

Cryopreservation of Spermatozoa from Freeze-Tolerant and -Intolerant Anurans

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Spermatozoa of the freeze-tolerant wood frog (*Rana sylvatica*) were used to develop a general protocol for the frozen storage of amphibian spermatozoa. Tolerance of spermatozoa to cryoprotective agents and freezing in suspension (-80°C) was determined from rates of sperm lysis and dual-fluorochrome vital dye assays. We tested the efficacy of four cryoprotectants (Me_2SO , methanol, glycerol, and ethylene glycol), two supplements (fetal bovine serum or glutathione), and combinations of these cryoprotectants and supplements. Me_2SO and fetal bovine serum were the most effective cryoprotectant and supplement, respectively, in reducing sperm lysis. Vital dye assays showed that viability was greatest for spermatozoa treated with both Me_2SO and fetal bovine serum. Thus, this combination was used to cryopreserve spermatozoa from the freeze-intolerant anurans, *Rana pipiens* and *Bufo americanus*. Recovery of viable spermatozoa was significantly greater for *R. sylvatica* (mean \pm SE = $81.2 \pm 9.6\%$) than for *R. pipiens* ($59.0 \pm 2.8\%$) and *B. americanus* ($47.8 \pm 4.1\%$), perhaps owing to inherent factors promoting its freeze tolerance. Nonetheless, our results support the feasibility of using gamete cryopreservation techniques in programs aimed at the captive propagation of amphibians. © 1998 Academic Press

Key Words: amphibian; spermatozoa; cryopreservation; vital dye assay; freeze tolerance; *Rana sylvatica*.

Environmental pollutants and habitat destruction threaten the existence of amphibians, whose life history and physiology render them particularly vulnerable to such perturbations. Considering the growing concern for global decline in amphibian populations (3, 17, 19, 26), we proposed that gamete cryopreservation may ultimately provide a means for countering further endangerment of these animals (16). Although not a permanent solution, the banking of frozen spermatozoa may afford temporary protection against extinction and provide a source of genetically diverse material for *ex situ* propagation and reintroduction efforts (11). Amphibians may be particularly well suited to such an approach (20). Indeed, cryopreservation of spermatozoa is widely used to propagate species for commercial purposes (4, 13, 18) and supply gene pools for managing endangered animals (8, 9). However, very little is known about the freezing viability of amphibian spermatozoa (2, 21–23).

Our initial investigation focused on *Rana sylvatica*, a naturally freeze-tolerant frog that may experience repeated freeze/thaw episodes while overwintering in its shallow, terrestrial hibernaculum. Some of the biophysical and physiological responses promoting freeze tolerance in *R. sylvatica* have been described (6). Glycogenolysis in the liver, which is triggered when freezing begins, provides an ample supply of glucose, the cryoprotectant of its cells and tissues. We recently found that spermatozoa of *R. sylvatica* survive *in vitro* freezing at ecologically-relevant temperatures (i.e., $\geq -4^{\circ}\text{C}$), even in the absence of cryoprotectant (7). This capacity is adaptive because *R. sylvatica* breeds in late winter, when cryoprotectant production capacity is low and freezing may occur (5).

Subsequent work explored the tolerance of *R. sylvatica* spermatozoa, in the presence of cryoprotectant, to very low temperatures (16). Spermatozoa were harvested from the testes of breeding frogs and frozen at -80°C in suspensions containing Me_2SO , glycerol, or glucose. Tolerance of freezing was assessed from the decrease in sperm count, which is indicative of sperm lysis, and by vital dye assays, which

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evaluated the integrity of membranes of the surviving cells. Freezing viability was markedly improved by 1.5 and 3.0 M Me_2SO and glycerol (but not 2.0 M glucose), although these solutions were mildly cytotoxic (16).

One purpose of the current investigation was to further refine the protocol for cryopreserving *R. sylvatica* spermatozoa. To limit cytotoxicity, we used a relatively low concentration (0.5 M) of the cryoprotectants Me_2SO , glycerol, methanol, and ethylene glycol, which were chosen based upon their efficacy in cryopreserving piscine spermatozoa (4, 18). We also tested fetal bovine serum (FBS) and glutathione, supplements that promote freezing viability of spermatozoa (2, 15, 24). Finally, we applied our most effective cryopreservation treatment to spermatozoa of freeze-intolerant anurans as an initial step in assessing the feasibility of implementing this approach with diverse amphibian taxa.

