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The effect of freezing rate on the quality of striped bass sperm

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ABSTRACT

Several studies have been conducted in an attempt to determine the optimal freezing rate for cryopreservation of striped bass (*Morone saxatilis*) sperm. In this study, the effects of freezing rate (−10 °C, −15 °C, −20 °C, and −40 °C/min) on gamete quality was examined, using Sybr-14 and propidium iodide to determine viability (sperm cell membrane integrity), ATP concentration using a luciferin-luciferase bioluminescence assay, and a CEROS computer-assisted sperm analysis system to characterize striped bass sperm motion. Adult male striped bass (N = 12) were sampled once a week for 5 weeks. Collected samples were extended, cryoprotected using a 7.5% (vol/vol) dimethyl sulfoxide final concentration solution, and frozen using a Planer Kryosave controlled-rate freezer. Samples were stored in liquid nitrogen for 49 days, and sperm quality was re-evaluated after thaw (same methods). Sperm cryopreserved at −40 °C/min resulted in means for total motility (10.06%), progressive motility (7.14%), ATP concentration (0.86 pmol/10^6 cells), and sperm viability (56.5%) that were greater ($P < 0.05$) than those for slower cooling rates. Therefore, −40 °C/min was the optimal freezing rate (among those tested) for cryopreservation of striped bass sperm.

1. Introduction

Hybrid crosses with members of the teleost family Moronidae are widely used in fishery management and aquaculture. The most common cross used for commercial purposes involves *in vitro* fertilization of eggs obtained from white bass (*Morone chrysops*) females with semen collected from striped bass (*Morone saxatilis*) males. Hybrid progeny have several highly desirable traits (from an industry perspective), including faster growth, hardiness, and adaptability to environmental conditions and stressors [1]. Although there is a lack of fundamental knowledge regarding the molecular basis of hybrid vigor, there have been promising results with recurrent selection of *Morone* broodstock, based on performance of their progeny [2].

To create this hybridization, semen is collected from spermiating striped bass during the spring spawning season, which can often be prolonged by giving a gonadotropin-releasing hormone agonist to induce spermiation and increase milt production [3], or with the human chorionic gonadotropin product Chorulon, which was recently approved by the US Food and Drug Administration as a spawning aid for all brood fish [4]. More than 220 species of finfish and shellfish are now farmed, with production rates exceeding 25 × 10^6 metric tons [5]. As part of the total production rate, the striped bass aquaculture industry has been estimated to be as high as fifth in volume and fourth in value of all food fish grown in the United States. [6]. To increase production rates, one of the major needs cited is efficient production and survival of larval fish to ensure that adequate numbers of fingerlings are available on a year-round basis [6,7]. The striped bass industry is very dependent on wild individuals for gametes, creating a challenge for development of domesticated striped bass and white bass brood stock [7], coupled with a genetic improvement program [4]. Cryopreservation is commonly used to preserve semen samples for long-term storage and for use with asynchronous and geographically isolated spawning populations, such as the white bass and striped bass [1]. To

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date, successful fertilization of *Morone* eggs has been achieved in several studies using cryopreserved striped bass sperm [8–10].

Multiple experiments have been conducted in an attempt to determine the optimal freezing rate for cryopreservation of striped bass semen [10–12], examining a range of freezing rates from −10 °C to −40 °C/min. The present study was designed to confirm the optimum freezing rate for striped bass sperm and to quantify additional information on the effects of cryopreservation on cell viability, motion, and energetics (ATP content).

2. Materials and methods

2.1. Collection of striped bass semen

Eight-year-old male striped bass (*Morone saxatilis*) (N = 12) were selected from a population maintained under computerized photo-thermal control (Lutron Version 2.72 Grafix Eye GRX-PRG, Coopersburg, PA, USA) at the University of Maryland's Crane Aquaculture Facility. For each of the 5 weeks, four different individuals were sampled each day, 3 days a week, to ensure that all 12 individuals were sampled once weekly. Fish were anesthetized in a 100 mg/L MS-222 (Finquel, Argent Chemical Laboratories, Redmond, WA, USA) bath, buffered to pH 8.0 with sodium bicarbonate. To prevent contamination, slight pressure was applied around the urogenital vent to expel urine and then dried with Kimwipe absorbent towels. Each semen sample (approximately 5 mL) was then placed into sterile, 50-mL conical tubes and immediately placed on ice. An aliquot (1 mL) of semen was then removed from each sample and used to determine baseline sperm concentration (total number of sperm per mL), viability, ATP, and motion characteristics before freezing.

