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Production of channel catfish with sperm cryopreserved by rapid non-equilibrium cooling ☆

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ABSTRACT

This report describes the feasibility of using vitrification for fish sperm. Vitrification can be used to preserve samples in the field and offers an alternative to conventional cryopreservation, although it has not been systematically studied for sperm of aquatic species. The overall goal of the project was to develop streamlined protocols that could be integrated into a standardized approach for vitrification of aquatic species germplasm. The objectives of the present study in channel catfish (*Ictalurus punctatus*) were to: (1) evaluate the acute toxicity of 5%, 10%, 20% and 30% methanol, *N*,*N*-dimethyl acetamide, dimethyl sulfoxide, 1,2-propanediol, and methyl glycol; (2) evaluate a range of devices commonly used for cryopreservation and vitrification of mammalian sperm; (3) compare vitrification with and without cryoprotectants; (4) evaluate the post-thaw membrane integrity of sperm vitrified in different cryoprotectant solutions, and (5) evaluate the ability of vitrified sperm to fertilize eggs. Cryoprotectant concentrations of higher than 20% were found to be toxic to sperm. Methanol and methyl glycol were the least toxic at a concentration of 20% with an exposure time of less than 5 min. We evaluated a method reported for human sperm, using small volumes in loops (15 μl) or cut standard straws (20 μl) with and without cryoprotectants plunged into liquid nitrogen. Cryoprotectant-free vitrification using loops did not yield fertilization (assessed by neurulation), and the fertilization rates observed in two trials using the cut standard straws were low (~2%). In general, fertilization values for vitrification experiments were low and the use of low concentrations of cryoprotectants yielded lower fertilization (<10%) than the use of vitrification solutions containing high cryoprotectant concentrations (as high as 25%). The highest neurulation obtained was from a mixture of three cryoprotectants (20% methanol + 10% methyl glycol + 10% propanediol) with a single-step addition. This was reflected in the flow cytometry data from which the highest membrane integrity using loops was for 20% methanol + 10% methyl glycol + 10% propanediol (~50%). We report the first successful sperm vitrification in fish and production of offspring from vitrified sperm in channel catfish. Although the fertilization values were low, at present this technique could nevertheless be used to reconstitute lines (especially in small aquarium fishes), but it would require improvement and scaling up before being useful as a production method for large-bodied fishes such as catfish.

Introduction

Vitrification is considered to be an attractive alternative to standard cryopreservation and has been used in mammals for the cryopreservation of spermatozoa, embryos, oocytes, stem cells, and organs [88]. The advantages of vitrification are that it does not require expensive equipment, it is simple and requires seconds for freezing, and it can be used to preserve samples in the field. In addition, it offers perhaps the greatest potential in overcoming the challenges for preservation of fish embryos [21]. At present, there have been two reports addressing vitrification (for basic research purposes) in fish sperm [2,56]. In addition, partial vitrification is likely to have occurred coincidentally in other studies addressing conventional cryopreservation (i.e. equilibrium cooling) (Table 1). Based on the advances in mammalian sperm vitrification, the discovery of cryoprotectant-free vitrification, and the finding that a wide range of cooling rates (160–250 °C/min) can attain sperm vitrification [37], we evaluated vitrification as an option to cryopreserve fish sperm. To develop a vitrification protocol, the first step was to identify suitable vitrification solutions by measuring the toxicity of cryoprotectants at various concentrations, exposure times, and temperatures. Cryoprotectants are not equally effective for vitrification, and do not have equivalent toxicity or

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Table 1
Previous studies in fish sperm that attempted to cryopreserve samples by plunging into liquid nitrogen. The cryoprotectants (CPA) used were, dimethyl sulfoxide (Me_2SO), glycerol (Gly), ethylene glycol (EG), propanediol (PROH), and methanol (MEOH). Despite the potentially rapid cooling rate, none of these studies likely resulted in substantial vitrification because of low CPA concentrations, large sample volumes, or poor heat transfer of containers. Studies listed in chronological order from 1968 to 2010.

Fish	Scientific name	Cryoprotectant	CPA (%)	Exposure time	Devices	Thawing (°C)	Assessment	Reference
Atlantic salmon	*Salmo salar*	Me_2SO, Gly, EG	2.5–27.5	30 min to 8 h	1-ml Ampoule and aluminium packet	5	No motility or fertility	[27]
Atlantic salmon	*Salmo salar*	EG	5	1 h	Aluminium packet	3	No motility or fertility	[26]
Atlantic salmon	*Salmo salar*	Me_2SO, Gly, EG	5–40	30 min to 6 h	1-ml Ampoule	ND[a]	Completely destroyed	[87]
Grey mullet	*Mugil cephalus*	Me_2SO, Gly	3.3–15	<30 min	Glass vial (0.2–0.3 ml)	20–37	"Some degree of fertility"	[30]
Grey mullet	*Mugil cephalus*	Me_2SO, Gly	6–30	ND	Glass vial (20 × 30 mm)	20–24	"Some degree of fertility"	[8]
Channel catfish	*Ictalutus punctatus*	Me_2SO, Gly, PROH	5–20	1 to 24 h	5-ml Vials	4	"Unsuccessful"	[19]
Common carp	*Cyprinus carpio*	Me_2SO, Gly, EG	2.5–15	5 s to 6 min	Ampoules in basket	0–60	Coagulation of sperm	[58]
Atlantic salmon	*Salmo salar*	Me_2SO, Gly	12.5	ND	2-ml Ampoules	38	7% motile, 80% fertilization	[62]
Cod	*Gadus morhua*	Me_2SO, Gly	12.5	ND	2-ml Ampoules	38	7% motile, 60% fertilization	[62]
Striped bass	*Morone saxatilis*	Me_2SO, Gly, EG, PROH	10–25	ND	0.5-ml Plastic tubes	ND	No fertilization	[42]
African catfish	*Clarias gariepinus*	Me_2SO, Gly, MEOH	5 & 12.5	20 min	Straws and vials	20	No motility	[81]
Elongate tigerfish	*Hydrocynus forskahlii*	Me_2SO, Gly	7 & 11	10 min	1-ml Cryotubes	25	No motility	[82]
Pacific herring	*Clupea pallasi*	Me_2SO, Gly, EG	5–15	10 min	1.8-ml Microcentrifuge	4–37	⩽95% fertilization	[71]
Razorback sucker	*Xyrauchen texanus*	MEOH	10	1.5–30 min	0.5-ml Straws	20–40	Straws burst at thawing	[84]
Sea bass[b]	*Dicentrarchus labrax*	Me_2SO	10	None	1.5-ml Cryovials	50	⩽70% fertilization	[16]
Brook trout[c]	*Salvelinus fontinalis*	Me_2SO, Gly	5	1–20 min	5-ml Straws	35–40	39% fertilization	[45]
Arctic charr[c]	*Salvelinus alpinus*	Me_2SO, Gly	5	1–20 min	5-ml Straws	35–40	39% fertilization	[45]
Rainbow trout	*Oncorhynchus mykiss*	Me_2SO	7	15 min	5-ml Macrotubes	25–80	⩽73% fertilization	[6]
Green swordtail	*Xiphophorus helleri*	Gly	14	10 min	0.25-ml French straws	40	Minimal motility < 1%	[29]
Bluefin tuna	*Thunnus orientalis*	Me_2SO, Gly, MEOH	10–30	5–20 min	0.25-ml Straw	20	No motility	[20]
Common carp	*Cyprinus carpio*	DMSO	10	10 min	0.25-ml Straw	70	No motility, jelly form	[32]
Rainbow trout	*Oncorhynchus mykiss*	Me_2SO, lipids, sugars	10	ND	Glass cell layer 0.1 mm	34–36	90% fertilization vs. fresh sperm control	[2]
Russian sturgeon	*Acipenser gueldenstaedtii*	Me_2SO, lipids, sugars	10	ND	Glass cell layer 0.1 mm	34–36	90% fertilization vs. fresh sperm control	[2]

[a] ND: not described.
[b] Cryovials immersed for 15 s were laid on a tray 2 cm above liquid nitrogen for 15 min and dropped into liquid nitrogen.
[c] Straws were cooled at 0–1 cm above the level of the liquid nitrogen.

osmotic effects [70]. The typical aim in vitrification protocols is to increase the speed of temperature change while keeping the concentration of cryoprotectants (although high) as low as possible [66]. Accordingly, the second step was to select a vitrification device that would minimize sample volume, and allow ultra-rapid cooling. This would allow the use of less concentrated cryoprotectants, and prevent heterogeneous ice formation [90]. Thus, vitrification should be performed in a kinetic way, reconciling concentration of the vitrificant with the rates of cooling and warming [41].

