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Assessment of gamete quality for the eastern oyster (*Crassostrea virginica*) by use of fluorescent dyes ☆

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Abstract

Evaluation of sperm motility is the single most widely used parameter to determine semen quality in mammals and aquatic species. While a good indicator for fresh sperm viability, post-thaw motility is not always effective at predicting fertilizing ability. Techniques using fluorescent dyes can assess functionality of mammalian sperm, but have not been widely applied in aquatic organisms. The eastern oyster *Crassostrea virginica* is an important mollusk in the United States, and cryopreservation protocols have been developed to preserve sperm and larvae to assist research and hatchery production. In this study, protocols were developed to assess sperm cell membrane integrity and mitochondrial function by flow cytometry and to assess viability of eggs by fluorescence microscopy. The fluorescent dyes SYBR 14 and propidium iodide (PI) (to assess membrane integrity) and rhodamine 123 (*R*123) (to assess mitochondrial membrane potential) were used to evaluate the quality of thawed oyster sperm previously cryopreserved with different cryoprotectant and thawing treatments. Membrane integrity results were correlated with motility of thawed sperm and mitochondrial membrane potential with fertilizing ability. Fluorescein diacetate (FDA) was used to assess cytotoxicity of cryoprotectant solutions and post-thaw damage to oyster eggs. The results indicated that membrane integrity ($P=0.004$) and thawing treatments ($P=0.04$), and mitochondrial membrane potential ($P=0.0015$) were correlated with motility. Fertilizing ability was correlated with cryoprotectant treatments ($P=0.0258$) and with mitochondrial membrane potential ($P=0.001$). The dye FDA was useful in indicating structural integrity of fresh and thawed eggs. Exposure of eggs, without freezing, to dimethyl sulfoxide yielded higher percentages of stained eggs and fertilization rate than did exposure to propylene glycol ($P=0.002$). Thawed eggs were not stained with FDA (<1%) and larvae were not produced.

Keywords: Gamete; Quality; Flow cytometry; Cryopreservation; Eastern oyster

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Assessment of gamete quality for aquatic species will become an important issue as cryopreservation advances in these organisms. Thus, development of protocols is necessary to assess functionality of thawed sperm, eggs or embryos by using effective and rapid techniques such as fluorescence microscopy and flow cytometry.

In most cases, motility has been the most commonly used quality assessment parameter to indicate viability of thawed sperm, despite variable correlations with fertility [16,34,35]. Freezing and thawing of sperm can impair cellular functions, thereby reducing fertility [14]. Indeed, sperm cells consist of several compartments bounded by the plasma membrane, acrosomal membrane, or mitochondrial membrane. Sperm cell competency requires that each of these membranes and compartments remain intact [13].

Cryopreservation of oyster sperm has been studied since the 1970s, and studies have investigated factors that decrease the fertilizing ability of thawed sperm [20,22]. Observations of survival, motility, and morphology of thawed sperm of the Pacific oyster, *Crassostrea gigas*, were performed by using light microscopy and scanning electron microscopy (SEM) [20,22]. Other technologies such as flow cytometry can quickly provide an estimation of sperm cell membrane integrity, and with proper protocols, can be relatively simple to perform [16,23]. Simultaneous evaluation of two or more characteristics of sperm by dual-staining techniques has been used with terrestrial species including turkey [9], boar, ram, rabbit, mouse, and bull [10,11], and with aquatic species [1,33].

Dual-staining techniques that utilize dye exclusion employ stains that cannot penetrate intact membranes, but are able to stain cells with damaged plasma membranes. These dyes are coupled with others such as fluorochrome SYBR 14 that are able to penetrate the sperm head and stain the nucleic acids of viable (membrane-intact) cells. Sperm stained with SYBR 14 emit green florescence resulting from positive staining of viable cells. The fluorochrome, propidium iodide (PI), can be used to identify non-viable cells because it can only penetrate damaged nuclear membranes and stain nucleic acids by intercalating between the base pairs. This dye emits red fluorescence resulting from positive staining of non-viable cells [12]. Another dual-staining technique can be achieved using rhodamine 123 (R123) and PI, which can be used to evaluate the mitochondrial function of cells [1,33]. Rhodamine 123 is a cationic, fluorescent dye widely used as an indicator of membrane potential. It provides high-resolution fluorescent images of mitochondria with little background and no apparent cytotoxic effects [17].

Flow cytometry has been used recently with fish sperm [4,7,33] and some invertebrates including sea urchin (*Evechinus chloroticus*), rainbow abalone (*Haliotis iris*), green-lipped mussel (*Perna canaliculus*), and white shrimp (*Litopenaeus vannamei*) [1,21]. This technology would be useful for oyster sperm.

