreading (+). Based on metabolic activity and cell spreading, a 10% concentration for each cryoprotectant was selected for use in all further studies.

Study 2: Effects of thawing temperature. For atrial cells, the thawing temperature and the type of cryoprotectant significantly affected the metabolic activity of thawed cells (Fig. 2). No interaction was found between thawing temperatures and the type of cryoprotectant ($P = 0.3159$). For each cryoprotectant, there was no significant difference in metabolic activity of cells thawed at the different temperatures. The metabolic activity of cells frozen and thawed without cryoprotectant was significantly less than that of cells cryopreserved with DMSO and glycerol. Overall, atrial cells cryopreserved in glycerol showed greatest metabolic activity at all thawing temperatures, followed by DMSO, and propylene glycol (Fig. 2).

For ventricular cells, the thawing temperature and the type of cryoprotectant significantly affected the metabolic activity of thawed cells (Fig. 2). Significant interaction was observed between thawing temperature and the type of cryoprotectant used ($P < 0.0001$). The metabolic activity of ventricular cells cryopreserved with glycerol and thawed at 25° C was significantly greater than at 45 or 70° C. The metabolic activity of ventricular cells cryopreserved with DMSO and thawed at 25° C was significantly greater than at 70° C. No significant difference was found in the metabolic activity of ventricular cells cryopreserved with PG and thawed at 25, 45, or 70° C. The metabolic activity of cells frozen and thawed without cryoprotectant was significantly less than that of cells cryopreserved with any cryoprotectant. Overall, ventricular cells cryopreserved in glycerol showed the greatest metabolic activity at all thawing temperatures compared to cells cryopreserved with DMSO and PG. (Fig. 2).

Thawing temperature did not affect atrial cell spreading in each cryoprotectant treatment, but did affect ventricular cell spreading. The best ventricular cell spreading (++++) was found in cells frozen with 10% glycerol and thawed at 25° C. Based on metabolic activity and cell spreading, thawing temperatures of 45° C for atrial cells and 25° C for ventricular cells were selected for use in all further studies.

Study 3: Effects of cooling rates. For atrial cells, the cooling rates and cryoprotectant types significantly affected the metabolic activity of thawed cells (Fig. 3). Significant interaction was observed between cooling rate and type of cryoprotectant used ($P < 0.0001$). The metabolic activity of cryopreserved atrial cells cooled at fast and medium rates was significantly greater than the metabolic activity of cryopreserved atrial cells cooled at a slow rate, regardless of the cryoprotectant. There was no significant difference in metabolic activity of atrial cells frozen without cryoprotectant at the different cooling rates (Fig. 3).

For ventricular cells, the cooling rates and cryoprotectant types significantly affected the metabolic activity of thawed cells (Fig. 3). Significant interaction was observed between cooling rate and type of cryoprotectant used ($P < 0.0001$). For DMSO, no significant difference in metabolic activity was found for all cooling rates. Similarly, for glycerol no significant differences in metabolic activity were found for all cooling rates. Only the metabolic activity of ventricular cells cryopreserved with PG and cooled at a medium rate was significantly greater than at the slow and fast cooling rates (Fig. 3).

The best atrial cell spreading was found at the fast cooling rate for the DMSO treatment (+++), and at the medium cooling rate for the glycerol (++++), and PG (++) treatments. For ventricular cells, the best cell spreading was found at the medium cooling rate for the DMSO (++), glycerol (++++), and PG (++) treatments. Based on metabolic activity and cell spreading, a medium cooling rate was selected for atrial cells and ventricular cells for Study 4.

Study 4: Comparison of cryoprotectants. Both atrial and ventricular cells cryopreserved with glycerol showed significantly greater metabolic activity and viability than cells cryopreserved with DMSO or PG or cells frozen without cryoprotectant (Fig. 4). For atrial cells, cell recovery was not significantly different for all treatments (Fig. 4). For ventricular cells, cell recovery was significantly lower in PG compared with DMSO and glycerol (Fig. 4). The atrial cells cryopreserved in glycerol showed 53 ± 5% of the metabolic activity and 92 ± 2% of the viability of nonfrozen cells (Fig. 4). The ventricular cells cryopreserved in glycerol showed 83 ± 5% of the metabolic activity and 96 ± 2% of the viability of nonfrozen cells (Fig. 4). The number of cells recovered following cryopreservation with glycerol was 84 ± 5% of the number of nonfrozen cells for atrial cells and 91 ± 5% for ventricular cells (Fig. 4).

The best spreading atrial cells were found with glycerol (++++), followed by DMSO (+++), and PG (++). Regardless of the cryoprotectant, however, atrial cells tended to clump (Fig. 5*b–d*). This phenomenon was also found in cells that were frozen without cryoprotectant and in cells that had not been frozen (Fig. 5*a*). Moreover, fragments of lysed cells were found in PG-treated cells (Fig. 5*d*). The best cell spreading of ventricular cells was found in glycerol-treated cells (++++) (Fig. 6*c*). Some clumps were also formed in ventricular cells that received no cryoprotectant

or were not frozen, but to a lesser extent than in atrial cells (Fig. 6*a*). Fragments of lysed cells were found in DMSO-treated and PG-treated ventricle cells (Fig. 6*b* and *d*).