The overall goal of the project was to develop streamlined protocols that could be integrated into a standardized approach for vitrification of aquatic species germplasm. The objectives of the present study were to: (1) evaluate the acute toxicity of 5%, 10%, 20% and 30% methanol, *N*,*N*-dimethyl acetamide, dimethyl sulfoxide, 1,2-propanediol, and methyl glycol; (2) evaluate a range of devices commonly used for cryopreservation and vitrification of mammalian sperm; (3) compare vitrification with and without cryoprotectants; (4) evaluate the post-thaw membrane integrity of sperm vitrified in different cryoprotectant solutions, and (5) evaluate the ability of vitrified sperm to fertilize eggs. We report the first successful sperm vitrification in fish and production of offspring from vitrified sperm in channel catfish (*Ictalurus punctatus*). Although the fertilization values were low, the feasibility of using vitrification for fish sperm was demonstrated.

The channel catfish was selected for this research because it is reasonably well studied in the field of cryopreservation. Work with channel catfish began in the 1970s [19] and subsequent research has refined protocols and moved towards standardization for high-throughput commercial-scale sperm cryopreservation for the closely related blue catfish (*Ictalurus furcatus*) [28]. In addition, early out-of-season induced spawning of channel catfish is an established technique that allows extended production of eggs and fry for research projects outside of the natural (1–2 months) spawning season [47,69], and synchronized conditioning of broodstock enables a predictable spawning schedule [95]. Furthermore, catfish is the largest foodfish aquaculture industry in the United States [89]. Although the small sperm volumes used in vitrification are not practical for commercial aquaculture production, catfish provided a useful working model for this

work in terms of reproductive availability and control, and can serve as a model for small-bodied fishes (<5 cm) which have minute sperm volumes such as the endangered Neosho madtom (*Noturus placidus*) [40], which is in the same taxonomic family (Ictaluridae), and important biomedical research models such as zebrafish (*Danio rerio*).

Materials and methods

Fish source and care

Healthy, mature (3–4 year-old) male channel catfish (1.3–2.6 kg) of current commercial stocks were obtained from Baxter Land Company Inc. (Arkansas City, AR) and maintained in aerated earthen ponds at the Louisiana State University Agricultural Center, Aquaculture Research Station. Fish were fed daily to satiation with a commercial diet (Aquaxcel, Cargill™, 45% protein), and were routinely screened for disease by the Louisiana Aquatic Animal Disease Diagnostic Laboratory at the LSU School of Veterinary Medicine. Temperature and dissolved oxygen were monitored twice daily, and dissolved oxygen was maintained around 5 ppm. Experiments were conducted during Spring (February–May in Southern Louisiana) each year from 2007 to 2010. Guidelines from the Institutional Animal Care and Use Committees of Louisiana State University were followed for animal care and use.

Acute toxicity of cryoprotectants

Sperm cannot be collected by stripping from ictalurid catfishes [92] and thus large, mature males must be killed for collection of testis by dissection [85]. Three males were killed with a blow to the head and their testes were removed. Adherent tissues were dissected away, and testes were blotted dry and weighed (5.3–9.5 g). Testes were crushed in Hanks' balanced salt solution [85] prepared at 300 mOsmol/kg (HBSS300) measured with a vapor pressure osmometer (model 5500 Wescor, Inc., Logan UT) to provide an initial dilution ratio of 1 g of testis per 3 ml of HBSS300. Sperm suspensions from individual males were strained through a series of filters, progressing from a sieve (mesh size of 0.5 mm) to a nylon screen with a final pore size of 200 μm. Sperm concentration was estimated by use of a hemacytometer (Hausser Scientific, Horsham PA) and diluted to a final concentration of 5×10^8 sperm/ml with HBSS300.

Five cryoprotectants, methanol (MEOH; Fisher Scientific, Fair Lawn NJ), *N*,*N*-dimethyl acetamide (DMA; Sigma–Aldrich, St. Louis, MO), dimethyl sulfoxide (Me_2SO; OmniSolv, France), 1,2-propanediol (PROH; Sigma–Aldrich), and methyl glycol (2-methoxyethanol, MG; Sigma–Aldrich) were used at final concentrations of 5%, 10%, 20% and 30% (v/v) (Table 2). Cryoprotectant solutions were prepared in HBSS300 at double the final concentration and kept cold (4 °C) before being added at that temperature to the sperm suspension at a ratio of 1:1 (200 μl of cryoprotectant solution: 200 μl of sperm suspension). The samples were held on ice during the acute toxicity experiment.

Sperm motility was estimated using dark-field microscopy (Optiphot-2, Nikon, Garden City, NY) at 200× magnification within 30 s of dilution with cryoprotectant solution. Catfish sperm (as for most freshwater fishes) is immobile in the testis and becomes activated when exposed to hypotonic solutions. The resultant peak motility is rapid and transient (<1 min). The addition of 20 μl of distilled water was used to activate 1 μl of sperm suspension placed on a glass slide without the use of a coverslip. The percentage of sperm swimming actively in a forward direction was estimated immediately (within 5 s) after addition of distilled water, then at 5 min intervals for 30 min, and finally at 60 min.

Table 2
Cryoprotectant concentrations expressed as percent (v/v), molarity, and the osmolality used for the acute toxicity experiment. The cryoprotectants were diluted in Hanks' balanced salt solution at 300 mOsmol/kg for osmolality measurement.

Cryoprotectant	Percent (v/v)	Molarity (mol/L)	Osmolality (mOsmol/kg)
Methanol	5	1.24	278
	10	2.47	271
	20	4.94	261
	30	7.41	234
Dimethyl acetamide	5	0.54	478
	10	1.08	828
	20	2.15	1413
	30	3.23	1507
Dimethyl sulfoxide	5	0.70	1193
	10	1.41	2015
	20	2.82	3349
	30	4.22	5118
Propanediol	5	0.68	1202
	10	1.36	2000
	20	2.72	2445
	30	4.08	2946
Methyl glycol	5	0.63	236
	10	1.27	281
	20	2.54	276
	30	3.81	258

Vitrification device configurations

A pilot study was carried out prior to the experiments to test eight vitrification devices (Fig. 1): 20-μm mounted cryoloop™ (0.5–0.7 mm) (Hampton Research, Aliso Viejo, CA), capillary tubes (70 μl, Fisherbrand, Pittsburgh, PA), gel-loading pipette tips (Dot Scientific, Burton MI), 0.25-ml French straws (IMV international, Minneapolis, Minnesota), cut standard straws (0.25-ml French straw with a ~20° bevel cut at the end), 10-μl polystyrene loops (Nunc™, Roskilde, Denmark), and 5-mm nichrome loops (~15 μl) (Cole-Parmer, Vernon Hills, IL). The parameters that were evaluated were: efficiency of loading and unloading of samples, sample storage, sample volume, speed of cooling and warming, visualization of glass formation, sample labeling, and cost per sample.