The assessment of quality of eggs is more challenging than that of sperm. Because of technical problems with flow cytometry, microscopy is an option for assessing egg quality. Dyes such as neutral red can be used to stain viable eggs, although the dye can be toxic to developing embryos [19]. The non-toxic, non-fluorescent, non-polar dye, fluorescein diacetate (FDA) can penetrate egg membranes and be hydrolyzed by intracellular esterases to the polar fluorescent compound fluorescein that accumulates in intact cells [3,29]. If the plasma membranes of oyster eggs were damaged due to cryopreservation, fluorescein would be extruded, disallowing intracellular fluorescence. Lack of fluorescence in oyster eggs would indicate that those eggs were not viable and that sperm would not be able to fertilize them. The analysis of eggs of aquatic species by flow cytometry is difficult. Although oyster eggs are <50 μm in diameter and could theoretically pass through an instrument, lipid droplets from broken eggs would disrupt sample flow.

The objectives of this work were to develop: (1) flow cytometric protocols to assess cell membrane integrity and mitochondrial membrane of thawed eastern oyster sperm and (2) fluorescence microscopy protocols to assess viability of eastern oyster eggs before and after addition of cryoprotectant and after thawing.

Materials and methods

Gamete preparation

Gametes were removed from oysters by the dry stripping method [2]. The gonads were gently disrupted and gonadal material was collected with a Pasteur pipette. A 10-μL sample was removed to measure osmolality with a vapor pressure osmometer (model 5500, Wescor Inc., Logan, Utah). Sperm or egg samples were placed in 50-mL beakers prior to suspension in an extender.

Sperm samples were washed through 70- and 15-μm screens with calcium-free Hanks' balanced salt solution (C–F HBSS, 600 mOsmol/kg, pH 8.0, 21 °C) [27], and motility was estimated by observing a 10-μL sample from the sperm suspension with dark-field microscopy at 200× magnification. Unlike sperm of most fishes, oyster sperm can remain continuously motile for hours or days and do not require the addition of an activating solution to elicit motility [27]. Sperm vibrating in place were not considered to be motile. Only males with progressively motile sperm (>90%) were selected for experimentation [27].

Egg samples were washed through a 70-μm Nitex screen (Aquacenter, Leland, Mississippi), collected on a 15-μm-mesh screen, and suspended in artificial seawater (ASW) (MX3-50, Hawaiian Marine Imports Inc., Houston, Texas), before use in the cytotoxicity and cryopreservation experiments.

Cryopreservation of oyster sperm

Sperm samples from three males (replicates) were suspended in six concentrations (0, 5, 10, 15, 20, and 25%) of propylene glycol (PG), equilibrated for 10 min, and placed in 0.5-mL French straws (IMV International Co., Minneapolis, Minnesota). To avoid direct contact of undiluted cryoprotectant with sperm cells and to decrease the osmotic shock, the cryoprotectants were diluted (1:1) in ultrapure deionized water (NANOpure Barnstead Unit, 18 MΩ resistivity and ~22 mOsmol/kg) and mixed with sperm in a proportion that resulted in the specific final concentration for each treatment. The samples were cooled at 2.5 °C/min until reaching −30 °C in a controlled-rate freezer (KRYO 10 series II, Planer Products, Sunbury-on-Thames, UK), and held for 5 min before being plunged into liquid nitrogen [28]. After 24 h, samples were thawed at 25 or 70 °C by immersing the straws in a water bath for 6–7 s until ice crystals were no longer visible. Thawed sperm were suspended in fresh C–F HBSS before motility observations, fertilization or staining preparation. Fertilization of fresh oyster eggs was performed according to [28], and fertilization percentage was recorded at the trochophore stage [28].

Evaluation of membrane integrity and mitochondrial membrane potential

Preliminary experiments

Three males were used to generate standard curves to assess membrane integrity and mitochondrial membrane potential of sperm cell. To produce non-viable cells and develop known ratios of viable and non-viable sperm to be used in the generation of the standard curve, 10-mL samples of suspended sperm from three males (final concentration 1×10^8 cells/mL) were exposed to the following treatments: (a) 1-mL samples were placed in a freezer (−20 °C) for 15 min. The samples were thawed at room temperature (21 °C), and this freeze–thaw process was repeated three times, (b) 1-mL samples were plunged directly into liquid nitrogen for 15 min and thawed at room temperature, (c) 1-mL samples were exposed to a heat (50 or 65 °C) in a water bath for 10 min and cooled to room temperature, or (d) 1-mL samples were exposed to 10% methanol for 15 min. After each treatment, the samples were evaluated for motility by light microscopy at 200× magnification.