2.2. Extender, cryoprotectant, and cryopreservation protocol

Freshly collected samples were extended 1:3 (v:v) using our lab's striped bass extender (NaCl 1400 mg, KCl 40 mg, $NaHCO_3$ 200 mg, glucose 100 mg, glycine 75 mM, and 100 mL deionized ultra-filtered water) [13] modified to an osmolality of 550 mmol/L using NaCl. Hyperosmotic extenders improved postthaw quality of cryopreserved striped bass sperm samples when quickly frozen after dilution [14,15]. Dimethyl sulfoxide was then added as the cryoprotectant to obtain a final concentration of 7.5% (vol/vol), which has been reported to help preserve sperm plasma membranes and protect mitochondrial function during cryopreservation [16]. After a 10-minute equilibration period, six 250-μL volume aliquots from a single individual were pipetted into 500-μL Cassou straws for each freezing rate and immediately heat-sealed. Each set of six straws was then placed into a programmable freezer (Planer Kryosave-Model KS30, Sunbury-on-Thames, Middlesex, UK) and frozen at −10 °C, −15 °C, −20 °C, or −40 °C/min. Once the straw's core temperature of −120 °C was reached (determined by a thermistor placed into an extra straw containing the treatment mixture), the straws were removed from the freezer and immediately placed into dewars containing liquid nitrogen for storage. The initial freezing rate used was rotated for each day of each sampling week (to account for potential differences in the time samples were frozen relative to when they were collected). In all cases, semen samples were frozen no longer than 10 minutes after being exposed to extender and cryoprotectant (equivalent to the time required to load and seal the straws after extension). Each sample was removed from storage 49 days after it was frozen, thawed for 6 seconds in a 40 °C water bath, and re-evaluated for postthaw sperm viability, ATP, and motion characteristics (using the same procedures as described earlier in the text).

2.3. Concentration, viability, and motion analysis

2.3.1. Concentration

To determine initial sperm concentrations of freshly collected semen, 10 μL of neat semen was added to 3990 μL of deionized ultra-filtered water (DIUF) in a 5-mL conical tube to activate the sperm (1:400 [v:v] dilution). To obtain accurate readings, sperm were held in DIUF for 1 minute until all movement had ceased. After gently homogenizing the sample, 15 μL of the diluted sample was placed on a Makler counting chamber (Sefi Medical Instruments, Haifa, Israel) and analyzed utilizing a CEROS (Version 12; Hamilton-Thorne, Beverly, MA, USA) computer-assisted sperm analysis (CASA) system.

2.3.2. Viability

Striped bass sperm were examined for viability using a LIVE/DEAD sperm viability kit (Life Technologies Invitrogen-L-7011, Carlsbad, CA, USA) before and after freezing. The same procedure was used for fresh and postthaw samples. For postthaw samples, Cassou straws were placed in a 40 °C water bath for 6 seconds; thereafter, the sample was expelled into a test tube and immediately diluted with our lab's striped bass extender (osmolality, 350 mmol/kg) to create a sperm concentration of 6.4×10^6 cells per mL. To each straw's diluted semen, 9.6 μM of propidium iodide and 0.14μM Sybr-14 molecular stain were added. The mixture was incubated for 5 minutes on ice, and then run on a BD-FACSVerse cytometer for analysis via BD-FACSuite software (Version 1.01; BD Biosciences, San Jose, CA, USA). For the purposes of this study, cells that fluoresced both green and red were considered moribund but counted as "dead," because a red fluorescence signal can only be emitted by a cell whose membranes have been compromised.

2.3.3. ATP

ATP concentration for each sample was determined with a firefly-luciferase bioluminescence procedure, using an ATP bioluminescent assay mix (FLAAM, Sigma-Aldrich, St. Louis, MO, USA). The same procedure was used for fresh and postthaw samples. For the postthaw samples, Cassou straws were placed in a 40 °C water bath for 6 seconds, after which the sample was expelled into a test tube in preparation for analysis.

2.3.3.1. ATP standard preparation. To each well of a white-bottomed, 96-well microtiter plate, 50 μL of DIUF was first added. Nine ATP standards (0.625, 1.25, 2.5, 5, 10, 20,

40, 80, and 160 pmol/mL) were created from an ATP disodium salt hydrate stock solution (FLAAS, Sigma-Aldrich) and 25 μL of each standard was added to wells 1 to 27 in triplicate.