MATERIALS AND METHODS

Specimens

Male *R. sylvatica* were collected from a breeding pond in southern Ohio in February 1997 and 1998. Frogs were vocalizing at the time of their capture; hence, their spermatozoa were likely at the peak of fertility. Frogs were chilled and transported to laboratory facilities where they were refrigerated (4°C) and kept on damp moss, unfed, within darkened plastic boxes. Male leopard frogs (*Rana pipiens*) were obtained commercially in May 1997 and March 1998, fasted, and kept chilled in boxes containing a pool of dechlorinated water at one end. Male American toads (*Bufo americanus*) were collected from a breeding pond in southwestern Ohio during May 1997 and April 1998 and kept chilled on damp moss. All specimens were used in experiments within 4 weeks.

Collection of Spermatozoa

Animals were euthanized by double pithing and dissected to expose the coelomic organs. Testes were removed, weighed to the nearest 0.1 mg, and macerated with Teflon-coated for-

ceps in isotonic suspension buffer (SB, in mM: 104.4 NaCl, 2.0 KCl, 6.1 Na_2HPO_4 , 1.0 KH_2PO_4 ; 230 mosmol/kg, pH 7.4). The resulting homogenate, standardized to 15 mg tissue/mL SB, was centrifuged (7g, 3 min) to sediment the tissue fragments. The exceptionally low centrifugal force was produced by elevating the sample tube within the rotor bucket, thus reducing the radius to the spindle. The supernatant containing spermatozoa was decanted, placed in microcentrifuge tubes, and held on ice until used.

Determination of Sperm Lysis and Viability

Sperm counts were made in duplicate using a Levy hemocytometer (improved Neubauer, 0.10 mm depth) according to standard procedures (14). Following gentle reflux pipetting, 20 μL of sperm suspension was observed under Nomarski differential contrast illumination (400 \times) on an Olympus BH-2 microscope. Sperm lysis caused by freezing/thawing was determined from the difference in cell counts of corresponding nonfrozen and frozen/thawed samples.

Membrane integrity of intact spermatozoa was assessed for nonfrozen and frozen/thawed samples using our modification of a dual-fluorochrome vital dye procedure (Molecular Probes, Inc., Eugene, OR, U.S.A.), which distinguishes intact from damaged plasma membranes (10). A 100- μL sample of sperm suspension was dyed by adding 1 μL of SYBR 14 solution (20 μM) and incubated at $\sim 20^\circ\text{C}$ for 15–20 min, after which 2 μL of propidium iodide solution (1.2 mM) was added and the suspension was again incubated for 15–20 min. When viewed using fluorescence microscopy with a 490-nm excitation wavelength, SYBR 14 stained viable cells green, whereas propidium iodide stained damaged spermatozoa red. Prepared samples were kept in the dark to prevent premature excitation. Sperm viability (i.e., the proportion of “viable” spermatozoa in the sample) was determined for ~ 300 cells in randomly selected fields.

Efficacy of Cryoprotectants and Supplements in Reducing Sperm Lysis

Spermatozoa harvested from *R. sylvatica* ($n = 9$) were suspended in SB containing 0.5 M cryoprotectant (Me_2SO , methanol, glycerol, or ethylene glycol), a supplement (FBS or glutathione), or combinations of these cryoprotectants and supplements. The final concentration of FBS (312 mosmol/kg, 3.0–4.5 % protein; Sigma) in suspensions was 50% (v/v). The final concentration of glutathione was 163 mM. SB was used as a diluent in all samples.

Sperm suspensions were apportioned into 50- μL aliquots held in 0.5-mL polypropylene microcentrifuge tubes and centrifuged (735g, 6 min) to gently pellet the cells. The cell-free supernatant was decanted and 25 μL of cryoprotectant and/or supplement solution was mixed with the spermatozoa, on ice, using reflux pipetting. After being incubated on ice for ~ 15 min, sperm suspensions were frozen to an equilibrium temperature of -80°C for 1 h by placing them directly in an ultracold freezer. Cooling rate of the samples, as determined in preliminary tests using a thermocouple thermometer, was $\sim 130^\circ\text{C}/\text{min}$. Frozen samples were rapidly thawed in warm water to the slush point and then held on ice until used (undiluted) ~ 45 min later. Nonfrozen sperm suspensions were incubated on ice until they were assayed.