Based on these experiments, heating to 50 °C was chosen to produce non-viable sperm to generate the standard curves. The exact physiological status of these heat-treated sperm was difficult to determine, but they are referred to herein as being "non-viable" in comparison to the untreated fresh sperm which are referred to as being "viable." Viable and non-viable sperm were mixed in five ratios (100:0, 75:25, 50:50, 25:75 or 0:100) and were stained with the fluorescent dyes SYBR 14 and PI (Molecular Probes, Eugene, Oregon) to determine membrane integrity, or with R123 (Molecular Probes, Eugene, Oregon) and PI to evaluate mitochondrial membrane potential. To optimize dye concentrations, sperm were stained at three concentrations for each dye: SYBR 14 at 100, 50, and 20 nM; PI at 24, 12, and 6 μM; and R123 at 0.1, 0.07, and 0.03 nM. A SYBR 14 solution stock was prepared that contained 1 mM in 100 μL of dimethyl sulfoxide (Me_2SO). The final concentrations chosen for further analyses were: 20 nM for SYBR 14, 12 μM for PI, and 0.03 nM for R123. A linear regression was used to determine the correlation between the predicted and measured ratios of viable and non-viable sperm for the standard curves generated for membrane integrity and for mitochondrial membrane potential when analyzed by flow cytometry. Additionally, epifluorescent microscopic observations were performed to corroborate the staining results.

Sample preparation for the evaluation of sperm membrane integrity

Based on the results of dye optimization, samples of thawed sperm from three males were prepared for

flow cytometric analysis. Aliquots of 20 μL of sperm were suspended in 1 mL of C–F HBSS (final concentration 2×10^6 sperm/mL) and were incubated with 20 nM SYBR 14 in the dark for 10 min at 24 °C, followed by a second incubation with 12 μM PI for 10 min.

Sample preparation for the evaluation of sperm mitochondrial membrane potential

As described above, aliquots of 20 μL of thawed sperm were suspended in 1 mL of C–F HBSS (final concentration 2×10^6 sperm/mL) and were incubated with 0.03 nM R123 in the dark for 10 min at 24 °C, followed by a second incubation with 12 μM PI for 10 min. After incubation, the samples were analyzed in a flow cytometer as described below.

Flow cytometry

Standard curves and experimental samples were analyzed by using a flow cytometer (FACSCalibur® Model, Becton–Dickinson, San Jose, California) equipped with an air-cooled 488-nm argon (blue) laser. FACS Comp® software provided by the manufacturer (Becton–Dickinson) was used to calibrate equipment settings. A total of 10,000 sperm cells were analyzed for each sample in triplicate. To collect membrane integrity data, density plots were generated with green fluorescence for membrane-intact sperm (SYBR 14) and with red fluorescence for membrane-damaged sperm (PI). To estimate mitochondrial membrane potential, density plots were generated with green fluorescence for functional mitochondria (R123) and with red fluorescence for membrane-damaged sperm (PI). Data were analyzed using Cell Quest Software (Becton, Dickinson). The data were acquired as the percentage of cells gated in the regions of the plot previously designated as viable or non-viable (Fig. 1).

Cytotoxicity of cryoprotectant solutions to eggs

Eggs from three ripe females were obtained as described above and pooled for subsequent experimentation. After preliminary trials, about 4000 eggs were held for 5 min in the following conditions: 0.86 M Me_2SO and 0.14 M sucrose (S); 1.75 M Me_2SO and 0.25 M S; 2.63 M Me_2SO and 0.38 M S; 3.50 M Me_2SO and 0.50 M S; 0.86 M PG and 0.14 M S; 1.75 M PG and 0.25 M S; 2.63 M Me_2SO and 0.38 M S; or 3.50 M PG and 0.50 M S. Cryoprotectant solutions were prepared in ultrapure deionized water as described for sperm. After treatment, the eggs were washed in fresh filtered ASW for 5 min.