Procedures for spawning, thawing, and fertilization

In the hatchery, fish are often injected with hormones to induce the final stages of oocyte maturation and to synchronize the readiness of multiple females for efficient stripping of eggs [63]. Female channel catfish were obtained from Baxter Land Company Inc. and maintained at the Aquaculture Research Station. During 2007 the females were induced to ovulate using standard procedures [47] by the intraperitoneal injection of 10 mg/kg of carp pituitary extract (lot numbers: 031109, 032209, 033109, Stoller Fisheries, Spirit Lake, IA) [5]. From 2008 to 2010, ovulation was induced by intraperitoneal injection of 100 μg/kg of luteinizing hormone-releasing hormone analog (2008, 2009: Syndel International Inc., Canada; 2010: Argent Laboratories, Redmond, WA) [5]. Final maturation of oocytes and time of ovulation was monitored by ultrasound [67]. Eggs were stripped by gentle abdominal pressure. Eggs from single females were separated into aliquots by placing a monolayer in a 100-ml tri-pour beaker (Fisher Scientific). Because egg size can vary, these aliquots had an average of 114 (±23) eggs in 2007, 167 (±15) eggs in 2008, 148 (±19) eggs in 2009, and 146 (±22) eggs in 2010.

Goblets containing cut standard straws or loops were removed from the liquid nitrogen canister and liquid nitrogen was allowed to drain from the goblets. Samples from Experiments 1–5 were

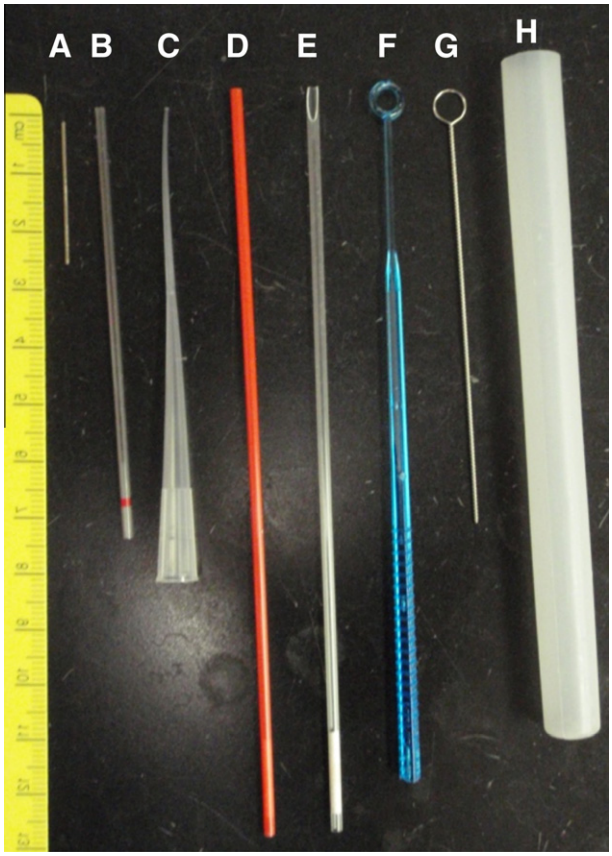

Fig. 1. Different apparatuses were screened to choose the most suitable device for further experiments. The parameters evaluated were: ease of handling, loading and unloading of sample, storage, volume, speed of cooling and warming, visualization of glass formation, labeling, and cost. The devices were: (A) 20-μm cryoloop, (B) capillary tube, (C) gel-loading tip, (D) 0.25-ml straw, (E) cut standard straw, (F) 10-μl polystyrene loop, (G) 5-mm nichrome loop. The devices were stored in 12-cm goblets (H).

thawed into conical tubes (15-ml, Corning, NY) containing 5 ml of HBSS300 warmed in a water bath at 40 °C. Samples from Experiment 6 were thawed into 1.5-ml microcentrifuge tubes containing 1 ml of HBSS300 warmed in a water bath at 40 °C. Tubes containing the straws or loops were gently agitated in the water bath for 10 s and the suspensions were mixed with egg aliquots. Water from the hatching system (10 ml for Experiments 1–5, or 5 ml for Experiment 6) was added at the same time as the sperm suspension to activate the gametes and avoid heat shock. Egg quality was evaluated by using fresh sperm collected that day from males of the same population for fertilization of replicate egg batches (egg quality control). To evaluate the occurrence of gynogenesis (a form of parthenogenesis or uniparental reproduction), vitrification solution or warm HBSS300 (40 °C) without sperm were added to aliquots of eggs in duplicate. The purpose of the gynogenetic controls was to be sure that chemical shock (vitrification solutions) or temperature shock (warm HBSS300) did not activate embryogenesis in unfertilized eggs by retention of the second polar body [43]. The percentage of embryos to reach neurulation (~30 h at 25 °C) was used as a conservative index of fertilization [85]. Neurulation corresponded to the optic-cup stage to pectoral fin-bud stage [76] or early stage V (organogenesis) [52]. The neurulated embryos were counted by viewing with the naked eye using back illumination, and fertilization rate was expressed as the percentage of neurulated embryos in relation to the total number of eggs (referred to as "neurulation").

Vitrification procedures

Experiment 1. Evaluation of cryoprotectant-free vitrification

Two apparatuses were chosen: nichrome loops and cut standard straws. Sperm were collected from four males as described above and diluted to 1×10^9 sperm/ml with HBSS300. For vitrification using cut standard straws, 20 μl of sperm suspension were loaded into the cut end of five straws by use of a micropipette. No equilibration time was needed because there were no cryoprotectants involved. After loading, straws were placed inside a goblet (10-mm Visotube, IMV, L'Aigle, France) attached to a cane, and submerged in liquid nitrogen. The time for this process was ~2 min. For vitrification using nichrome loops, a film (~15 μl) of sperm sample was suspended inside the loop and individual loops were plunged into liquid nitrogen. The time required for loading and freezing for each loop was ~50 s. After the film was frozen, the loop was placed in a goblet attached to a cane (five nichrome loops per goblet), and stored in liquid nitrogen.

Experiment 2. Evaluation of two apparatuses with a single cryoprotectant

The apparatuses selected were polystyrene loops and cut standard straws, and MG was used as the cryoprotectant. Sperm were collected from four males as described above and diluted to 1×10^9 sperm/ml with HBSS300. Sperm suspensions were diluted 3:2 with the cryoprotectant solution to achieve final concentrations of 20% methyl glycol and 1.2×10^7 cells/ml for cut standard straws and 6×10^6 cells/ml for loops. Equilibration time was held to a minimum (i.e. <5 min). For the purposes of these studies we defined equilibration time as having two components: (1) time of exposure to cryoprotectants of the sperm samples before freezing and (2) processing time from the first addition of cryoprotectant until plunging into liquid nitrogen. If a step-wise addition of cryoprotectants was used, exposure time could vary in each step. Cut standard straws (containing 20 μl of sample) and polystyrene loops (containing a thin film ~10 μl of sample) were submerged in liquid nitrogen as described above within 5 min of dilution with cryoprotectant. The samples in cut standard straws (five per goblet) and polystyrene loops (four per goblet) were stored in liquid nitrogen.

Experiment 3. Evaluation of different concentrations using a single cryoprotectant

Methanol was tested at final concentrations of 10% and 20% in HBSS300 using cut standard straws. Sperm suspensions from four individual males were vitrified using the procedure described above for MG and cut standard straws. The difference from Experiment 2 was that for 10% MEOH the sperm suspensions were diluted 4:1 with cryoprotectant solution.

Experiment 4. Evaluation of higher cryoprotectant concentrations by combination of cryoprotectants in a three-step addition

A combination of 5% MEOH, 10% MG, and 20% PROH was tested using nichrome loops. Sperm were collected from three males as described above and adjusted to a concentration of 1×10^9 sperm/ml with HBSS300. Dilutions of MEOH, MG and PROH in HBSS300 were prepared individually to yield a concentration of 40% each. The sperm suspension was diluted 1:1 with 40% MEOH and the exposure for this first addition was 2 min. Methyl glycol was added at a ratio of 1:1, and after 1 min PROH was added at a ratio of 1:1. A thin film of sample was suspended in individual nichrome loops, which were submerged into liquid nitrogen within 2 min of the addition of PROH (total equilibration time from the first addition was ~5 min). Loops were stored in goblets (five per goblet) attached to canes in liquid nitrogen.