2.3.3.2. Sample preparation. A sample aliquot equivalent to 40×10^6 sperm per mL was first created using an ATP assay mix dilution buffer (FLAAB, Sigma-Aldrich) as a diluent. Immediately after diluting, 10 μL of Phosphatase Inhibitor Cocktail (P5726, Sigma-Aldrich) consisting of sodium vanadate, sodium molybdate, sodium tartrate, and imidazole, was added. Addition of an inhibitor cocktail has been shown to help preserve ATP normally dephosphorylized by acid, alkaline, and tyrosine phosphatases [17]. The inhibited samples were maintained at room temperature for 30 minutes and subsequently placed into a −80 °C freezer until all samples were collected and prepared for the 5-week duration. Thereafter, frozen samples were placed in a boiling water bath for 10 minutes. After boiling, the samples were centrifuged for 5 minutes at 23,000 × *g*, and the resulting supernatants removed into separate 1.5-mL snap-cap vials. An aliquot (25 μL) of each was then added in triplicate to the remaining empty wells. To each well containing a standard or sample, 100 μL of working assay mixture (consisting of ATP assay mix and ATP assay mix dilution buffer) was added to initiate the luciferin-luciferase reaction. The entire plate was then analyzed using a SpectraFlour bioluminescence plate reader (Tecan, Morrisville, NC, USA). Samples were analyzed within 5 minutes after the working assay mixture was added to each well. The standard curve was calculated from the fluorescence readings and the mean luminescence for each sample was converted to $\log_{10}$ for determination of ATP concentration in pmol/10^6 cells [18].

2.3.4. Motion analysis

For motion analysis, 15 μL of DIUF was first added to a Makler counting chamber (Sefi Medical Instruments, Haifa, Israel) and inserted onto a Bionomic (Hamilton-Thorne) temperature-controlled stage mounted onto a negative phase contrast microscope (CX41, Olympus, Tokyo, Japan). The DIUF was allowed to chill to 4 ± 2 °C. The addition of sperm to DIUF results in simultaneous activation and using the CEROS (Hamilton-Thorne) CASA, allowed for rapid tracking of sperm motion. After the Makler reached temperature, an undiluted fresh or postthaw semen sample (containing 350–500 sperm) was added to the Makler chamber and the contents quickly mixed. Sperm activity was then immediately recorded and analyzed using the CEROS CASA. For each sample, a single 0.5-second acquisition was made within 5 seconds after activation. Parameters measured included: total motility; progressive motility (defined as cells exhibiting a path velocity >80 μm/s and straightness >80); and curvilinear velocity (measured in μm/s). Image capture settings were adjusted to a frames/s rate of 60 Hz and the number of frames adjusted to 30. Because striped bass sperm are smaller than those of mammalian species, the minimum cell size was adjusted to two pixels with the minimum CEROS contrast adjusted to a setting of 45.

2.4. Statistical analyses

Data analysis was performed using SAS 9.2 (SAS Institute, Cary, NC, USA). A repeated measures, mixed model analysis of variance was used, with $P < 0.05$ considered significant [19]. Homogeneity of treatment variance and normal distribution of residual errors were examined before selection of the covariance structure. An LSMEANS statement using the DIFF option was used for comparison of least squares means. For the statistical model, freezing rate was considered a fixed effect and week of semen sampling for each fish as a repeated measure. All variables were tested for rate by week interactions. For motility, progressive motility, and viability, fresh and thawed semen were analyzed separately, because of marked differences in variances between fresh and thawed sperm.

3. Results

3.1. Viability

The mean percentages of viable striped bass sperm over the course of the 5-week spawning period were significantly higher in treatments using faster freezing rates (Fig. 1). Over the same interval, there was 96.60 ± 0.10% (mean ± SEM) viable fresh sperm (not shown). The −40 °C/min freezing rate yielded the greatest mean percentage of viable sperm (56.50 ± 0.10%) after thawing (significantly different from all other freezing rates). In contrast, the −10 °C/min freezing rate had the lowest mean percentage of sperm with intact cell membranes (34.54 ± 0.10%). Mean viability values obtained from the intermediate rates (−15 °C and −20 °C/min) were better than the slower rate, but not statistically different from each other. There were no significant rate by week interactions for viability, or for any other of the variables evaluated.

