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Effects of dimethyl sulfoxide and glycine on cryopreservation induced damage of plasma membranes and mitochondria to striped bass (*Morone saxatilis*) sperm[☆]

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Abstract

Intact plasma membrane and functional mitochondria are important attributes for the fertilization capacity of fish sperm. In the present study, dimethyl sulfoxide (Me_2SO) and glycine were investigated in an effort to improve plasma membrane integrity and mitochondrial function in cryopreserved striped bass (*Morone saxatilis*) sperm. Prior to freezing, no concentration of Me_2SO (2.5, 5 or 10%) was found to affect ($P > 0.05$) the integrity of plasma membranes after sperm were exposed for 10 min. However, mitochondrial function decreased ($P < 0.01$) with increasing Me_2SO concentration. Both fluorescent staining and microscopic examination of the ultrastructure of post-thaw plasma membranes indicated that with increasing Me_2SO concentration, plasma membranes were better protected, and 10% Me_2SO had the highest percentage of sperm with plasma membranes intact. However, sperm mitochondrial function decreased ($P < 0.05$) with increasing Me_2SO concentration. The inverse relationship between plasma membrane integrity and mitochondrial function, given the Me_2SO concentration, suggests that care must be taken to select Me_2SO concentration that will maximize the protection of both plasma membranes and mitochondrial function. The addition of glycine to the cryomedia increased ($P < 0.05$) the percentage of sperm with post-thaw functional mitochondria and ATP content. However glycine did not provide ($P > 0.05$) protection to post-thaw plasma membrane integrity. The highest percentage of sperm with both intact plasma membranes and functional mitochondria was obtained with 7.5% Me_2SO and 75 mM glycine.

Keywords: Sperm; Striped bass; Glycine; Plasma membrane integrity; Mitochondrial function

Structural and functional integrity of the plasma membranes and mitochondria as well as motility are critical to fertilization in teleost fish sperm. The percentage of motile sperm is the most common attribute used to evaluate fish sperm quality. This assay, however, may not be well correlated with the fertilizing capacity of semen [7,16]. Other attributes, such as cell viability (defined as cells with intact plasma membranes), and mitochondrial function, have been chosen as

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indicators to evaluate fish sperm quality, especially for the cryopreserved sperm cell whose plasma membranes and mitochondria could be damaged during the freeze-thaw process [25,26,31]. Among the methods that are used to investigate cellular damage, electron microscopy can provide detailed information on the subcellular ultrastructure and thus help elucidate morphological change [8,19,20,35,38]. Fluorescent staining has been recently shown to provide rapid assessment of the integrity of plasma membranes and mitochondrial function in fish sperm [25,26,31].

In recent years, the effects of cryoprotectants and cryopreservation procedures on the cellular structure of spermatozoa have been intensively studied. Aberrations of the normal sperm morphology have been observed due to cryopreservation. The plasma membrane is one of the main structures affected by cryopreservation. Damage to plasma membranes has been observed when sperm were exposed to cryoprotectants before freezing [19,35], with a greater percentage of sperm losing the integrity and normal function of their plasma membranes during the freezing and/or thawing processes. The morphology of mitochondria was also affected especially during the freezing and thawing phases [8,19,20,25,26,35]. Damage to the mitochondria may be responsible for the decrease in the percentage of motile sperm as well as decreased duration of motility in post-thaw sperm. Mammalian sperm have a high degree of mitochondrial activity, with sufficient levels of ATP to provide motility in many species for more than 1 h [14]. In contrast, the ATP levels in fish sperm are only sufficient to maintain motility for 30 s to several minutes [27,32]. Thus, cryopreservation methods that protect mitochondrial function and preserve ATP of fish sperm become extremely important to the efficacy of the cryopreservation protocol.

