

EVALUATION OF GLYCEROL AND DIMETHYL SULFOXIDE FOR THE CRYOPRESERVATION OF SPERMATOOZOA FROM THE WOOD FROG (*RANA SYLVATICA*)

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Summary

Conventional cryopreservation techniques were applied to spermatozoa from the freeze-tolerant wood frog as an initial step toward developing protocols for the frozen storage of amphibian gametes. In chilled, but unfrozen, samples, a high concentration (3.0 M) of glycerol or dimethyl sulfoxide (Me₂SO) in the suspension medium was not harmful to spermatozoa. However, 2.0 M glucose promoted loss of viability as assessed by fluorescence vital dyes assays. Control suspensions prepared in isotonic saline contained 1.86×10^4 spermatozoa/ μ L, of which $71.6 \pm 4.1\%$ were viable. No cells survived freezing at -80°C without cryoprotective additives, whereas samples containing 1.5 or 3.0 M glycerol or Me₂SO yielded $>1.2 \times 10^4$ intact spermatozoa/ μ L upon thawing.

Key Words: amphibian, spermatozoa, cryopreservation, *Rana sylvatica*

Introduction

The life history and physiology of amphibians render them highly sensitive to environmental contaminants and the loss of critical habitats. A growing literature has documented increasing concern about declines of amphibian populations on a global scale (2, 8, 10). Captive breeding and propagation programs that rely on artificial fertilization, and in some cases cryopreserved gametes, have proved successful with some animal species of special concern (6). Whereas a few investigators have reported the effects of freezing on amphibian spermatozoa (1, 11, 12), no work has produced an effective protocol for cryopreserving these cells. Although not a permanent solution, the banking of frozen gametes may provide temporary protection against extinction and provide a source of genetically diverse germ material for *ex situ* propagation and reintroduction efforts.

We chose the freeze-tolerant wood frog (*Rana sylvatica*) as a model in these experiments, as our previous study showed that *R. sylvatica* spermatozoa readily survive freezing at high, ecologically-relevant temperatures ($\geq -4^\circ\text{C}$), even with little or no cryoprotectant (J. Costanzo, J. Mugnano, H. Wehrheim, R. Lee, unpublished data). For our initial assessment of the feasibility of cryopreserving frog spermatozoa, we adapted techniques that were developed for use with piscine spermatozoa (3, 9) because the reproductive biology of frogs is generally comparable to that of freshwater fishes. Empirically derived protocols for sperm cryopreservation have been refined and are easily practiced by fisheries biologists in the field without specialized equipment. The goal of the work reported here was to make an initial assessment of the feasibility of adapting such protocols for use with amphibian spermatozoa.

Materials and Methods

Wood frogs were collected in southern Ohio during the peak of their breeding period, in late winter. They were kept unfed on damp moss in darkened plastic boxes at 4°C and used within a few weeks. Testes were dissected from euthanized frogs, weighed to within ± 0.1 mg, and macerated in phosphate-free suspension buffer (SB, in g/L: 6.5 NaCl, 0.25 KCl, 0.15 CaCl₂, 0.1 NaHCO₃, 0.54 D-glucose; 220 mosmol/kg, pH 7.4) using Teflon-coated forceps. The tissue density of the homogenate was standardized to 15 mg/mL SB. This crude homogenate was centrifuged (7 g for 3 min) and the supernatant containing spermatozoa was held briefly on ice in 0.5-mL polyethylene centrifuge tubes until used.

Tolerance of spermatozoa to experimental treatment was inferred from the degree of spermolysis (decrease in count of visibly intact cells) and membrane integrity of intact cells, as determined using vital dye assays. Sperm counts were made in duplicate using a hemocytometer viewed under Nomarski differential interference contrast illumination (400 \times) on an Olympus BH-2 microscope. Membrane integrity was assessed using our modification of a dual-fluorochrome procedure (FertiLight, Molecular Probes, Inc., Eugene, OR). A 100- μ L aliquant of sperm suspension was dyed with 1 μ L of a solution of SYBR 14 (20 μ M) and incubated at $\sim 20^\circ\text{C}$ for 15-20 min, after which 2 μ L of propidium iodide solution (1.2 mM) was added and the suspension incubated an additional 15-20 min. Using fluorescence microscopy with a 490 nm excitation wavelength, spermatozoa with intact membranes fluoresced green, while spermatozoa with damaged membranes fluoresced red. The proportion of viable cells in a 10- μ L aliquant of dyed suspension was determined for ~ 200 cells observed in randomly-selected fields.

Survival of spermatozoa in chilled cryoprotectant solutions

To determine whether cryoprotectants were toxic, sperm suspensions prepared in SB from each of 5 animals were divided into aliquants of a predetermined volume to which stock reagents of glucose, glycerol, or Me₂SO were added to achieve a final volume of 250 μ L and concentrations of 2.0 M, 3.0 M, and 3.0 M, respectively. Cryoprotectants (CPAs) were added gradually into chilled samples which were kept on ice for ~ 20 min before sperm counts were made. SB was added to a predetermined volume of the sperm suspensions to produce a 250 μ L sample with a sperm density similar to that in samples treated with CPAs.