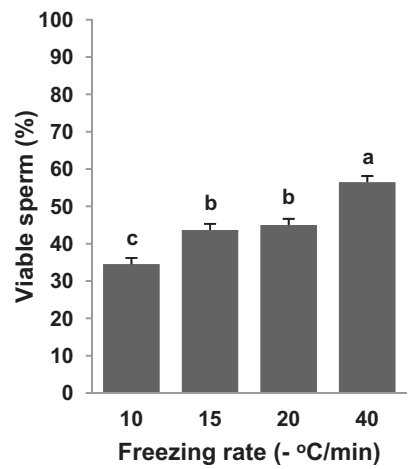

Fig. 1. Mean (±SEM) percentages of viable postthaw male striped bass (N = 12) sperm exhibiting green fluorescence (maximum absorption at 488 nm and emission at 518 nm) when cryopreserved at various freezing rates over the 5-week spawning period. Means without a common letter differed ($P < 0.05$).

3.2. ATP

ATP concentration in striped bass sperm was determined for freshly collected and postthaw samples over the final 3 weeks of the 5-week spawning period (Fig. 2) when semen samples were at or approaching peak quality and concentration for the entire population. Fresh samples had an average ATP concentration of 1.18 ± 0.007 pmol/10^6 sperm (not shown). Sperm cryopreserved using the fastest freezing rate (−40 °C/min) had significantly higher concentrations of ATP (0.86 ± 0.007 pmol/10^6 cells) than all other freezing rates (which were not significantly different from each other).

3.3. Motion analysis

For each freezing rate, total motility for every postthaw sperm sample was greater than the corresponding sample's estimate of progressive motility (Fig. 3). Overall, total motility was 54.64 ± 0.085% and progressive motility was 42.81 ± 0.085% for fresh samples (not shown). For postthaw sperm that were frozen at the −40 °C/min rate, total and progressive motility (10.06 ± 0.085% and 7.14 ± 0.069%, respectively), were significantly better than sperm frozen at any of the three slower rates (no significant differences among the three slower rates for either motility end point; Fig. 3).

4. Discussion

Motility, viability, and ATP quantification are some of the most widely used standards to determine sperm quality and have been correlated with fertilization success (see later in text). For the purpose of our study, an "optimal" freezing rate was defined as the rate that produced the highest percentages of total and progressive motility, percentage viability, and ATP concentration in postthaw striped bass sperm. Unfortunately, we were unable to examine faster freezing rates, because our programmable freezer was only able to create consistent, repeatable freezing rates up to

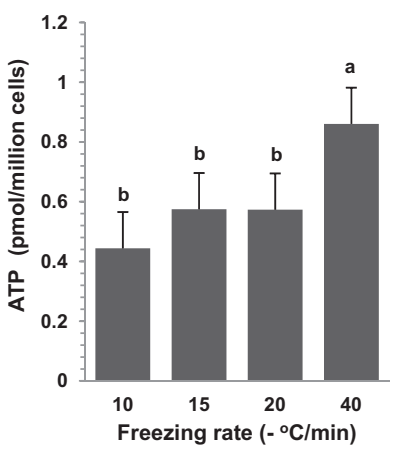

Fig. 2. Mean (±SEM) ATP concentration of postthaw male striped bass (N = 12) sperm when cryopreserved at various freezing rates over a 3-week interval. Means without a common letter differed (P < 0.05).

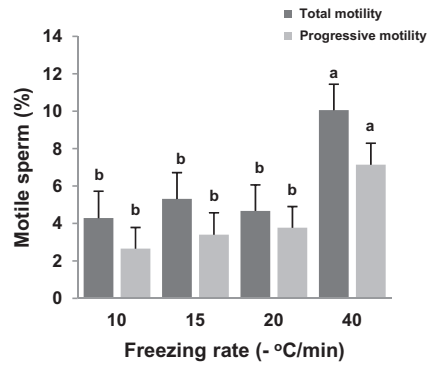

Fig. 3. Mean (±SEM) percentage of total and progressive motility estimates for postthaw male striped bass (N = 12) sperm when cryopreserved at various freezing rates over a 5-week spawning period. Progressive motility was defined as cells with path velocity >80 μm/s and straightness >80%. Means without a common letter differed (P < 0.05).

−40 °C/min. Based on the present results, using the well-documented, preferred cryoprotectant DMSO along with appropriate isosmotic extenders for striped bass semen, a freezing rate of −40 °C/min was optimal for cryopreservation of striped bass sperm.

For viability, there was an increasing percentage of striped bass sperm with intact cell membranes or viable sperm as the freezing rate increased, with the highest membrane integrity obtained from the −40 °C/min freezing rate. Because the freezing process has been previously shown to cause varying degrees of damage to sperm cell membranes [20], axonemes [21], and mitochondria [22] because of osmotic changes and ice crystal formation, it is vital for the optimal freezing rate to demonstrate the capacity to produce sperm with intact membranes.