Eggs were suspended in fresh ASW (519 mOsmol/kg, pH 8.0, 21 °C) at a concentration of 35 eggs per mL and fertilized with fresh sperm from two pooled males. Fertilized eggs were incubated at 21 °C in 50-mL disposable centrifuge tubes (Costar, Corning, New York) with loose tops. Twelve hours after fertilization, the numbers of trochophore larvae per mL were counted in a Sedgewick–Rafter chamber (Hausser Scientific Partnership, Horsham, Pennsylvania). To assess cytotoxic effects using viability as an endpoint, 1-mL egg samples in 1.5-mL microcentrifuge tubes were incubated with a final FDA

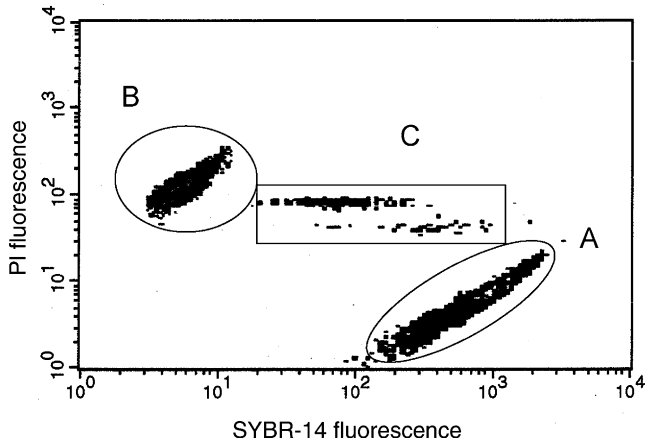

Fig. 1. Flow cytometric analysis of sperm of the eastern oyster *Crassostrea virginica* to evaluate plasma membrane integrity. Sperm were incubated with SYBR-14 for 10 min and then with PI for another 10 min and analyzed by flow cytometry. (A) Sperm with intact membranes; (B) non-viable, membrane-damaged sperm cells; and (C) sperm cells in transition between membrane functionality and non-functionality.

concentration of 6×10^{-6} M. The stock solution of FDA contained 1 mg of dye (6×10^{-3} M) per mL of acetone and was stored at −20 °C. After incubation for 15 min at 21 °C in the dark, eggs were examined with an epifluorescence microscope (Microphot-SA, Nikon Inc., Garden City, New York) equipped with a high-pressure mercury lamp (Model HB 10110AF Nikon Inc., Garden City, New York) and an excitation filter (bypass 450–490 nm) for green fluorescence. A subsample of 200 eggs from each treatment was examined, and the number of fluorescent (viable) and non-fluorescent eggs (non-viable) was recorded.

Cryopreservation of eastern oyster eggs

Eggs (~3×10^6) were obtained from two individual ripe females and ~40,000 eggs per female were suspended in 20 mL of ASW. An average of 5000 eggs was suspended in 0.5 M Me_2SO, 1.75 M Me_2SO, or 2 M Me_2SO or combined with 0.25 M sucrose, and placed in 0.25-mL French straws (IMV International Corp., Minneapolis, Minnesota). The eggs were equilibrated in the cryoprotectants for 5 min before being plunged directly into liquid nitrogen (fast cryopreservation) or cooled in a controlled-rate freezer at a rate of 1.5 °C per min until reaching a final temperature of −30 °C which was held for 5 min before plunging into liquid nitrogen (slow freezing). After 2 days, the straws were thawed in a water bath at 35 °C for 10 s. Eggs were collected on a 10-μm Nitex screen and were rinsed with filtered ASW for 10 min. A sample of ~200 eggs per treatment was placed in microcentrifuge tubes, stained with FDA, and examined as described above. Fertilization ($n = 2000$) was performed as described above and fertilization percentage was recorded. All experiments were terminated when the larvae were at D-stage (24 h).

Data analysis

Analyses of multiple linear regressions were used to relate membrane integrity, cryoprotectant concentration, or thawing rate with motility or fertilizing ability ($n = 3$). Other multiple linear regressions were used to relate mitochondrial membrane potential, cryoprotectant concentration, or thawing rate with motility or fertilizing ability ($n = 3$). Sperm data were arcsine square root transformed before analysis. To find the best model for each regression, a stepwise regression was used. A value of $P < 0.05$ was chosen as the level of significance. Egg data were analyzed with one-way analysis of variance using categorical data with 24 comparisons ($P = 0.002$). This method compared each Me_2SO and PG treatment paired by molarities, alone, or as overall results compared to the control. Statistical analyses were performed with SAS software for Windows® (SAS Institute, Cary, North Carolina).

Results

Quality of thawed sperm

The only treatment that consistently produced non-motile, non-viable, and non-agglutinated sperm was heating at 50 °C. This treatment was used for the generation of standard curves for sperm viability ($R^2 = 0.9122$) and mitochondrial function ($R^2 = 0.8293$). When viewed by fluorescence microscopy, motile sperm displayed a bright green fluorescence after staining with SYBR 14. Mitochondria of motile sperm stained with R123 also displayed a bright green fluorescence. Non-motile, non-viable (heat-treated) sperm stained with PI showed a bright red fluorescence. Mixtures of viable and non-viable sperm displayed both green and red fluorescence (Fig. 2).