Experiment 5. Evaluation of higher cryoprotectant concentrations by combining cryoprotectants in a two-step addition

Methanol at 10% in combination with 20% MG, and the proprietary glass formation enhancers 1% X-1000™, and 1% Z-1000™ (21st Century Medicine, Fontana, CA) were tested in polystyrene loops and cut standard straws. Sperm were collected from three males as described above, and adjusted to 1×10^9 sperm/ml with HBSS300. Methanol was diluted in HBSS300 to yield a concentration of 40%. A double-strength cryoprotectant solution containing 40% MG, 2% X-1000™, and 2% Z-1000™ was prepared in HBSS300. The sperm suspensions were diluted 1:1 with the 40% MEOH solution and the exposure for this first addition was 5 min. The cryoprotectant solution containing 40% MG, 2% X-1000™, and 2% Z-1000™ was added to the sperm suspension at a ratio of 1:1. Samples were immediately loaded into cut standard straws or polystyrene loops and individually submerged into liquid nitrogen within 1 min (~50 s) of the addition of the cryoprotectant solution (total equilibration time from the first addition was ~6 min). Samples were stored in goblets (five cut standard straws or three loops per goblet) as described above.

Experiment 6. Evaluation of vitrification solutions in one-step addition

Three vitrification solutions were tested: 20% MEOH + 10% MG + 10% PROH; 20% MEOH + 20% MG; and 20% MEOH + 20% MG + 0.25 M trehalose. Sperm were collected from three males as described above and adjusted to 1×10^9 sperm/ml with HBSS300. Double-strength cryoprotectant solutions were prepared in HBSS300 and diluted with sperm suspension at a ratio of 1:1. Samples were immediately loaded (within 15 s) into polystyrene loops, and submerged in liquid nitrogen within 1 min (~50 s) after the addition of the vitrification solutions (equilibration time, ~1 min). Glass formation was assessed by observing the appearance of the vitrified sample (a milky appearance indicated ice crystal formation). Although direct visualization of vitrification resulted in a transparent form, some microscopic ice crystals could have been present. Loops were stored in goblets (three per goblet) in liquid nitrogen.

Assessment of membrane integrity

Sperm samples from three males were vitrified using two apparatuses: polystyrene loops, and 0.25-ml straws. Six treatments were evaluated: (1) no cryoprotectant, (2) 10% MEOH, (3) 20% MEOH, (4) 20% MG, (5) 20% MEOH + 10% MG + 10% PROH, and (6) 20% MEOH + 20% MG. Cryoprotectant solutions were prepared at double-strength in HBSS300 and diluted 1:1 with sperm at 1×10^9 sperm/ml. Individual loops and straws were submerged into liquid nitrogen, within 1 min of addition of cryoprotectant solutions (equilibration time, ~1 min). The samples were stored in liquid nitrogen for 20 days before flow cytometry analysis. To thaw the sperm, each loop was warmed directly in 495 µl of HBSS300 at room temperature (24 °C), and straws were thawed at 40 °C for 5 s. Thawed sperm from the straws was further diluted by adding 5 µl of sperm suspension to 495 µl of HBSS300. The sperm concentration for the diluted thawed samples was held between 5×10^6 to 1×10^7 cells/ml.

To evaluate membrane integrity, fresh and thawed sperm were filtered through 35-µm nylon mesh and duplicate aliquots of 250 µl were stained with the fluorescent dyes SYBR-14 and propidium iodide (PI) (live/dead sperm viability kit, Molecular Probes, Eugene, OR). Final concentrations of the fluorescent dyes were 100 nM SYBR-14 and 12 µM PI, and samples were incubated in the dark for 10 min at room temperature prior to analysis. Flow cytometry was performed using an instrument (C6 Accuri Cytometers Inc., Ann Arbor, MI) equipped with a 488-nm, 50-mW solid-state laser. Flow cytometer performance was assessed using fluorescent validation beads (Spherotech, Accuri Cytometers Inc.) to ensure that coefficient of variation values were <3.0% (calculated based on full peak height) for the fluorescence detectors (FL1, FL2, FL3, and FL4). Each microcentrifuge tube was flicked gently three times with a finger prior to analysis to ensure suspension of the cells, and 10 µl of sample were analysed at a flow rate of 35 µl/min using CFlow® software (version 1.0.202.1, Accuri Cytometers Inc.). Green fluorescence (SYBR 14) was detected with a 530 ± 15 nm bandpass filter (FL1), and red fluorescence (PI) was detected with a >670 nm longpass filter (FL3). Events were viewed on forward-scatter (FSC) vs. side-scatter (SSC) plots, and a gate (used to define target cells within the total event population) was drawn around the sperm population to exclude non-sperm events. Gated events were viewed on a scatter plot showing FL1 vs. FL3 with fluorescence compensation based on the computed median fluorescence values of the different populations to reduce spectral overlap. Sperm that stained with SYBR 14 alone were considered to have an intact membrane, and those that stained with both SYBR 14 and PI or PI alone were considered to be membrane-compromised.

Statistical analysis

The fixed treatment variables were: cryoprotectant (T), concentration (C), and incubation time (I). Acute toxicity data were analyzed as a factorial ($5T \times 4C \times 8I$) randomized block design. The channel catfish males were grouped in a block to remove variation among individual motility from the error term. The dependent variable was sperm motility (%). The control (fresh sperm) was excluded from the model, but was used as a reference to ensure sperm viability. Analysis was conducted using a mixed ANOVA procedure for all interactions among T, C, and I. Membrane integrity data were analyzed using a mixed ANOVA procedure with apparatus and cryoprotectants as fixed treatments and membrane intact (%) as a dependent variable. The control (fresh sperm) was excluded from the model, but was used as a reference for viable sperm. Statistical differences were determined at an $\alpha = 0.05$ level using Tukey's adjustment. Statistical analyses were performed using SAS software (Statistical Analysis System Inc., version 9.1; SAS institute, Cary, NC).

Results

Acute toxicity of cryoprotectants

When low concentrations (5%) of cryoprotectants were used, there was no difference ($P = 0.52$) in the type of cryoprotectant, and the sperm motility remained high (40–60%) (Fig. 2). When the concentrations were increased to 10%, two cryoprotectants (MEOH and MG) were the least toxic with no difference between them ($P = 0.25$), and motility remained high (~50%). Time played a key role in the toxicity of cryoprotectants. When 10% was used, motility did not change ($P = 0.96$) within the first 10 min of exposure for each cryoprotectant used. After 10 min, two groups of cryoprotectants could be distinguished. The first group (MEOH and MG) was the least toxic (~50% motility), while the second group (Me_2SO and PROH) was more toxic (<20% motility). After the cryoprotectant was increase to 20% (around half of the concentration needed for vitrification) motility was reduced in all treatments (Fig. 2). At 5 min after the addition of 20% of cryoprotectants, the average motility for MEOH was 33 ± 3%, followed by MG (22 ± 12%). Sperm in the other cryoprotectants (Me_2SO, DMA, and PROH) had low motilities (<10%). When the cryoprotectants were increased to 30% all ($P = 0.69$) motility was eliminated within 10 min (Fig. 2).