Striped bass (*Morone saxatilis*) sperm are used to fertilize the eggs of white bass to produce the hybrid sunshine bass for the rapidly growing striped bass industry in the US [34]. A viable protocol for cryopreservation of striped bass sperm is under development to solve problems relating to the fact that striped bass and white bass spawning seasons are asynchronous [9,10,13]. Cryopreserved sperm from striped bass can be shipped and stored on site where white bass females are environmentally conditioned to spawn year-round in hatcheries. In previous studies, dimethyl sulfoxide (Me_2SO) was found to be the best cryoprotectant for striped bass sperm when compared to glycerol, propylene glycol, methanol, and dimethylacetamide [13,16]. Glycine has been used successfully as a non-permeating cryoprotectant to cryopreserve spermatozoa, and to improve post-thaw motility in variety of species, including bovine [29], goat [17], and fish [9,21]. However, the mechanism by which glycine increases post-thaw motility is unclear.

To better understand and potentially improve the cryopreservation protocol for striped bass sperm, the objective of the present study was to evaluate the effect of Me_2SO and glycine as cryoprotectants, on plasma membrane integrity, mitochondrial function, and ATP content of cryopreserved striped bass sperm.

Materials and methods

Chemicals and reagents

The live/dead sperm viability kit, containing Sybr14 and propidium iodide (PI), was purchased from Molecular Probes (Eugene, Oregon, USA). Rhodamine 123 (Rh123), gonadotropin-releasing hormone analog, paraformaldehyde, glutaraldehyde, and osmium tetroxide were purchased from Sigma Chemical (St. Louis, MO, USA). Bioluminescent kits were purchased from Promega (Madison, WI, USA). All other chemicals and reagents were purchased from Fisher Scientific International (Atlanta, GA, USA).

Semen collection and cryopreservation

Three-year-old domestic striped bass males were maintained in an 8600 L circular tank, part of a recirculating water system at the University of Maryland's Crane Aquaculture Facility. In the spring, 12 males (2.2 ± 0.18 kg) were evenly distributed into three 1600 L circular tanks, and held at 15 ± 1 °C for the 5 week study. Each fish was given a cholesterol cellulose implant [33] containing 150 µg of gonadotropin-releasing hormone analog [11], inserted into the dorsal lymphatic sinus, as previously described for striped bass [36]. Three days after administering the implant, the fish were anesthetized in a 70 mg/L quinaldine bath [37] and urine was removed by applying gentle pressure around the urogenital vent. Aliquots of 2 mL semen from each male were collected directly into 50 mL sterile conical tubes and placed immediately on ice. Fresh semen from each male was then diluted with the same volume of a simple extender (14 g/L NaCl, 0.4 g/L KCl, 0.25 g/L $NaHCO_3$, 1 g/L glucose, and pH 7.6). To test the effect of Me_2SO concentrations, the extended samples were subsequently diluted 1:1 (v/v) with the same extender containing 2.5, 5 or 10% Me_2SO (final concentration). Based on our previous study [9], two glycine concentrations of 0 (control) and 75 mM were examined in the presence of 5, 7.5 or 10% Me_2SO (final concentration). For pre-freezing, sperms were exposed to Me_2SO for 10 min at 4 °C and immediately stained with fluorescent dyes. For cryopreservation, the final sperm mixture was quickly pipetted into 500 µL cryo-straws in aliquots of 150 µL. The equilibration time was 10 min. Cryo-straws containing sperm samples were frozen using a programmable freezer (Planer Products, Sunbury-on-Thames, England) with a previously optimized freezing protocol of: −40 °C/min from 5 °C until −120 °C and then plunged into liquid nitrogen [13]. Samples were thawed in a 35 °C water bath for 8 s. Post-thaw sperm were immediately stained with fluorescent dyes.