The highest percentages of thawed sperm with intact membranes were seen with samples cryopreserved with 10% PG and thawed at 25 °C (38% viable sperm), and with 10% PG and thawed at 70 °C (30% viable sperm) (Fig. 3). The highest percentages of thawed sperm with functional mitochondria were seen with 5% PG and thawed at 25 °C (68% functional), and with 10% PG and thawed at 70 °C (48% functional) (Fig. 4).

A significant correlation was found for motility with membrane integrity ($P = 0.004$) and for motility with thawing temperatures ($P = 0.04$). A significant correlation was also found for motility with mitochondrial membrane potential ($P = 0.0015$).

A significant correlation was found for fertilizing ability with cryoprotectant solutions ($P = 0.0258$) and mitochondrial membrane potential ($P = 0.001$).

Cytotoxicity of cryoprotectants and cryopreservation of eggs

Fluorescein diacetate was a useful indicator of egg structural integrity, as stained eggs were fertilized with fresh sperm and developed normally (Fig. 5). This suggested that FDA did not have toxic effects on

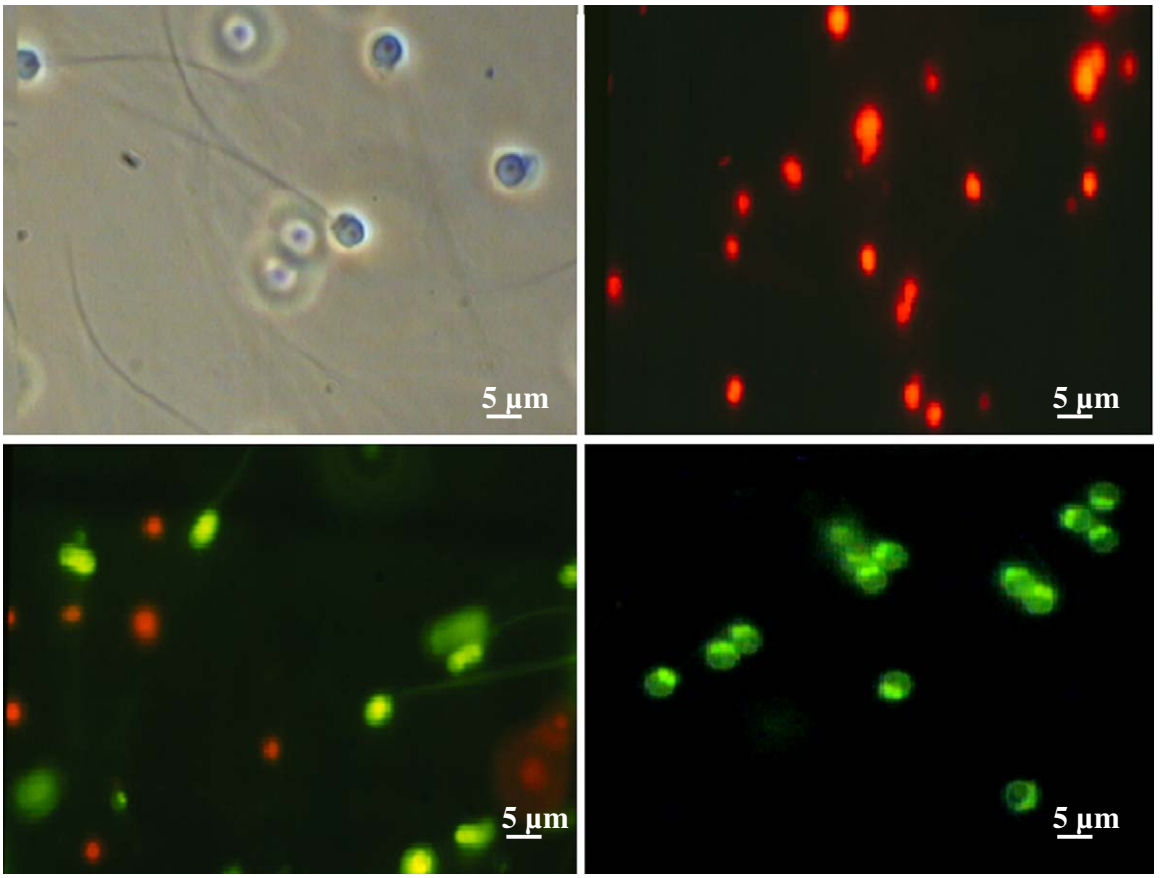

Fig. 2. Eastern oyster sperm stained with fluorescent dyes. Upper left panel, unstained sperm (phase-contrast microscopy); upper right, nuclei of non-viable sperm stained with propidium iodide; bottom left, nuclei of viable sperm (green) stained with SYBR-14 and nuclei of non-viable sperm (red) stained with propidium iodide; and bottom right, viable mitochondria stained with rhodamine 123. All samples were observed at 1,000-x magnification.