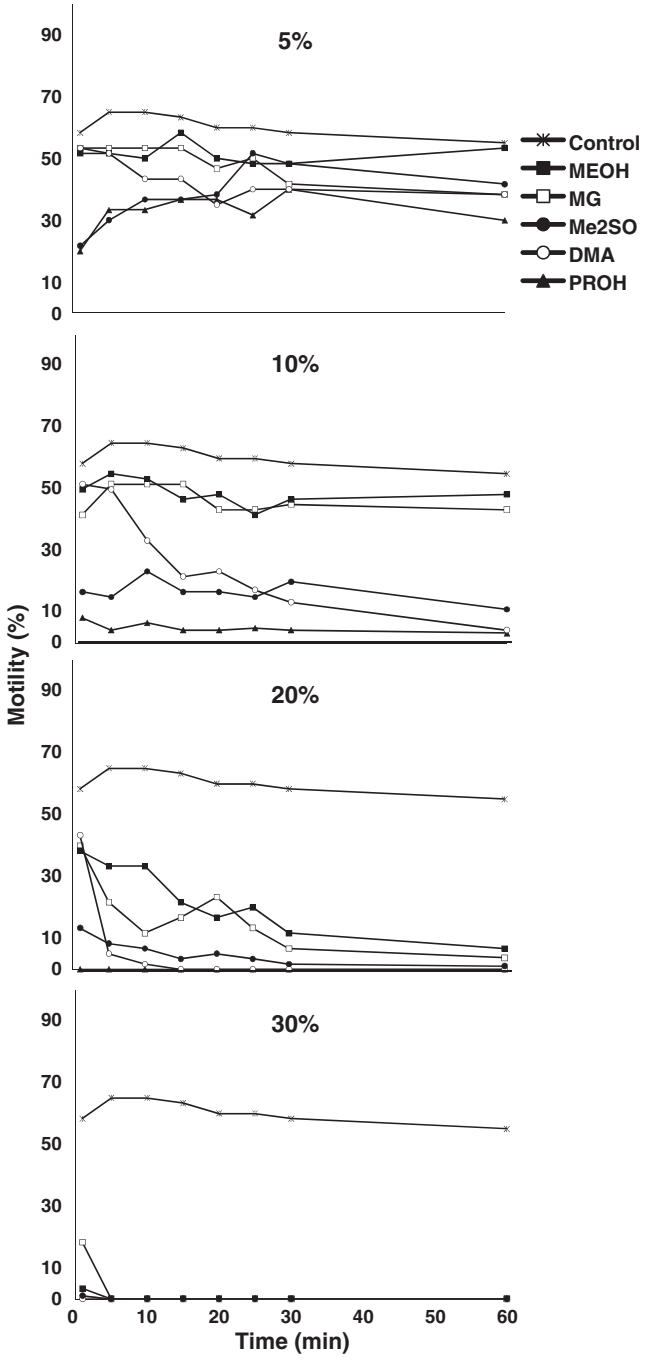

Fig. 2. Acute toxicity to sperm of channel catfish of 5%, 10%, 20% and 30% of five cryoprotectants. Each point represents the mean of three replicates. The cryoprotectants used were, methanol (MEOH), methyl glycol (MG), dimethyl sulfoxide (Me_2SO), dimethyl acetamide (DMA), and propanediol (PROH).

Vitrification device configurations

From the pilot study it was determined that each device had unique characteristics that influenced their suitability and practicality for use in vitrification. The volume held by the 20-μm cryoloop™ (<1 μl) was too small to be practical for use in fertilization trials for channel catfish. Expulsion of samples from the gel-loading tips and capillary tubes was difficult because of the high viscosity of the vitrification solutions. A device that proved to be advantageous was the cut standard straws, which offered the standard advantages of the straws (e.g. ease of labeling, handling, and storage) while decreasing the sample volume for faster cooling, and the cut edge provided easy loading and unloading of the small volumes. The nichrome and polystyrene inoculation loops also proved to be effective and easy to use. The polystyrene loops provided the additional advantage that they could be easily cut to lengths that would fit into goblets. Therefore, the vitrification devices chosen for further study were the cut standard straw, nichrome loop, and polystyrene loop.

Vitrification procedures

Experiment 1. Evaluation of cryoprotectant-free vitrification

Some twitching and vibration of sperm was observed after thawing, but no true progressive post-thaw motility was observed in any experiments. Mean neurulation (fertilization) for all experiments was low. None of the gynogenetic controls produced fertilization. Cryoprotectant-free vitrification in nichrome loops did not yield fertilization, and cryoprotectant-free vitrification in cut standard straws yielded low levels (<2%) of fertilization in 2 of 16 trials (Table 3).

Experiment 2. Evaluation of two apparatuses using a single cryoprotectant

Cut straws yielded more reproducibility (neurulation was observed in 16 of 16 trials) than polystyrene loops (neurulation in 13 of 16 trials) (Table 3). In addition, mean neurulation values were higher (~5%) in cut standard straws than polystyrene loops, but were still low. This higher fertilization could be related to the higher volume (20 vs. 10 μl) held by cut standard straws which translated into more sperm per egg (Table 3).

Experiment 3. Evaluation of different concentrations using a single cryoprotectant

Higher concentrations of cryoprotectants yielded higher mean neurulation values in all experiments when comparing 10% and 20% MEOH in cut standard straws, 10% had higher reproducibility with neurulation observed in 11 of 15 trials (Table 3).

Experiment 4. Evaluation of higher cryoprotectant concentrations by combination of cryoprotectants in a three-step addition

Neurulation in the fresh sperm control for Experiment 4 was low (~21%) indicating poor egg quality. The vitrification solution (5% MEOH + 10% MG + 20% PROH) formed almost completely transparent glass (~80% by visual assessment). Although the glass was clearer when the solution was vitrified in a single step than after the stepwise additions, there was no benefit in adding the cryoprotectants in different steps, as the neurulation remained low (<10%) in all experiments (Table 3).

Experiment 5. Evaluation of higher cryoprotectant concentrations by combining cryoprotectants in a two-step addition

The egg quality assessed using fresh sperm for fertilization was low (<50% neurulation). Glass formation was enhanced by the use of polymers (X-1000™ and Z-1000™) with the observation of an almost complete transparency (~90% by visual assessment). There was no benefit in adding the cryoprotectants in a two-step addition (Table 3), as neurulation remained low (<10%) for the two-step addition using either polystyrene loops or cut standard straws.

Experiment 6. Evaluation of vitrification solutions in one-step addition

Complete glass formation was observed in 20% MEOH + 10% MG + 10% PROH, 20% MEOH + 20% MG, and 20% MEOH + 20% MG + 0.25 M trehalose treatments vitrified in loops (Fig. 3). The combination of cryoprotectants was difficult to evaluate due to poor egg quality, demonstrated by the low neurulation with fresh sperm. The egg quality had a direct relationship with the fertiliza-

Table 3
Apparatus, cryoprotectants, sperm number per container, number of trials, and mean fertilization used for vitrification experiments in channel catfish performed from 2007 to 2010. Containers used were 5-mm nichrome loops (15 μL), cut standard straws (20 μL), and polystyrene loops (10 μL). Fertilization rate was expressed as the percentage of neurulated embryos in relation to the total number of eggs.