Fluorescence staining analysis

The live/dead sperm viability kit was utilized to examine the integrity of plasma membranes. Sperm stained with Sybr14, but not stained with PI, were classified as non-viable (PI−) [5]. Rh123 staining was used to evaluate mitochondrial function. Rh123 positive (+), represented cells with functional mitochondria [6]. To estimate sperm viability, 100 µL (10 µM) of Sybr14 was added to a 900 µL pre-freezing or post-thaw sperm sample and incubated at 20 °C for 10 min, and 5 µL (2.4 mM) of PI was added and the sample was incubated for an additional 10 min prior to analysis. The final concentrations of Sybr14 and PI were 1 and 12 µM, respectively. To estimate mitochondrial function, 100 µL (13 µM) of Rh123 was added to a 900 µL sperm sample, incubated at 20 °C for 10 min. To estimate both plasma membrane integrity and mitochondrial function, 100 µL (13 µM) of Rh123 was added to a 900 µL sperm sample, incubated at 20 °C for 10 min, and 5 µL (2.4 mM) of PI was then added and the sample was incubated for an additional 10 min prior to analysis. The final concentrations of Rh123 and PI were 1.3 and 12 µM, respectively. Approximately, 50–80 spermatozoa were analyzed with an epifluorescent microscope (Zeiss, Berlin, Germany) at 400× for each treatment group from each male. Rhodamine B filter (Zeiss, BP546/LP590) was used to detect PI+ cells, and fluorescein filter (Zeiss, BP 450–490/LP 520) was used to observe Sybr14+ and Rh123+ cells.

Measurement of ATP content

To extract Adenosine 5′- triphosphate (ATP), 200 µL semen samples were mixed with 400 µL extraction medium containing 2% trichloroacetic acid and 2 mM EDTA for 15 min on ice. Samples were subsequently centrifuged at 8000*g* for 10 min using a Heraeus Model 400-R centrifuge (Hanau, Germany) at 4 °C. Supernatants were pipetted into 100 µL vials, and kept on ice before analyses were performed. ATP content in 100 µL supernatant was evaluated by bioluminescence. Luminescence was read with a luminometer (Berthold Analytical, Munich, Germany). The number of sperm per milliliter was determined using Makler counting chamber (TS Scientific, PA, USA).

Scanning electron microscopy

Fresh, pre-freezing, and post-thaw sperm were fixed with a paraformaldehyde–glutaraldehyde–osmium tetroxide solution, following the method of Lahnsteiner and Patzner [18]. Briefly, sperm samples were mixed with the solution (semen:fixative = 1:2 v/v), which consisted of 10%

paraformaldehyde, 5% glutaraldehyde, and 2% osmium tetroxide. After fixing the sample on ice for 15 min, samples were washed in double distilled water and dehydrated with a series of ethanol concentrations. Samples were then dried using critical point drying and subsequently coated with gold–platinum. Samples were observed and photographed with an Amray 1820D scanning electron microscope.

Statistical analysis

The statistical analysis was performed with SAS version 8.0 (SAS Institute, Cary, NC, USA). Data are presented as means ± standard error of the mean (SEM). Each experiment was repeated five times ($n = 5$). Data were analyzed by analysis of variance (ANOVA) with subsequent Duncan multiple range test.

Results

Effect of Me_2SO on plasma membrane integrity, mitochondrial function, and ATP content

Prior to freezing, no effect ($P > 0.05$) of Me_2SO concentration on plasma membrane integrity was detected. The percentage of fresh sperm having intact plasma membranes were more than 95% after exposure to 2.5, 5, or 10% Me_2SO for 10 min (Fig. 1). However, there was a Me_2SO effect ($P < 0.01$) on post-thaw plasma membrane integrity. Higher Me_2SO concentrations tested provided better protection of plasma membranes. Sperm cryoprotected with 10% Me_2SO had the highest (88 ± 2.5%) post-thaw plasma membrane integrity, while only 9 ± 1.4% and 30 ± 4.3% of the cells retained intact plasma membranes with 2.5 and 5% Me_2SO, respectively (Fig. 2). These results were supported by observations of the ultrastructure of sperm using scanning electron microscope (Fig. 4), which illustrated morphological changes to the head of the sperm. With 2.5% Me_2SO (Fig. 4B) there were significant irregularities observed to the surface membrane of the sperm head, while 10% Me_2SO resulted in the smoothest surface (Fig. 4D).