Viability of Cryopreserved Spermatozoa

The purpose of this experiment was to further investigate the cryoprotective efficacy of Me_2SO and methanol, which were particularly effective in the initial experiment. Spermatozoa harvested from *R. sylvatica* ($n = 5$) were prepared in SB containing 0.5 M Me_2SO or methanol, or in SB containing cryoprotectant and either FBS or glutathione, and frozen and thawed as described above. Nonfrozen sperm suspensions (SB) held on ice served as controls. For each sample, we determined sperm count and viability. The count of viable sperm in samples was calculated as the product of sperm count and the corresponding viability rate. Recovery of viable spermatozoa after freezing was

determined as the ratio of the viable count in the frozen/thawed sample to that in the corresponding nonfrozen suspension.

Cryopreservation of Spermatozoa from Freeze-Intolerant Anurans

The cryopreservation treatment judged most successful with spermatozoa of *R. sylvatica*, $\text{Me}_2\text{SO}/\text{FBS}$, was used to cryopreserve testicular spermatozoa from *R. pipiens* ($n = 5$) and *B. americanus* ($n = 6$). Sperm lysis, sperm viability, viable sperm count, and recovery rate were determined for frozen/thawed suspensions of spermatozoa from these species and compared to values for *R. sylvatica*.

Statistical Analyses

The effects of cryoprotectants and supplements on the tolerance of spermatozoa to freezing/thawing were analyzed using two-factor analyses of variance (ANOVA; Tukey multiple comparisons). Elsewhere, mean values were compared using one-factor ANOVA. Values are shown as the means \pm SE.

RESULTS

Efficacy of Cryoprotectants and Supplements in Reducing Sperm Lysis

The number of intact spermatozoa remaining in frozen/thawed suspensions was strongly influenced by both cryoprotectant ($F_{3,87} = 12.1$, $P < 0.0001$) and supplement ($F_{2,86} = 30.6$, $P < 0.0001$; Fig. 1). Generally, Me_2SO provided better protection against sperm lysis than the other agents, whereas methanol, glycerol, and ethylene glycol produced similar results. When used in conjunction with each cryoprotectant, FBS yielded higher cell survival than either glutathione or no supplement (Fig. 1).

Efficacy of the two supplements, FBS and glutathione, in reducing sperm lysis was examined by comparing relative sperm counts in frozen/thawed suspensions containing supplement alone (Table 1). Samples containing FBS lost significantly fewer spermatozoa during freezing/thawing than samples containing glutathione. Based on the results for SB, glutathi-

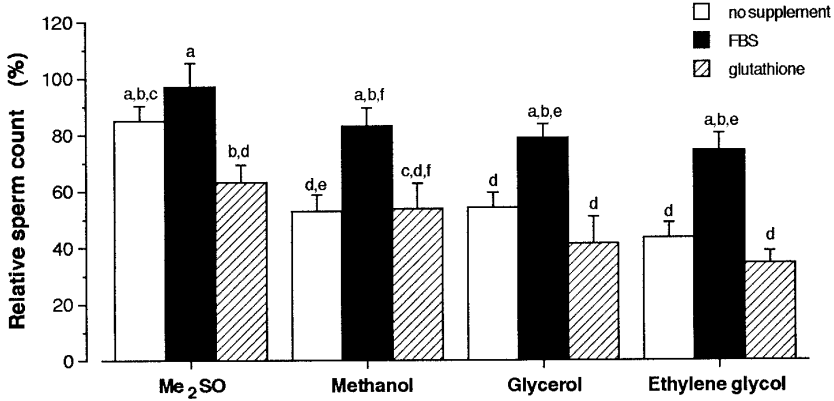


FIG. 1. Relative counts of *R. sylvatica* spermatozoa frozen/thawed in the presence of permeating cryoprotectant (Me₂SO, methanol, glycerol, or ethylene glycol), with or without supplements (FBS or glutathione), expressed as a percentage of the count in the corresponding nonfrozen suspension ($0.78 \pm 0.14 \times 10^4$ cells/ μ L SB). Mean values representing data from $n = 6-9$ frogs/group are depicted by the height of each column; SE values are shown by the vertical bars.

one provided no protection against sperm lysis (Table 1); however, this supplement was used in the subsequent experiment because of its potential value in protecting cells against membrane damage.