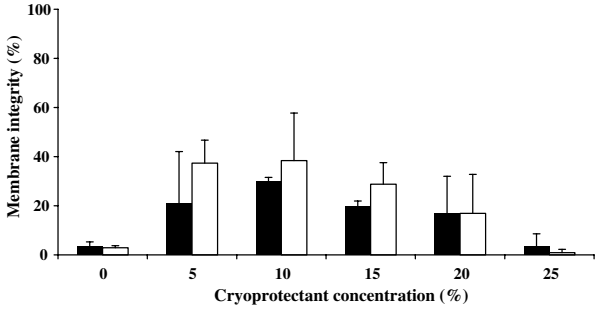

Fig. 3. Percentage of eastern oyster sperm cells with intact membranes (stained green with SYBR-14) cryopreserved with propylene glycol after thawing at 25 °C (white bars) or 70 °C (black bars).

the early survival and development of embryos. Each of the Me_2SO treatments resulted in more staining of eggs than did the corresponding PG treatments ($P = 0.002$) except for PG (3.50 M) and sucrose (0.50 M), and only Me_2SO (0.86 M) and sucrose (0.14 M) resulted in significantly higher staining of eggs than the control (no cryoprotectant).

Of the cryoprotectants, Me_2SO (0.86 and 1.75 M) and sucrose (0.14 and 0.25 M) were the least toxic to non-frozen eggs. The fertilization rates (50%) of these eggs were equal to those of control treatments (50%), and the eggs fertilized developed normally to D-stage larvae. The Me_2SO (3.50 M) and sucrose (0.50 M) resulted in lower fertilization (17%) than

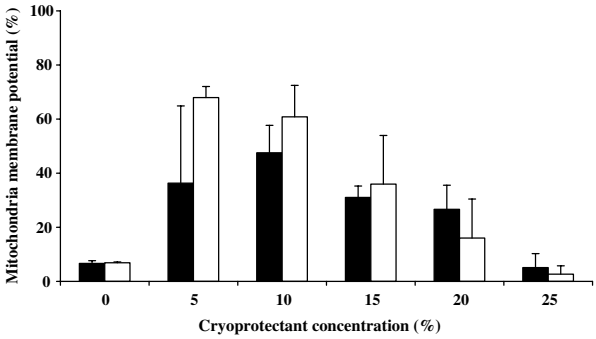

Fig. 4. Percentage of eastern oyster sperm cells with evident mitochondrial membrane potential (stained green with rhodamine 123) cryopreserved with propylene glycol after thawing at 25 °C (white bars) or 70 °C (black bars).

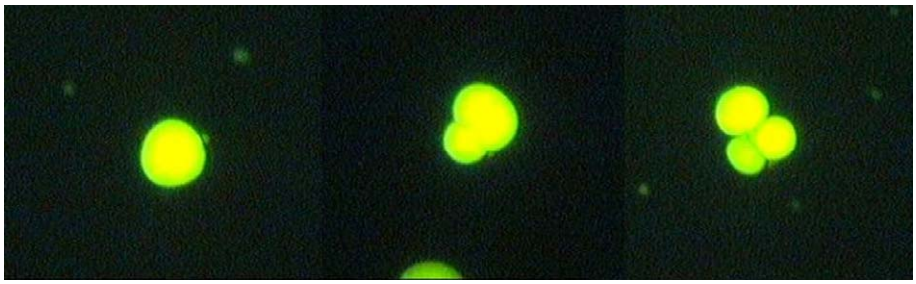

Fig. 5. Fertilized eastern oyster egg and embryos stained with fluorescein diacetate. Left panel, fertilized egg (first polar body); middle, two-cell stage embryo; right, four-cell stage embryo.

Table 1
Osmotic pressure of solutions, percentage of eastern oyster eggs stained with fluorescein diacetate (FDA) after exposure to different combinations of cryoprotectants and sucrose, ($n = 200$), and the percentage of larvae produced after fertilization ($n = 2000$): Me_2SO, dimethyl sulfoxide; PG, propylene glycol; S, sucrose