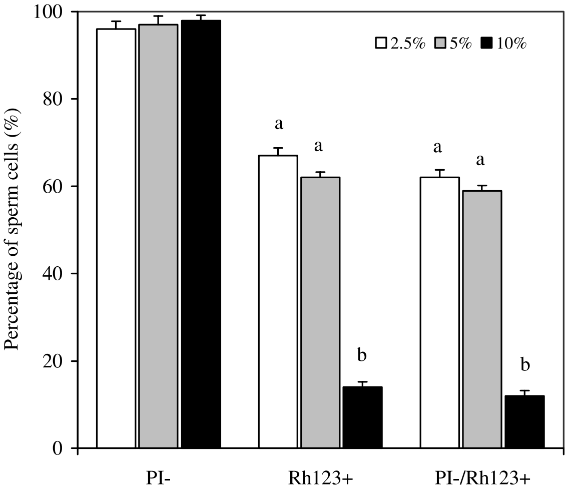

Fig. 1. The effect of Me_2SO concentrations (2.5, 5, and 10%) on either pre-freezing plasma membrane integrity, or mitochondrial function, or both, in striped bass spermatozoa. The results were expressed as the percentage (means ± standard error) of cells with PI−, Rh123+, and PI−/Rh123+, respectively. Bars with different letters are different ($P < 0.05$) within each of PI−, Rh123+, and PI−/Rh123+.

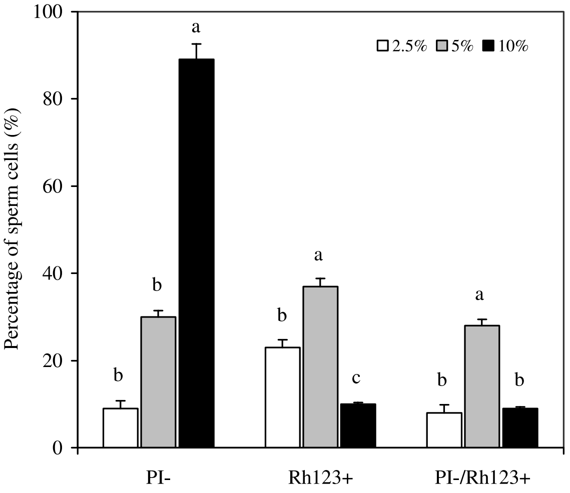

Fig. 2. The effect of Me_2SO concentrations (2.5, 5, and 10%) on either post-thaw plasma membrane integrity, or mitochondrial function, or both, in striped bass spermatozoa. The results were expressed as the percentage (means ± standard error) of cells with PI−, Rh123+, and PI−/Rh123+, respectively. Bars with different letters are different ($P < 0.05$) within each of PI−, Rh123+, and PI−/Rh123+.

A different response was observed for Me_2SO's effect on mitochondria when compared with plasma membranes. Sperm cryopreserved with 10%

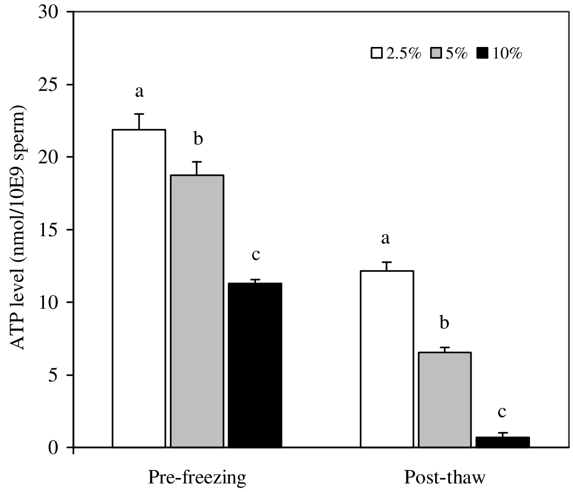

Fig. 3. The effect of Me_2SO concentrations (2.5, 5, and 10%) on ATP content of striped bass spermatozoa (means ± standard error) at pre-freezing and post-thaw stages. Bars with different letters are different ($P < 0.05$) within pre-freezing or post-thaw.