Efficacy of Cryoprotectants and Supplements in Promoting Sperm Viability

Nonfrozen suspensions of spermatozoa in SB contained $0.77 \pm 0.14 \times 10^4$ intact cells/ μ L, of which $64.7 \pm 2.8\%$ were deemed viable using vital dye fluoroscopy. Thus, these control samples contained $0.50 \pm 0.08 \times 10^4$ viable cells/ μ L.

Sperm lysis in samples from *R. sylvatica* did not vary as a function of cryoprotectant ($F_{1,25} = 2.3$, $P = 0.14$) or supplement ($F_{2,26} = 2.4$, $P = 0.11$; Fig. 2A). However, both factors strongly affected sperm viability (cryoprotectants, $F_{1,25} = 17.2$, $P = 0.0004$; supplements, $F_{2,26} = 31.2$, $P < 0.0001$). Fluoroscopy revealed that FBS was particularly effective in reducing membrane injury to frozen/thawed spermatozoa (Fig. 2B). For example, viability of cells frozen in suspensions containing Me₂SO or methanol with FBS was 42 and 35%, respectively, only moderately lower than that of nonfrozen samples. Cryoprotectant and supplement also

TABLE 1

Counts of *R. sylvatica* Spermatozoa ($\times 10^4/\mu$ L) in Nonfrozen and Frozen/Thawed Suspension Buffer (SB) or SB Containing Fetal Bovine Serum (FBS) or Glutathione

Treatment	Nonfrozen	Frozen/thawed	Recovery ^a
SB	1.27 ± 0.20^a	0.02 ± 0.006^a	1.3 ± 0.3^a
SB + FBS	1.40 ± 0.21^a	0.74 ± 0.11^b	54.1 ± 4.3^b
SB + glutathione	0.93 ± 0.18^a	0.05 ± 0.02^a	6.4 ± 2.0^a
$F_{2,23}$	1.2	30.3	105.8
P	0.33	<0.0001	<0.0001

Note. Within each column, mean (\pm SE) values identified by different roman, superscripted letters differed significantly. Means are based on $n = 6-9$ frogs/group.

^a Cell count in frozen/thawed suspension expressed as a percentage of the count in the corresponding nonfrozen suspension.

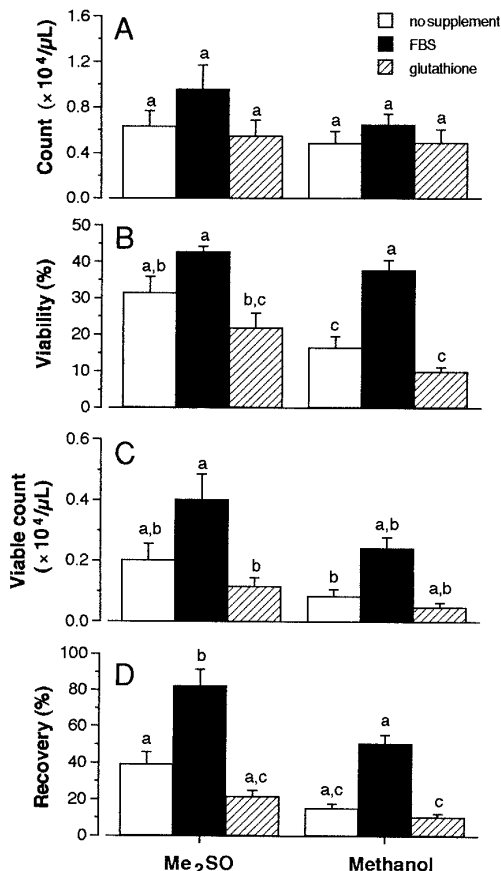


FIG. 2. Cryopreservation of *R. sylvatica* spermatozoa in frozen/thawed suspensions containing Me₂SO or methanol, with or without a supplements (FBS, glutathione), showing: (A) counts of intact spermatozoa; (B) sperm viability (percentage of cells exhibiting functional membranes in vital dye assay); (C) viable sperm count (sperm count \times viability rate); and (D) recovery (viable sperm count in frozen/thawed suspensions expressed as a percentage of the viable sperm count in the corresponding nonfrozen suspension, $0.50 \pm 0.08 \times 10^4$ cells/ μL SB). Mean values representing data from $n = 5$ frogs/group are depicted by the height of each column; SE values are shown by the vertical bars.