Cryoprotectant (molarity)	mOsmol/kg (mean ± SD)	Stained with FDA (%)	Fertilization (%)
Control (no cryoprotectant)	519 ± 0	86.5	50
Me_2SO (0.86 M) and S (0.14 M)	1934 ± 92	93.5	50
Me_2SO (1.75 M) and S (0.25 M)	>2000[a]	82.0	50
Me_2SO (2.63 M) and S (0.38 M)	>2000	79.0	21
Me_2SO (3.50 M) and S (0.50 M)	>2000	27.5	17
PG (0.86 M) and S (0.14 M)	1499 ± 52	18.0	2
PG (1.75 M) and S (0.25 M)	1874 ± 29	31.5	2
PG (2.63 M) and S (0.38 M)	1857 ± 12	34.0	2
PG (3.50 M) and S (0.50 M)	>2000	47.5	1

[a] The highest value that the vapor pressure osmometer could register was 2000 mOsmol/kg.

the three other treatments. Eggs suspended in PG yielded the lowest percent fertilization (1–2%), and all PG treatments were significantly different from fertilization without cryoprotectant ($P = 0.002$). This indicated that PG was not a suitable cryoprotectant for eastern oyster eggs (Table 1).

In the cryopreservation experiment, the cryoprotective treatment that yielded the highest percentage of positively stained eggs before cryopreservation was 2 M Me_2SO (82%). No fertilization was observed after thawing (Table 2). Fertilization of control eggs was 31% (Table 2). After thawing, the cooling rate that yielded the highest percentage of fluorescent eggs (<1%) was the slow cooling rate (−1.5 °C/min), (data not shown). Overall, none of the freezing methods yielded embryonic development.

Discussion

Sperm evaluation is generally performed subjectively by microscopy using visual assessments of

Table 2
Percentage of eastern oyster eggs stained with fluorescein diacetate (FDA) ($n = 200$) after suspension in three cryoprotectant solutions before cryopreservation, and percentage of larvae produced from thawed eggs ($n = 2{,}000$): Me_2SO, dimethyl sulfoxide; S, sucrose

Treatment	Stained with FDA (%)	Fertilized eggs (%)
Control (no cryoprotectant, no freezing)	70	31
Me_2SO (0.5 M)	68	0
Me_2SO (2 M)	82	0
Me_2SO (1.75 M) and S (0.25 M)	72	0

morphology and motility and is thus dependent upon the ability and experience of the operator [16]. These methods are simple and quick and remain the parameters of choice to assess the degree of sperm damage produced by cryopreservation in aquatic species [8]; however, sperm motility is only one parameter important for fertilization.

Microscopic examination using scanning or transmission electron microscopy has been valuable; however, these techniques only provide partial information and are time consuming and expensive [8]. New approaches to functionally assess thawed spermatozoa by using fluorescent probes have been developed and can provide information regarding the ability of sperm to tolerate freezing and thawing. These approaches have been successfully used in mammals [11]; however, studies reporting the use of fluorescent probes to assess sperm functionality after thawing in aquatic organisms are scarce, especially in invertebrates [21]. Applications for these probes in the cryopreservation of oyster gametes have been recognized. Thus, the use of fluorescent dyes to evaluate invertebrate gamete quality could be used to assess post-thaw sperm fertilizing ability and egg quality.

In the present study, we found that fluorescent dyes can be used to assess sperm function of the eastern oyster. Motility was highly correlated with membrane integrity and with mitochondrial membrane potential; however, fertilizing ability of sperm was not correlated with membrane integrity. Analogous to mammalian sperm, oyster sperm cells have an acrosome, which is a lysosome-like vesicle at the tip of the sperm head that plays an important role in fertilization. One of the possible reasons for no correlation between cell membrane integrity and fertilization is that the acrosome can undergo spontaneous reaction or receive structural damage at some point in the cryopreservation process, which prevents entry of the sperm into the egg. In fact, studies on the morphology and ultrastructure of thawed sperm of Pacific oysters have revealed that freezing can affect or destroy acrosomal morphology [20,22]. Thus, it is likely that motile sperm with an intact plasma membrane but with a damaged acrosome are not capable of fertilizing eggs. The dyes used in this study responded to plasma membrane damage and mitochondrial membrane potential but not to acrosomal damage. Flow cytometric techniques have been used to evaluate the acrosome reaction in mammals by use of a fluorescent-labeled-lectin (*Pisum sativum*) agglutinin [13]. Optimizing techniques to evaluate the acrosome reaction as a parameter to determine fertilizing ability of oyster sperm await further development.