Apparatus	Cryoprotectant	Neurulation		Sperm per container	Sperm-to-egg ratio
		By # trials	Mean ± SD[a]		
Experiment 1 (4 males; 4 females)					
Nichrome loop	None	0 of 16	0	1.5×10^7	6.4×10^5
Cut standard straw	None	2 of 16	2 ± 1	2.0×10^7	8.4×10^5
Control		32 of 32	56 ± 19	1.0×10^8	8.4×10^5
Experiment 2 (4 males; 4 females)					
Polystyrene loop	20% MG[b]	13 of 16	2 ± 1	6.0×10^6	2.0×10^5
Cut standard straw	20% MG	16 of 16	5 ± 2	1.2×10^7	5.1×10^5
Control		32 of 32	56 ± 19	1.0×10^8	8.4×10^5
Experiment 3 (4 males; 4 females for 10%MEOH and 2 females for 20%MEOH)					
Cut standard straw	10% MEOH[c]	11 of 15	3 ± 3	1.6×10^7	6.0×10^5
Cut standard straw	20% MEOH	5 of 8	9 ± 5	1.2×10^7	4.5×10^5
Control		8 of 8	65 ± 9	1.0×10^8	7.5×10^5
Experiment 4 (3 males; 1 female)					
Nichrome loop	5%MEOH + 10%MG + 20%PROH[d]	7 of 9	4 ± 3	1.9×10^6	6.1×10^4
Control		9 of 9	21 ± 7	1.0×10^8	6.4×10^5
Experiment 5 (3 males; 2 females)					
Polystyrene loop	10%MEOH + 20%MG + 1%X[e]+1%Z[f]	8 of 12	3 ± 2	2.5×10^6	5.4×10^4
Control		18 of 18	21 ± 7	1.0×10^8	7.1×10^5
Cut standard straw	10%MEOH + 20%MG + 1%X + 1%Z	14 of 18	4 ± 3	5.0×10^6	1.6×10^5
Control		16 of 16	51 ± 9	1.0×10^8	6.6×10^5
Experiment 6 (3 males; 2 females)					
Polystyrene loop	MMP[g]	5 of 6	11 ± 11	5.0×10^6	9.1×10^4
Polystyrene loop	MEMG[h]	5 of 6	4 ± 3	5.0×10^6	9.1×10^4
Polystyrene loop	MEMGT[i]	4 of 6	4 ± 2	5.0×10^6	9.1×10^4
Control Female 1		9 of 9	83 ± 5	1.0×10^8	6.1×10^5
Polystyrene loop	MMP	2 of 6	3 ± 2	5.0×10^6	1.2×10^5
Polystyrene loop	MEMG	3 of 6	2 ± 1	5.0×10^6	1.2×10^5
Polystyrene loop	MEMGT	4 of 6	1 ± 1	5.0×10^6	1.2×10^5
Control Female 2		8 of 9	17 ± 4	1.0×10^8	7.8×10^5

[a] Mean and standard deviation from the samples that had neurulation.
[b] Methyl glycol.
[c] Methanol.
[d] 1,2-Propanediol.
[e] X-1000™.
[f] Z-1000™.
[g] 20% MEOH + 10%MG + 10% PROH.
[h] 20% MEOH + 20%MG.
[i] 20% MEOH + 20%MG + 0.25 M trehalose.

tion trials. For example, 20% MEOH + 10% MG + 10% PROH treatment for female one yielded a mean neurulation of 11% while female two yielded 3%. For fresh sperm, female one had significantly higher neurulation (83%) than did female two (17%) ($P < 0.001$). The highest neurulation rates (as% success) were observed using 20% MEOH + 10% MG + 10% PROH in polystyrene loops (as high as 25%), followed by 20% MEOH using cut standard straws (as high as 15%).

Assessment of membrane integrity

There was a significant difference ($P < 0.001$) between loops and 0.25-ml straws in the percentage of membrane-intact sperm after vitrification. There was no significant difference ($P > 0.05$) among cryoprotectant-free vitrification and vitrification using 10% MEOH, 20% MEOH, and 20% MG. Membrane integrity using the vitrification solutions 20% MEOH + 20% MG and 20% MEOH + 10% MG + 10% PROH in a one-step addition was significantly higher ($P < 0.001$) than for the other treatments. The highest percentage of membrane-intact sperm was for 20% MEOH + 10% MG + 10% PROH in loops (50 ± 4%) followed by 20% MEOH + 20% MG in loops (45 ± 6%). In contrast, the percentage of intact sperm was lower for 20% MEOH + 10% MG + 10% PROH (21 ± 7%) and 20% MEOH + 20% MG in straws (11 ± 8%). There was no significant difference ($P = 0.8$) between 20% MEOH + 10% MG + 10% PROH in loops and 20% MEOH + 20% MG in loops. In general, as the cryoprotectant concentration increased, yielding glass-forming solutions, the membrane integrity also increased (Fig. 3).

Discussion

The technique used in general to attain vitrification is rapid non-equilibrium cooling, which differs from traditional slow-cooling cryopreservation protocols in that dehydration and cryoprotectant permeation take place before cooling begins [78]. This phenomenon can be enhanced by the use of high concentrations of cryoprotectants (40–60%) and an increase in cooling rate (>1000 °C/min) [48,50,78], although neither high cryoprotectant concentration nor increased cooling rates are essential for vitrification to occur. Partial or total intracellular vitrification can occur during conventional equilibrium cooling, and may be responsible for some degree of survival of cryopreserved samples [91]. Vitrification is now widely used to cryopreserve oocytes and embryos of several mammalian species [90]. In fishes, vitrification has been applied to cryopreservation of embryos, although results from some of these studies have been controversial [13]. Embryo survival has been reported but not successfully replicated in zebrafish [9], Japanese seaperch (*Lateolabrax japonicas*) [83], winter flounder

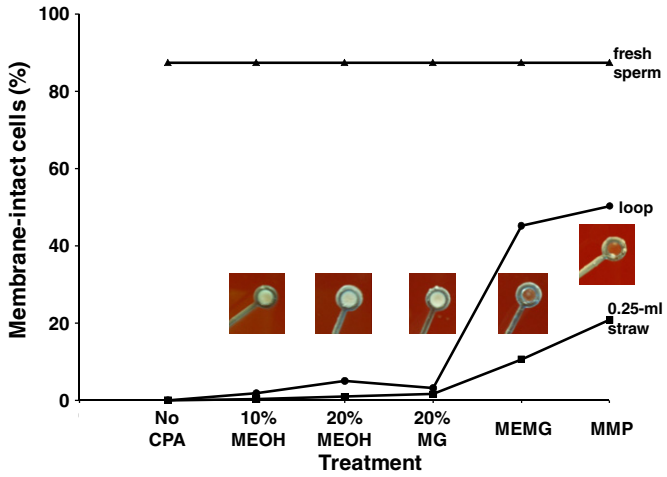

Fig. 3. Membrane integrity of thawed sperm from three channel catfish as determined by flow cytometry. Sperm were cryopreserved by using 10-μl polystyrene loops or 0.25-mL straws (250 μl sample volume). The treatments used were cryoprotectant-free (No CPA), 10% methanol (10% MEOH), 20% methanol (20% MEOH), 20% methyl glycol (20% MG), 20% methanol + 20% methyl glycol (MEMG), and 20% methanol + 10% methyl glycol + 10% propanediol (MMP). Assessment of ice crystals (milky color) or glass formation (clear) was evaluated visually for the loops with different treatments. Individual cryoprotectants with < 20% concentration appeared milky indicating ice crystal formation, while the mixture of cryoprotectants (final concentration 40%) appeared transparent indicating glass formation.

(*Pseudopleuronectes americanus*) [74], and Japanese flounder (*Paralichthys olivaceus*) [10]. The limited success in fish embryo vitrification could be due to the large size (>1 mm in diameter), complexity, and low permeability of the multicellular embryo, which could inhibit the entrance of cryoprotectants into the various compartments [73,101]. With the exception of two studies in which the investigators observed vitrification in fish sperm by use of cryomicroscope [2] or evaluated motility, membrane and mitochondrial integrity [56], to our knowledge there have been no studies that specifically addressed fish sperm vitrification or intended to develop streamlined protocols, although some cryopreservation studies have coincidentally produced vitrification (Table 1).

Acute toxicity of cryoprotectants

Choosing the least toxic permeable cryoprotectant is one of the first steps in developing a cryopreservation protocol. Cryoprotectant toxicity and osmotic effects can be seen as limiting factors for cryopreservation by slow-cooling and vitrification [14]. The toxicity of a cryoprotectant is related to its concentration, the duration of exposure, and temperature. Most cryoprotectants tend to have toxic and hypertonic effects when used at concentrations that are effective for successful vitrification [100].