Me_2SO had the lowest percentage of post-thaw functional mitochondria. The highest percentage of sperm with post-thaw functional mitochondria was achieved with 5% Me_2SO. This Me_2SO concentration also resulted in the highest percentage ($28 \pm 3.5\%$) of sperm with both intact plasma membranes and functional mitochondria post-thaw (Fig. 2).

The ATP content decreased ($P < 0.01$) when sperm were exposed to Me_2SO and subsequently dropped after the freeze-thaw process. By comparison with the level in fresh sperm (32.2 nmol/10^9 sperm), only 38, 20, and 2% ATP content retained after cryopreservation with 2.5, 5, and 10% Me_2SO, respectively (Fig. 3).

Effect of glycine on plasma membrane integrity, mitochondrial function, and ATP content

The addition of glycine to the cryoprotectant media did not provide ($P > 0.05$) any additional protection to the plasma membranes during the freeze-thaw process at all Me_2SO concentrations tested (Figs. 5A–C). However, glycine increased ($P < 0.05$) the number of sperm that exhibited normal mitochondrial function (Figs. 5A–C) and ATP content (Fig. 6) during cryopreservation.

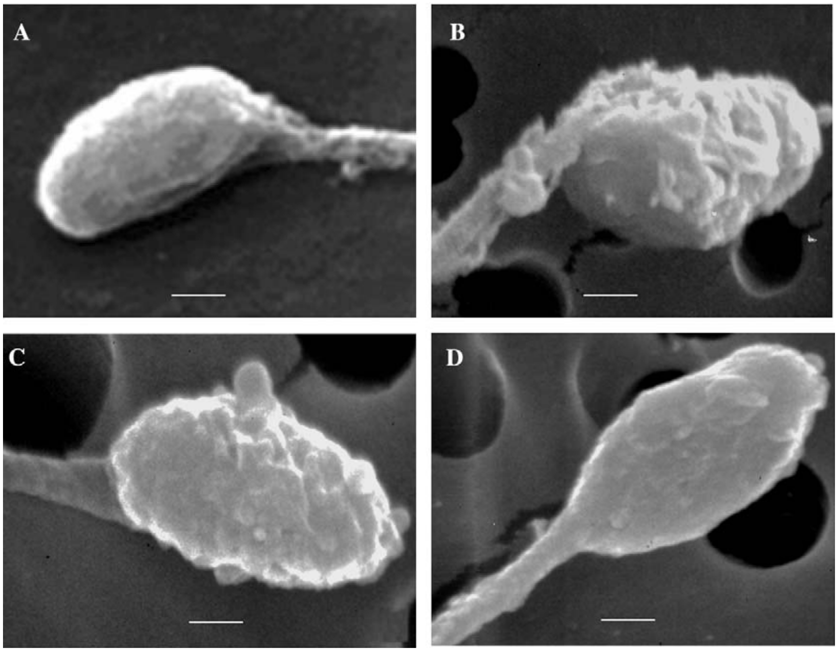

Fig. 4. Ultrastructure of striped bass spermatozoa (40,000×). (A) fresh spermatozoa; (B) 2.5% Me_2SO post-thaw; (C) 5% Me_2SO post-thaw; and (D) 10% Me_2SO post-thaw. Bar = 0.5 μm.