Postthaw sperm ATP concentrations in species such as the sea bass *Dicentrarchus labrax* [23] and carp [24] were positively correlated with sperm cell motility and fertilization capacity. In the present study, sperm from male striped bass cryopreserved using the −40 °C/min rate had significantly greater concentrations of ATP after thawing. Preservation of these limited ATP resources is paramount, because of several limiting factors unique to striped bass sperm. Compared with mammalian and other teleost species, striped bass sperm contain a relatively small amount of ATP, commensurate with the presence of only two mitochondria [10]. In addition, striped bass sperm appeared unable to replenish endogenous energy and to use exogenous energy sources for motion, as demonstrated by a previous study in which demembranated striped bass sperm incubated with excess exogenous ATP were unable to maintain normal duration of motility [15]. This was in contrast to a study performed using demembranated rainbow trout sperm cells, in which duration of motility was increased (from 30 seconds to approximately 20 minutes) with the addition of ATP to the extender solution [25]. These differences highlighted not only the inability for striped bass sperm to create new ATP via mitochondrial function and oxidative phosphorylation [26] when activated, but to date, have also not been shown to be able to actively transport or use the energy substrates in any

measurable way, when they have been provided to the cells (before or after freezing) via extenders or cryomedia.

This experiment was apparently the first to use a CEROS CASA system, not only to quantify striped bass sperm motion characteristics after thawing in a controlled and repeatable manner, but to use the data in a comparative method to evaluate the best freezing rates for cryopreservation of striped bass sperm. Total and progressive motilities were significantly higher in samples frozen using the −40 °C/min rate than all other rates. Striped bass sperm frozen at −40 °C/min had the highest ATP concentrations and the highest percentages of cell membrane integrity was consistent with this freezing rate yielding the highest percentages of motile sperm. Although there was a significant difference between postthaw motility from the −40 °C/min rate and other freezing rates, all cryopreserved samples were very low (<10%) when compared with the corresponding fresh samples (see section 3.3).

An unusually high degree of straightness (%) was observed from fresh and postthaw striped bass sperm samples, surpassing many species, e.g., horse [27], turkey [28], and even in other teleost species such as the Siberian sturgeon [29] and Chinook salmon [30]. However, it was not possible to determine whether freezing rate had an effect on this particular motion analysis characteristic in striped bass sperm. The use of CASA to determine motility might provide an explanation for the differences between the results published by earlier sources [10–12], although a direct comparison is difficult because of the much smaller concentration of cells needed for CASA analysis compared with previous methods.

In previous studies, motility was determined subjectively, using only visual assessment of an entire screen of sperm (in some cases, recorded and viewed more than once). In contrast, CASA enables collection of unbiased, controlled data, and multiple other characteristics (i.e., straight line velocity, curvilinear velocity, straightness, path velocity, etc.) that cannot be evaluated subjectively. Because successful fertilization might require multiple, positive sperm characteristics (motility, cell membrane integrity, ATP concentration, etc.) in order to successfully transfer its genetic information to the egg, it is important that studies examining the optimal conditions under which sperm are cryopreserved and stored use multiple methods of evaluation. Interestingly, when striped bass sperm have been cryopreserved in the presence of DMSO and at freezing rates of −10 °C, −20 °C, −30 °C, and −40 °C/min, the fastest rate (−40 °C/min) yielded significantly higher postthaw motility than the slower rates tested and when used in fertilization trials, provided a fertilization rate that was not statistically different from fresh semen control samples [10].

4.1. Conclusions

The results of previous studies examining the effects of freezing rates on striped bass sperm cells were not concordant with previous recommendations of both slower and faster freezing rates [10–12]. Whereas the present results of −40 °C/min were comparable with those obtained by He and Woods [10], their study was limited by a pooled sample of three individuals that were only sampled over the course of a single week during the spawning season. In addition, sperm cell quality was based solely on ATP concentration and measurements of motility obtained without CASA. Therefore, the current study was performed to reaffirm the optimal freezing rate, using multiple assay techniques, including CASA motion characterization, to further define and delineate differences in sperm quality of striped bass sperm collected from a substantially larger sample size (N = 12) over an extended interval (5 weeks). For future studies, it would be desirable to examine the effect of controlled, repeatable freezing rates faster than −40 °C/min with DMSO as the cryoprotectant, and novel cryoprotectants on postthaw quality of striped bass sperm.

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