Cryopreservation studies in channel catfish began in 1976 [19], and previous studies of acute cryoprotectant toxicity in channel catfish sperm evaluated low concentrations (<15%), and long exposure times (>10 min to days) [11,85]. The high concentrations of cryoprotectant used in vitrification required a new evaluation of acute toxicity and shorter exposure times. Concentrations of higher than 20% for six cryoprotectants were evaluated in this study and were found to be toxic to sperm. Methanol (5 M) and methyl glycol (2.6 M) were the least toxic at a concentration of 20% with an exposure time of less than 5 min. Methanol at 10% was the cryoprotectant of choice for channel catfish cryopreservation in previous studies [12]. Methyl glycol has been used to cryopreserve sperm from some freshwater fishes [53,93], but this is the first study to evaluate methyl glycol as a cryoprotectant for channel catfish sperm. One possible reason that methanol and methyl glycol yielded higher survival at higher concentrations is because they produce less osmotic damage than other cryoprotectants. The osmolality of methanol and methyl glycol at 20% in HBSS300 is close to 300 mOsmol/kg (the osmolality of HBSS300) [85], while the rest of the cryoprotectants tested in this study at 20% had osmolalities of greater than 1500 mOsmol/kg.

In general, sperm from freshwater fishes become motile in response to a reduction of osmotic pressure (hypotonic to blood plasma) while sperm from marine fishes become active with an increase of osmotic pressure (hypertonic, sea water >1000 mOsmol/kg) [86]. Activation of sperm motility in channel catfish occurs in the range of 35–270 mOsmol/kg, with complete activation occurring at 132 mOsmol/kg and below [3]. Sperm from channel catfish are not adapted to deal with high osmotic pressures and this could explain the low survival observed at higher concentrations of some cryoprotectants.

Cryoprotectant-free vitrification

Sperm cells are damaged primarily due to osmotic (solution) effects at slow cooling rates and intracellular ice formation at high cooling rates. These paired observations taken together are termed the "two-factor hypothesis" or "Oak Ridge curve" [49]. Recent publications have stated that there was no evidence of intracellular ice formation in human or horse sperm cooled at 3000 °C/min, and it was proposed that the cell damage observed was a result of an osmotic imbalance encountered during thawing [60,61]. Intracellular vitrification can be achieved relatively easily in cells such as spermatozoa because of their small size and high content of soluble macromolecules (such as proteins and sugars) that make the intracellular matrix highly viscous compared with oocytes and embryos [34,35]. Early attempts to vitrify mammalian spermatozoa resulted in low or no survival [79]. However, a breakthrough came in 2002, when human sperm were vitrified without conventional cryoprotectants by cryopreserving samples in thin films in copper cryoloops [65]. The idea of using loops dated back to 1942, when human sperm were vitrified in films on wire loops by plunging into liquid nitrogen, resulting in sperm survival as high as 67%, but "negative results" were obtained with sperm from rat, mouse, guinea pig, rabbit, and bull [25]. In another experiment, platinum loops were used to vitrify human sperm without cryoprotectants, but no motile sperm were observed after thawing [68]. It has been suggested that survival of vitrified sperm without cryoprotectants could be due to the presence of large amounts of osmotically inactive water bound to macromolecular structures, such as DNA and histones, or the presence of high molecular weight components in sperm that affect the viscosity and glass transition temperature of the intracellular cytosol [37,72].

Compared to those of mammals, fish sperm are small; for example, in most externally fertilizing teleost species the length of the sperm nucleus is <5 μm, and the midpiece length is 2–4 μm (although the flagellum is 30–40 μm or longer) [46]. Attempts to cryopreserve fish sperm samples at slow cooling rates without cryoprotectants have yielded low survival (~1% post-thaw motility cooled at 40 °C/min) [11] or no survival [8,80,94,99], most likely due to the large sample volume (>0.25 ml) and slow rate of cooling which lead to injuries by long exposure to concentrated solutions (i.e. the solution effect). Attempts have been made to plunge samples into liquid nitrogen to increase the cooling rate, although none of these publications made reference to vitrification (Table 1). One previous study in which ampoules (0.2–0.5 ml) of undiluted sperm of Pacific oyster (*Crassostrea gigas*) were plunged into liquid nitrogen without use of cryoprotectants reported fertilization rates as high as 40% [31] although total vitrification was not a likely result given the large volume and use of ampoules.

In the present study we evaluated a method reported for human sperm [65], using small volumes in loops plunged into liquid nitrogen without cryoprotectant. Fresh human sperm vitrified in this matter without addition of media yielded motilities of ~20% after warming [65]. In the present study, two apparatuses were used for cryoprotectant-free sperm vitrification in channel catfish. Vitrification using loops did not yield neurulation, and low rates were also observed using cut standard straws. The loops that were used in this experiment (5 mm; 15 μl) were similar to the ones used for humans (5 mm; 20 μl) [65], and to our knowledge there are no previous publications that used cut standard straws to vitrify sperm. A similar method (open straw) was described previously to vitrify human sperm by adding 1 μl to the open end of a 0.25-ml straw, which was placed inside a 90-mm straw that was hermetically sealed [38]. In the present study, cryoprotectant-free vitrification using a 20-μl sample in cut standard straws yielded low fertilization (~2%) in two trials. Cut standard straws have been used to vitrify human blastocysts [39], and they are easy to work with and can be used for different sample volumes. In addition, if there are concerns about cross-sample contamination, the cut standard straws can be inserted into a 0.5-ml straw that can be closed at both ends [39]. Recently sperm from rainbow trout (*Oncorhynchus mykiss*) was vitrified without cryoprotectants using the microdrop (20 μl) method [56]. Motility and membrane integrity after vitrification ranged from 70% to 90%. Although no fertilization attempts were made, it was the first report of cryoprotectant-free vitrification in fish sperm.

Previous attempts in twenty studies to cryopreserve aquatic species sperm by plunging them into liquid nitrogen have produced inconsistent results (Table 1). While one study using sperm from the Pacific herring (*Clupea pallasi*) yielded fertilization (assessed by neurulated embryos) as high as 95% [71], the majority of the studies have been unsuccessful. Complete vitrification was not attained in the previous studies because of the low concentrations of cryoprotectant (<30%; most <15%), and large volumes (0.25–5 ml) used. For example, in the Pacific herring study, DMSO was used at 15% with 1.8-ml polypropylene microcentrifuge tubes. The minimum concentration of DMSO in 0.25-ml straws that will vitrify when cooled by plunging into liquid nitrogen is 39% [1]. This indicates that that study did not result in complete vitrification. The high fertilization in the Pacific herring study was probably due to partial intracellular vitrification, because sperm from marine fishes have greater survival (as high as 80% vs. ~50% for freshwater fishes) during cryopreservation [44], and because of chemotaxis where the sperm are not active in seawater but become activated when coming in contact with the egg chorion [59]. Chemotaxis is an important factor in egg fertilization because herring eggs contain proteins that facilitate the union of the gametes. The high fertilization of the previous study could be explained by the unique fertilization strategy employing these proteins that guide the sperm into the micropyle (an opening in the egg chorion). The low success reported in most other studies that submerged samples into liquid nitrogen is likely due to a combination of insufficient cryoprotectant concentration, long pre-freeze exposure times, large sample volumes, and use of containers that inhibit heat transfer which translates into slow cooling rates (Table 1). Thus, although using a potentially rapid cooling method (plunging in liquid nitrogen), none of these studies were designed to directly address vitrification. One study used a high cryoprotectant concentration (40% EG) that had the potential to achieve glass formation, but the exposure time was long (2 h), and the sperm likely experienced damage due to cryoprotectant toxicity before cooling began [87]. Recently, one study attained an ultra-rapid cooling rate (3000–4000 °C/min) by using 10% Me_2SO and 10 μl samples of sperm from rainbow trout and Russian sturgeon (*Acipenser gueldenstaedtii*) in a 100-μm thick glass cell ("thin-layer freezing"). No ice crystals were observed by examination with a cryomicroscope. Sturgeon sperm cryopreserved by this method resulted in 90% egg fertilization (fresh sperm was defined as 100%) [2]. Although the focus of that study was to evaluate the formation of ice microparticles at different cooling rates with the use of different additives (i.e., egg yolk, sugars, and lipids), it nonetheless produced vitrification. That report focused on basic research rather than protocol development, and lacked practical details such as a description of the cooling method, post-thaw motility, fertilization assessment methods, percentage of fertilization from the control group, and number of females used.