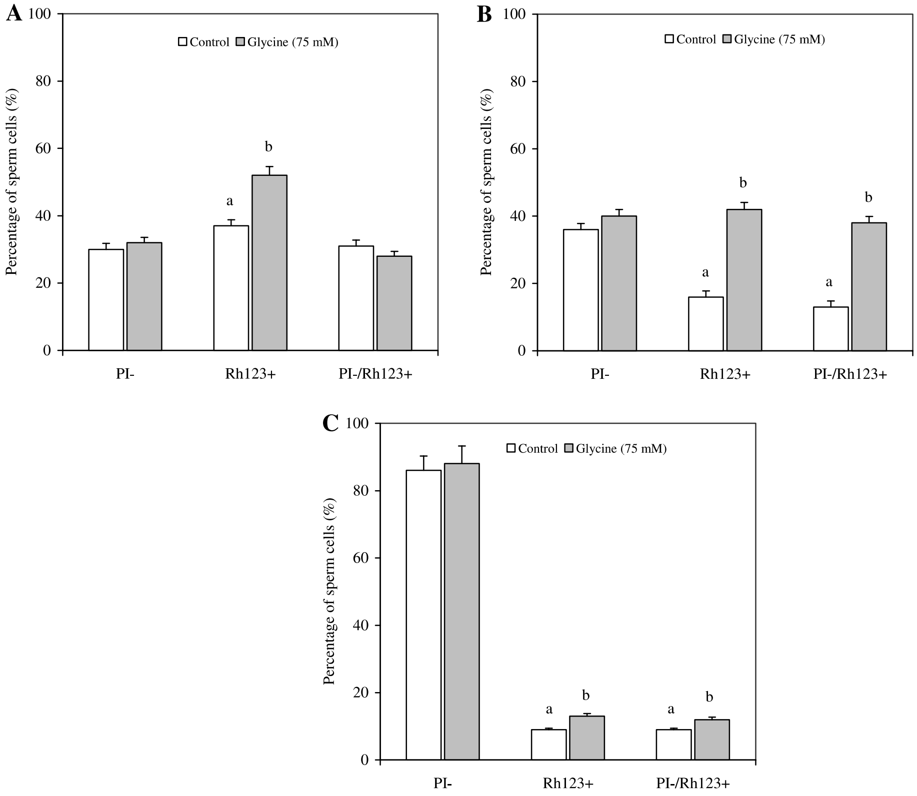

Fig. 5. The effect of glycine on either post-thaw plasma membrane integrity, or mitochondrial function, or both, in striped bass spermatozoa in the presence of 5% (A), 7.5% (B), and 10% (C) Me_2SO, respectively. The results were expressed as the percentage (means ± standard error) of cells with PI−, Rh123+, and PI−/Rh123+, respectively. Bars with different letters are different ($P < 0.05$) within each of PI−, Rh123+, and PI−/Rh123+.

Glycine also increased ($P < 0.05$) the percentage of sperm with both intact plasma membranes and functional mitochondria in the presence of 7.5 and 10% Me_2SO, however, no glycine effect was observed with 5% Me_2SO. The highest percentage ($38 \pm 2.6\%$) of sperm with both intact plasma membranes and functional mitochondria was achieved by 75 mM glycine and 7.5% Me_2SO (Fig. 5B).

Discussion

It has been reported that Me_2SO can damage plasma membranes [15,22] and denature proteins at room temperature due to osmotic shock and its high toxicity to cells [4]. In this study, no significant damage to the plasma membranes of striped bass sperm was observed after cells were exposed to Me_2SO concentrations as high as 10%, at 4 °C for 10 min. A similar result was observed in Atlantic croaker sperm prior to freezing with 15% Me_2SO [8]. In contrast, Lahnsteiner [19] found significant damage to the plasma membranes of grayling (*Thymallus thymallus*) sperm using 10% Me_2SO. This suggests that the degree of damage caused by Me_2SO before freezing may vary with different species. Our results demonstrated that significant damage to plasma membranes occurred during the freezing and thawing stages of cryopreservation, and that the degree of damage was Me_2SO concentration dependent. The unusual morphology of post-thaw sperm cryopreserved with 2.5% Me_2SO suggests that this concentration, at 10 min equilibration time, may have been too low to protect sperm from damage associated with