Discussion

Conditions were developed to successfully cryopreserve oyster heart cells. Using these conditions, atrial cells cryopreserved, thawed, and cultured for 3 d showed 53 ± 5% of the metabolic activity, 84 ± 5% of the number, and 92 ± 2% of the viability of nonfrozen cells. Ventricular cells yielded even better results, showing 83 ± 5% of the metabolic activity, 91 ± 5% of the number, and 96 ± 2% of the viability of nonfrozen cells. To our knowledge, these are the first effective cryopreservation conditions that have been reported for oyster somatic cells.

Successful cryopreservation of somatic cells from bivalve molluscs other than oysters has recently been reported by Odintsova and Tsal (1995) and Le Marrec-Croq et al. (1998). Odintsova and Tsal (1995), in the first report of the cryopreservation of primary cell cultures of a marine invertebrate, cryopreserved dissociated cells from mantle and gill tissues of the north Pacific mussel, *Mytilus trossulus,* and from the digestive gland and embryonic tissues of the Japanese scallop, *Mizuhopecten yessoensis.* No difference in the viability of cells dissociated from nonembryonic tissues cryopreserved with DMSO (5 and 10%) or glycerol (10%) was found. The viability of cells from scallop digestive gland, and mussel mantle and gills, after thawing ranged from 77 to 89%, compared to a range of 90–94% for nonfrozen cells and a range of 2–10% for cells frozen without cryoprotectant. Ribonucleic acid (RNA) synthesis in mantle and digestive gland cells cryopreserved with 10% glycerol, however, was lower than in cells cryopreserved with 10% DMSO. The mantle cells cryopreserved in 10% DMSO or 10% glycerol showed 68 and 44%, respectively, of the RNA synthesis of nonfrozen cells. Unexpectedly, the RNA synthesis in digestive gland cells cryopreserved with 10% DMSO or 10% glycerol was 210 and 147%, respectively, of the RNA synthesis of nonfrozen cells. Cells from dissociated scallop embryos were also successfully cryopreserved. The viability of cells from dissociated veliger larvae cryopreserved with 10% DMSO or 10% glycerol was greater than 90% and comparable to the viability of nonfrozen cells. The viability of cells from dissociated trocophore larvae cryopreserved in 10% DMSO (8% viable) or 10% glycerol (35% viable) was lower than the viability of nonfrozen cells (86%). Le Marrec-Croq et al. (1998) were also successful in cryopreserving dissociated heart cells from the great scallop, *Pecten maximus,* in 5, 10, 12, and 15% DMSO or 12 and 15% glycerol. The viability of thawed cells ranged from 70 to 90% and was similar under all conditions tested, while cell attachment was best for cells cryopreserved with 12% DMSO. These researchers showed that cells cryopreserved in 12% DMSO and thawed and cultured for 7 d exhibited morphology, protein synthesis, and DNA and lipid levels close to those observed in nonfrozen cells. It is clear from these two studies and our results that primary cell cultures of bivalve molluscs can be effectively cryopreserved with glycerol or DMSO.

In our work, eastern oyster cells cryopreserved with glycerol showed the best results in preliminary studies (data not shown) and in the four studies described here, in terms of metabolic activity, cell spreading, and cell viability. Our results also demonstrated that glycerol-treated heart cells tolerated a wider range of cooling and thawing rates than did DMSO-treated and PG-treated cells. Glycerol is, therefore, a useful cryoprotectant for heart cells of eastern oysters. In cryopreserving animal cells, glycerol has not been used as often as DMSO (Freshney, 1994) because glycerol is viscous and difficult to remove after thawing. DMSO is typically more toxic to animal cells than glycerol is, but can penetrate cell membranes faster due to its smaller molecular weight (Leung, 1991). At a specific concentration, decreasing the exposure time or increasing the cooling rate can minimize the toxicity of cryoprotectants to cells. In our work, atrial cells treated with 10% DMSO and cooled at a fast rate showed a higher metabolic activity than that of cells cooled at a slow rate. Although PG was found to be an effective cryoprotectant for embryos of *Crassostrea gigas* (Gwo, 1995) and trochophore larvae of *Crassostrea virginica* (Paniagua-Chavez et al., 1998), neither PG-treated atrial nor ventricular cells showed better metabolic activity and cell spreading than did DMSO-treated or glycerol-treated cells; and often results were worse. These results indicate that the effective cryoprotectant for sperm, embryos, and larvae may not be the same as for dissociated somatic cells of embryos.

Finally, this study yielded some interesting observations relevant to the nature of the cells studied. A percentage (~5%) of both cell types regularly survived freezing and thawing in the absence of cryoprotectant. This resistance to cryopreservation has been reported for other bivalve somatic and embryonic cells (Odintsova and Tsal, 1995), and could be related to natural adaptations. In fact, hemolymph constituents providing cryoprotection have been identified from the intertidal mussel *Mytilus edulis* (Loomis et al., 1988). Eastern oysters are intertidal and can experience rapid changes in temperature and salinity in nature, including exposure to freezing temperatures. Oyster cells may, therefore, be able to better survive freezing and thawing in the absence of cryoprotectant than cells from most animals, because they are more tolerant to changes in osmotic pressure.

In conclusion, the first effective cryopreservation conditions for oyster heart cells have been established. Atrial cells were best cryopreserved with 10% glycerol, using a medium cooling rate, and thawing at 45° C. For ventricular cells, 10% glycerol, a medium cooling rate, and thawing at 25° C yielded the best results. As oyster heart primary cell cultures contain heterogeneous cell populations, and will also vary with population of oysters and time of yr, response to cryopreservation for each culture can be expected to be variable even with optimal conditions. However, these conditions will assist in vitro studies of oysters by increasing the availability and uniformity of heart cells for primary culture.

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