strongly influenced the viable sperm count in frozen/thawed suspensions (cryoprotectants, $F_{1,25} = 9.3$, $P = 0.006$; supplements, $F_{2,26} = 14.4$, $P < 0.0001$; Fig. 2C). Samples treated with Me₂SO and FBS contained more viable cells than samples containing methanol alone or methanol combined with glutathione, although similar results were obtained with methanol

combined with FBS. Recovery of viable spermatozoa after freezing/thawing was strongly influenced by both cryoprotectant and supplement (cryoprotectants, $F_{1,25} = 24.3$, $P < 0.0001$; supplements, $F_{2,26} = 45.6$, $P < 0.0001$), being twofold higher in suspensions containing Me₂SO/FBS than in methanol/FBS samples (Fig. 2D). Thus, the Me₂SO/FBS cocktail was selected for use in the subsequent experiment.

Cryopreservation of Spermatozoa from Freeze-Intolerant Anurans

We detected no differences among the three species in the sperm counts of either nonfrozen or frozen/thawed suspensions (Table 2). Sperm viability was moderately higher in samples from *R. pipiens* than in samples from *R. sylvatica* and *B. americanus*, although counts of viable sperm were statistically indistinguishable among species. Nevertheless, recovery of viable spermatozoa in frozen/thawed suspensions was 1.4–1.7 times greater for *R. sylvatica* than for the freeze-intolerant species (Table 2).

DISCUSSION

In the present study, we used the level of sperm lysis in frozen/thawed suspensions as a coarse indicator of the cryoprotective efficacy of Me₂SO, methanol, glycerol, and ethylene glycol. Sperm lysis was especially low in suspensions containing Me₂SO (15%) and moderate (44–54%) in samples treated with equimolar concentrations of the other agents. These results concur with previous findings that cryoprotection of anuran spermatozoa is conferred by Me₂SO (2), methanol (21), glycerol (22, 23), and ethylene glycol (2). However, our results differed from those of Barton and Guttman (1972), who obtained higher survival of toad (*Bufo*) spermatozoa frozen in ethylene glycol compared to those frozen in Me₂SO or glycerol.

Marked differences were observed in the ability of the two supplements, used in conjunction with permeating cryoprotectant, to inhibit sperm lysis. FBS generally enhanced the efficacy of each cryoprotectant, whereas results obtained for suspensions containing glutathione were similar to those for samples without sup-

TABLE 2

Counts of Intact Spermatozoa, Sperm Viability (Percentage of Intact Sperm Exhibiting Functional Cell Membranes in Vital Dye Assay), and Viable Sperm Count in Nonfrozen and Frozen/Thawed Suspensions Containing Spermatozoa from Three Species of Anurans

	<i>R. sylvatica</i> (n = 5)	<i>R. pipiens</i> (n = 5)	<i>B. americanus</i> (n = 6)	$F_{2,15}$	P
Sperm count ($\times 10^4/\mu\text{L}$)					
Nonfrozen	0.78 \pm 0.14 ^a	1.20 \pm 0.24 ^a	1.46 \pm 0.31 ^a	1.9	0.19
Frozen/thawed	0.96 \pm 0.21 ^a	1.17 \pm 0.22 ^a	1.62 \pm 0.33 ^a	1.6	0.23
Sperm viability (%)					
Nonfrozen	64.7 \pm 2.8 ^a	90.8 \pm 2.0 ^b	77.6 \pm 4.6 ^a	12.2	0.001
Frozen/thawed	42.7 \pm 1.6 ^{a,b}	54.6 \pm 3.0 ^a	33.7 \pm 4.3 ^b	9.8	0.003
Viable sperm count ($\times 10^4/\mu\text{L}$) ^a					
Nonfrozen	0.50 \pm 0.08 ^a	1.09 \pm 0.20 ^a	1.19 \pm 0.30 ^a	2.5	0.12
Frozen/thawed	0.40 \pm 0.08 ^a	0.65 \pm 0.12 ^a	0.59 \pm 0.20 ^a	0.7	0.52
Recovery (%) ^b	81.2 \pm 9.6 ^a	59.0 \pm 2.8 ^b	47.8 \pm 4.1 ^b	8.1	0.005

Note. Within each row, mean (\pm SE) values identified by different roman, superscripted letters differed significantly.