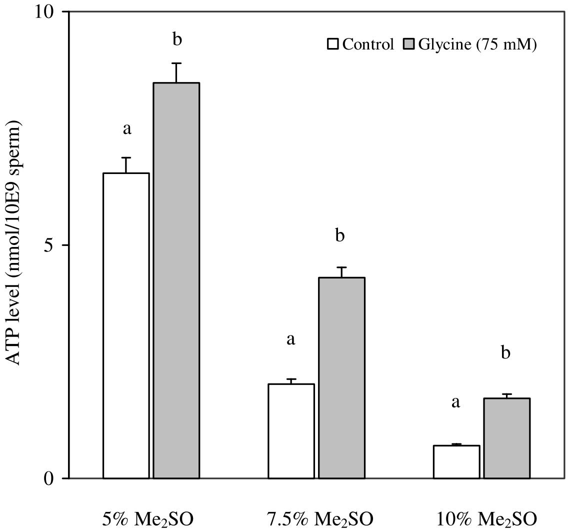

Fig. 6. The effect of glycine on post-thaw ATP content of striped bass spermatozoa (means ± standard error). Bars with different letters are different ($P < 0.05$) within the same concentration of Me_2SO.

ice crystal formation during the freezing and thawing phases.

The addition of Me_2SO has been shown to activate striped bass sperm kept quiescent in extenders [10], and with increasing Me_2SO concentration, increased percentages of sperm were activated (unpublished data). Me_2SO activation of sperm for other species has also been reported [28]. Premature activation consumes cellular ATP reserves. In general, ATP significantly decreased with increasing Me_2SO concentration at both prefreezing and post-thaw stages, however 5% Me_2SO had a higher percentage of post-thaw spermatozoa with functional mitochondria than 2.5% Me_2SO. These data suggest that striped bass sperm mitochondria may not be able to produce additional ATP reserves following Me_2SO induced activation.

Our data demonstrated that Me_2SO concentration had different effects on plasma membranes and mitochondria. When considering both post-thaw plasma membrane integrity and mitochondrial function, functional mitochondria became the limiting factor for 7.5 and 10% Me_2SO. However, plasma membrane integrity was the limiting factor for 2.5 and 5% Me_2SO. To obtain a higher percentage of sperm with both intact plasma membranes and functional mitochondria, the limiting factor associated with the Me_2SO concentration must be carefully identified.

It has been previously hypothesized that glycine helps maintain structural stability of the plasma membrane during the freeze-thaw process [1,19]. Anchordoguy et al. [1] suggested that amino acids could form a layer on the sperm surface by interacting with the phosphate groups in the sperm plasma membrane phospholipids. This layer may affect the permeability of Me_2SO into the cells and help protect the sperm plasma membrane from cold shock. Our results did not demonstrate that glycine provided any additional protection to plasma membranes, however, it significantly improved mitochondrial function and ATP content of striped bass sperm. This partially explains why glycine increased the percentage of sperm with both intact plasma membranes and functional mitochondria at both 7.5 and 10% Me_2SO, but not 5% Me_2SO.

The mechanism by which glycine protects mitochondria and their ATP content, but not the plasma membrane of sperm, is not clear. However, there have been at least two possible hypotheses suggested in published articles. The first hypothesis, reported by Flipse [3], was that glycine may pass through the plasma membrane of sperm to provide some positive effect on the mitochondria. He observed that ^{14}C-labeled glycine in a bovine sperm extender entered bovine sperm cells and was metabolized. Me_2SO has been shown to increase the transport efficacy of foreign DNA into bacteria [12]. Compared to the several thousand bases of DNA molecules, glycine is much smaller and may be more easily transported into the sperm cells in the presence of Me_2SO. A second hypothesis, associated with the glycine receptors reported for several mammalian species including: mouse [30], pig [24], hamster [23], and human [2], suggests that there may be glycine receptors on the plasma membrane and that the binding of glycine molecules to the receptors triggers signal transduction, which ultimately protects mitochondrial function and preserves ATP content. The glycine receptor plays an essential role in the acrosome reaction initiated by glycine or by the zona pellucida of human egg [2]. However, there is no evidence to indicate that teleost sperm have glycine receptors. In one of our previous study [9], glycine showed a significant dose effect on post-thaw motility. This result could be interpreted as contradictory to the receptor hypothesis, since the dose effect of glycine would be more unlikely to be observed if receptors were exposed to glycine molecules whose concentration was at least millimolar. In future studies, monitoring pathways of glycine molecules as well as their ultimate impact on mitochondrial enzymes may provide valuable information.

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