In general in the present study, the use of low concentrations of cryoprotectants yielded low fertilization, while use of vitrification solutions containing high cryoprotectant concentrations increased fertilization. The concentration of cryoprotectants needed for vitrification of mammalian embryos is high (>40%) and near the maximum tolerated by these cells [55]. There are a number of ways to reduce the concentration of individual cryoprotectants required for vitrification. For example, the application of high hydrostatic pressure, addition of non-permeating polymers or agents, combination of cryoprotectants, stepwise addition of cryoprotectants, and limiting of exposure time at high concentrations to a minimum [15]. In this study, the two most suitable cryoprotectants, based on acute toxicity (methanol and methyl glycol), were mixed to obtain additive effects of each agent. Methanol has a high rate of permeability and relatively low toxicity, but almost pure methanol (99.8%) exhibits little or no vitrification [1], while methyl glycol is considered to be a good glass former (it will vitrify at 40%) and may be useful in vitrification solutions [74]. In addition to combining the cryoprotectants, addition of proprietary polymers such as X-1000™ and Z-1000™ has been used to inhibit ice formation [96,97] and was able to enhance glass formation at lower concentrations of cryoprotectants in the present study. Despite this, the neurulation rates of sperm vitrified using these polymers remained low in the present study. Similarly, trehalose has been used to cryopreserve sperm in fish species [57,77], but in this study did not improve the fertilization success of vitrified samples.

Another way to reduce osmotic damage while using high concentrations is by stepwise addition of the cryoprotectants. In this study, three different addition methods were evaluated, but fertilization rates were low in all trials, suggesting that there was no advantage in adding the cryoprotectants in successive steps.

Overall, the highest neurulation obtained (25%) was from a mixture of three cryoprotectants (20% MEOH + 10% MG + 10% PROH) with a single-step addition. The reasons for this were most likely that the mixture reduced the aggregate toxicity of the cryoprotectants (although total osmolality was 1700 mOsmol/kg), but the exposure time was held to a minimum (<1 min). This was reflected in the flow cytometry data from which the highest membrane integrity using loops was for this cryoprotectant mixture (~50%). In addition, variation in the fertilization of eggs from two females with sperm vitrified using the same mixture of cryoprotectants emphasized the importance of egg quality, which when higher than 50% neurulation (in fresh sperm controls) showed fertilization for vitrified samples. Another variable in Experiment 6 was the use of a volume (5 ml) of activation solution that was smaller than in the other experiments (10 ml). Smaller volumes allowed more sperm contact with the egg, and a higher effective sperm-to-egg ratio.

Assessment of membrane integrity

Membrane integrity is commonly used assay to estimate viability of cryopreserved sperm [18]. Recognition of the benefits of evaluating plasma membrane functionality in fish sperm cryopreservation dates back to 1966 [17]. Since then different protocols have been tested in aquatic organisms [75]. The most common

fluorophore combination used is SYBR-14 and PI [54], which provides simultaneous information on the proportions of membrane-intact and membrane-compromised cells. This method can be analyzed by fluorescence microscopy or by flow cytometry [54] which is a precise, sensitive, accurate, and rapid method of multiparameter, single-cell analysis [51]. Previous studies in human sperm vitrification have evaluated DNA integrity, mitochondrial activity, and acrosomal status by use of flow cytometry. These studies concluded that sperm vitrification was similar to or better than standard cryopreservation [33,36,37].

In the present study, we evaluated membrane integrity as an additional means of assessing the effectiveness of vitrification. The first hypothesis that was tested was whether glass formation improved the survival of the sperm. The highest neurulation rate (25%) was observed using sperm cryopreserved with the vitrification solution containing 20% MEOH + 10% MG + 10% PROH, but some fertilization ($\leqslant$15%) was observed using sperm frozen with low cryoprotectant concentrations (20% MEOH). By assessing membrane integrity at different concentrations of cryoprotectants, we observed a direct relationship between viability and cryoprotectant concentration. In general, increasing the concentration of cryoprotectants led to more glass formation and increased sperm viability. This can be explained because vitrification can be partial or total. Total vitrification includes the glass formation of the whole sample including the extracellular and intracellular fractions, while partial vitrification includes the glass formation of intracellular water [90,91]. In this study, total vitrification (e.g. complete transparency) yielded higher survival. A study on vitrification of primordial germ cells in zebrafish concluded that there was no marked relationship between the appearance of ice formation during cooling and the cell survival rate [23]. But in a subsequent study, ice crystal formation lowered the survival of cells (to 1–26%) compared to glass formation (80%) [22].

The second hypothesis tested was whether there was any difference in vitrification using the same concentrations of cryoprotectants but different sample volumes. We compared the membrane integrity of sperm vitrified using loops (10 μl) and 0.25-ml straws (250 μl). When using vitrification solutions, the membrane integrity of sperm in loops was more than twice that of sperm vitrified in straws. This is most likely because the smaller volumes had faster cooling and warming rates, and lower concentrations of cryoprotectants were required to achieve glass formation [90]. Increasing the concentration of cryoprotectants could improve vitrification in straws, but it is possible that the increased toxicity may not be tolerated by the sperm.

Conclusions

This report demonstrates the feasibility of using vitrification for fish sperm. Overall, fertilization (neurulation) values were low, and thus while the current technique could be used to reconstitute lines (especially in small aquarium fishes), it would require improvement and scaling up before being useful as a production method for large-bodied fishes such as catfish. Recently, vitrification has been used in fish studies to cryopreserve blastomeres [7], testicular cells (i.e. spermatogonial stem cells) [4], and primordial germ cells by whole- embryo freezing [23,24]. In addition, live fish have been produced by transplanting primordial germ cells recovered from vitrified embryos [22]. This illustrates the importance and potential applications of vitrification as a feasible cryopreservation method. Vitrification is a simple, fast, and inexpensive method for preserving genetic resources that does not require equipment, and can be performed in the field. Because of the minute volumes needed to attain ultra-rapid cooling, vitrification is best suited for small fish or fishes that yield only small volumes of sperm.

Future work in sperm vitrification should focus on small (aquarium) fish, some of which (e.g. zebrafish) are extremely important and widely used biomedical models, and many of which are highly endangered and are typically overlooked in conservation programs. According to the International Union for Conservation of Nature and Natural Resources (IUCN) Red List, 65% of the ray-finned fish (Class Actinopterygii) listed as imperiled are less than 20 cm in length. Sperm vitrification could offer a new option for conservation biology in imperiled aquatic species. Samples can be preserved in remote locations such as on the riverbank or a boat, in a remote fish hatchery, or in developing countries, without the need for sophisticated procedures or equipment. In addition, vitrification could be a useful technique to preserve the genetic resources from aquatic model organisms. Laboratories around the world have produced tens of thousands of mutant, transgenic, and wild-type fish lines. Maintaining these valuable genotypes as live populations is expensive, risky, and beyond the capacity of even the largest stock centers [64]. Currently more than 20,000 lines of zebrafish require preservation in germplasm repositories (http://zfin.org) [98]. Vitrification could assist this process, and offers opportunities for gene banking of other materials such as oocytes, embryos, larvae, and stem cells not possible with conventional cryopreservation. This work provides a model for development of generalized protocols for aquatic species sperm and that could be integrated into a standardized approach for vitrification of aquatic species germplasm.

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