^a Sperm count \times viability.

^b Viable sperm count in frozen/thawed suspension as a percentage of the viable sperm count in the corresponding nonfrozen suspension.

plement (Fig. 1). Further study revealed that, unlike glutathione, FBS effectively reduced sperm lysis in frozen/thawed suspensions, even in the absence of permeating cryoprotectant (Table 1).

Vital dye assays provided a more refined assessment of the viability of *R. sylvatica* spermatozoa cryopreserved with Me₂SO or methanol alone or these agents combined with either FBS or glutathione. Mean viability of spermatozoa in nonfrozen suspensions (SB) was ~65%, slightly lower than that (72%) determined previously for *R. sylvatica* using an identical method (16). Viability of spermatozoa was reduced in all frozen/thawed suspensions, but to a lesser extent in samples treated with Me₂SO (Fig. 2B), perhaps owing to this agent's beneficial effect on the plasma membrane (1). Recovery of viable sperm in samples treated with 0.5 M Me₂SO in the present study was ~40%, substantially higher than that (13–18%) obtained with 1.5 or 3.0 M Me₂SO (16), suggesting that use of the lower concentration alleviated some of this agent's cytotoxicity.

Viability of spermatozoa in frozen/thawed samples was markedly enhanced by the addition

of FBS, which may protect cells against membrane damage by virtue of its viscous character or the specific action of its constituents. In particular, proteins and lipids may be important in this regard (12, 27). When used in combination, Me₂SO and FBS provided superior protection against cryoinjury to *R. sylvatica* spermatozoa than other treatments, as 81% of the viable cell population was recovered in frozen/thawed samples (Fig. 2D).

Antioxidants, such as butylated hydroxytoluene (BHT), may effectively moderate cryoinjury to spermatozoa by influencing the thermal phase of membrane lipids (27). Accordingly, glutathione used in conjunction with cryoprotectant improved fertility rates of cryopreserved fish spermatozoa (15). However, our results suggest that glutathione failed to protect *R. sylvatica* spermatozoa from membrane damage during cryopreservation (Fig. 2). This conclusion should remain tentative until alternative concentrations may be tested.

Use of the cryopreservation cocktail yielding the best results with *R. sylvatica* spermatozoa (Me₂SO + FBS) was successful with spermatozoa from two freeze-intolerant anurans (Table

2). However, recovery of viable spermatozoa in frozen/thawed samples from these species (48–59%) was lower than that (81%) for *R. sylvatica*. A similar result was obtained when the viability of spermatozoa frozen to relatively high (ecologically relevant) temperatures was compared between *R. sylvatica* and *R. pipiens* (7). This pattern may reflect innate cellular adaptations to tissue freezing in *R. sylvatica*, which apparently is a normal occurrence during overwintering. Such differences may be manifested through variation in membrane structure or physiology and its influence on cell permeability to cryoprotective agents. Refinement of a generalized cryopreservation protocol for amphibians will require comparative study of fundamental permeability characteristics of spermatozoa plasma membranes. Such work should involve both temperate and tropical species.

Our success in using a dual-fluorochrome vital dye procedure with spermatozoa from three species of anurans suggests that this technique may be generally useful for gauging viability of cryopreserved amphibian spermatozoa. Determining the functional integrity of spermatozoa using vital dye assays may be preferential to other assessments, such as motility rate (10, 25). Fertility trials are needed to determine whether cryopreserved spermatozoa judged viable using this method are indeed functional. However, our preliminary work showed that *R. sylvatica* spermatozoa, frozen (-80°C , 1 h) in SB containing 0.5 M glycerol, were activated to motility by subsequent hypotonic exposure and ultimately fertilized ova from two females, which then developed normally.

In the present study, spermatozoa were frozen briefly to expedite the collection of data. Additional research is needed to determine whether cryopreserved frog spermatozoa can tolerate longer-term storage, although our initial study showed no difference in viability for spermatozoa frozen 1–30 h or 58 weeks (16). Future work should endeavor to improve technical aspects of this cryopreservation protocol, particularly by determining optimal incubation time, cooling/warming rates, and methods for post-thaw activation.

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