The most difficult problem in the cryopreservation of gametes of aquatic organisms has been with unfertilized eggs [5,6,24]. After 40 years of effort, a recent report of successful cryopreservation of oyster eggs has been documented [36]. The most common problem identified in the cryopreservation of mammalian oocytes has been osmotic injury [15]. The same problem was identified for eggs of the Pacific oyster. To decrease the osmotic stress on oyster eggs, the cryoprotectants were prepared in Milli-Q water (reported as 18 MΩ resistivity and ~0 mOsmol/kg) as this yielded the best fertilization rates using this protocol [36]. In our study, the cryoprotectant solutions were also prepared in ultrapure deionized water (18 MΩ resistivity and ~22 mOsmol/kg). The final osmotic pressure of these solutions was between 1400 and 1800 mOsmol/kg for PG and S and >2000 mOsmol/kg for Me_2SO and sucrose (Table 1). Better fertilization and intact membranes, however, were found for eggs suspended in Me_2SO and S (50%) than in PG and S (<2%) showing that osmotic stress was not the problem in this species. For the Pacific oyster, the addition and removal of cryoprotectants in stepwise fashion was an important factor [36]. In our study, the concentration and type of cryoprotectant were more important than their addition and removal (Table 1).

The effect of Me_2SO on cellular structure and cytoskeletal organization has been examined in detail [39]. In mammalian oocytes, Me_2SO is responsible for shifting the polymerizing center for microtubule organization from the chromosomes toward the centrioles [39]. Propylene glycol has been shown to induce microtubule proliferation and disorgani-

zation of the meiotic spindle in oocytes of mouse [18] and rabbit [38]. Propylene glycol was chosen for our experiments because of its successful use in cryopreservation of human oocytes [37], and because PG yielded good results in the cryopreservation of oyster sperm [28]. Fertilization and development of eastern oyster eggs suspended in PG before cryopreservation, however, were inferior to those of eggs suspended in Me_2SO and similar to the results of the Pacific oyster [36]. Exposure of rabbit oocytes to propylene glycol at 1.5–2 M resulted in complete loss of microfilaments [38]. The effect of these cryoprotectants on oyster eggs requires further study.

Other than the type and concentration of cryoprotectant, cooling and thawing rates are responsible for cellular damage. For eggs of the Pacific oyster, cooling rates and holding temperatures were crucial [36] and the optimal cooling rate was slow (0.3 °C/min). Such rates should be tested for the eastern oyster because no fertilization or positively stained eastern oyster eggs were found after thawing when a cooling rate of 1.5 °C/min was used in the present study.

In mammalian oocytes, cooling rates and Me_2SO have each been shown to modify the properties of the zona pelucida and, by so doing, to influence the fertilizing ability of oocytes. Significant reductions in granule number have been observed in the cortex of mouse oocytes treated with 1.5 M Me_2SO at room temperature [32]. Moreover, Me_2SO and PG can each induce a significant premature exocytosis of cortical granules from the human oocyte [32]. The extent of these effects in oyster eggs has not been studied and could provide a useful model and an opportunity to explore mechanisms to enhance cryopreservation of oyster eggs.

Cooling sensitivity can be species specific, and accordingly, protocols designed for one group of organisms may not necessarily work for other groups. In mammals, changes of the cell complexity, such as elongation and disruption of vesicular smooth endoplasmic reticulum and disappearance of fibrillar inclusions, have been detected after oocytes were cooled or frozen [30,31]. The interactions between the lipid phase of cells and elements of the cytoskeleton are complex and confer another level of complexity to the process of cryopreservation. Hardening of the lipids can cause deformation and disruption of the plasma membrane or cytoskeleton, which plays an important role in the process of cell division. Cooling sensitivity in oyster eggs and those of aquatic organisms in general have not been studied as thoroughly as in mammals. Therefore, the effect of cooling on oyster egg organelles and cell division are important in understating the cryobiology mechanisms in eggs of aquatic organisms.

The developmental stage of the oocyte can influence cryopreservation success. In oysters, oocytes taken directly from the gonad are usually at the first prophase of meiosis [25]. They undergo germinal vesicle breakdown spontaneously in natural seawater or by application of serotonin and are arrested again at the first metaphase of meiosis [25,26]. Different stages of egg maturation can be found in samples from stripped gonads [25]. If the developmental stages of oyster eggs are associated with the success of cryopreservation, as in human or mouse oocytes, procedures for collection of eggs or synchronization of egg maturation should be considered. In this study, eggs were obtained from stripped females, and it is likely that maturation of eggs were not synchronized, resulting in cryopreservation of eggs in different maturation stages. In the future, eggs synchronized at different stages should be studied to examine the effects of cryopreservation.

In conclusion, techniques using fluorescent dyes can be used in assessing post-thaw gamete quality of eastern oysters. Techniques using fluorescent dyes to evaluate acrosome integrity in oysters are needed because the acrosome plays an important role in fertilization. Development and use of alternative techniques to detect cortical granule release or cytoskeleton system disruption could be valuable to understand damage of oyster oocytes after cryopreservation.

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