Effect of freezing on sperm survival

Survival of spermatozoa in 1.5 and 3.0 M glycerol or Me₂SO solutions was assessed by comparing counts of intact cells, and the proportions of cells judged to be viable in vital dye assays, with unfrozen (chilled) controls. Suspensions of spermatozoa from each of 5 animals were prepared in SB and divided into aliquants of a predetermined volume to which was added SB (control), glycerol, or Me₂SO to produce a final volume of 250 μ L at concentrations of 1.5 M or 3.0 M. Stock cryoprotectants were introduced dropwise to chilled suspensions which were gently mixed and held on ice. Each 250- μ L sample was divided into two 125- μ L aliquants. One aliquant treated with each CPA, as well as a control aliquant (SB), was reserved on ice ~ 20 min and then counted to establish pre-freeze cell density. The remaining samples were cooled at $\sim 130^\circ\text{C}/\text{min}$ by immersion in an ethanol/dry ice slurry and then stored frozen at -80°C . After a brief period of storage (1-30 h), samples were rapidly warmed in a water bath (30°C) to the slush point and then used (undiluted) to determine sperm count and viability.

An additional test was conducted to determine the effects of long-term storage. After being frozen in the ethanol/dry ice slurry, samples were transferred to a freezer (-80°C) where they were stored for 58 weeks. Samples were then thawed and assayed as described above.

In vitro freezing of intact testes

To assess the feasibility of cryopreserving spermatozoa *in situ*, intact testes were frozen in cryoprotectant solutions. One testis (~ 15 mg) from each of 4 animals was incubated 40 min in 100 μ L of ice-cold SB containing either 3.0 M glycerol or 3.0 M Me₂SO, and then frozen (as

above) at -80°C for 5 d. The remaining testis of each pair served as a control and was immediately used to prepare a sperm suspension in SB, which was then assayed for cell count and viability. After being rapidly warmed to near 0°C , the cryopreserved testes were used to prepare sperm suspensions in SB (15 mg/mL) and similarly assayed.

Results and Discussion

Survival of spermatozoa in chilled cryoprotectants

Densities of spermatozoa in SB ranged from $1.4\text{--}2.2 \times 10^4$ cells/ μL (mean \pm SE = $1.7 \pm 0.1 \times 10^4$ cells/ μL , $n=5$). Glucose was included in these trials because it is mobilized from hepatic glycogen reserves and serves as a cryoprotective agent in *R. sylvatica*. In spring, glucose reaches a concentration of up to 0.1 M in the blood of frogs from our population (4, 5). However, the count of intact sperm in samples treated with 2.0 M glucose were significantly (ANOVA, Bonferroni multiple comparisons: $F = 6.1$, $P = 0.004$) lower than that in SB. Given that glucose caused extensive spermolysis ($47.4 \pm 4.1\%$), this agent was omitted from most subsequent experiments. Counts of spermatozoa incubated in 3.0 M glycerol and Me_2SO were not significantly different from the SB controls.

Sperm counts in unfrozen SB samples contained $1.9 \pm 0.2 \times 10^4$ spermatozoa/ μL (mean \pm SE, $n = 5$), of which $71.6 \pm 4.1\%$ were scored "viable" using vital dye assays. Although neither glycerol nor Me_2SO induced significant spermolysis in unfrozen samples, vital dye assays suggested that these agents were mildly cytotoxic. Viability rates of cells ranged from 50% in samples containing 3.0 M glycerol to $\sim 60\%$ in 1.5 M and 3.0 M Me_2SO samples (Table 1); however, only the former differed statistically (ANOVA, Bonferroni multiple contrasts: $F = 3.13$, $P = 0.037$) from the SB control. Despite this trend, the addition of cryoprotectant did not significantly (ANOVA: $F = 2.56$, $P = 0.07$) reduce the number of viable spermatozoa relative to the SB samples.

Given that amphibian spermatozoa are activated to motility by exposure to a hypotonic environment, it was not unexpected that spermatozoa suspended in cryoprotectant (hypertonic) solutions remained immotile. This inactivity, coupled with the inhibitory effect of cold, may fortuitously promote the conservation of metabolic reserves during frozen storage of spermatozoa.

Effect of freezing/thawing on sperm survival

Spermatozoa did not tolerate freezing without cryoprotective additives, as virtually no intact cells were observed in frozen/thawed SB samples. In samples containing glycerol or Me_2SO , sperm counts were $>1.2 \times 10^4$ cells/ μL and generally unaffected by freezing/thawing (Table 1), although viability of these cells was markedly reduced. Generally, frozen/thawed samples contained 66–87% fewer viable spermatozoa relative to their unfrozen counterparts (Table 1), and 9–16% of the number in corresponding unfrozen SB samples, $1.4 \pm 0.2 \times 10^4$ viable cells/ μL . Cryospermolysis and additional cell injury may have been manifested had the spermatozoa been subsequently exposed to a hypotonic medium.

Concentrations of cryoprotectants used in this experiment were selected on the basis of previous work with amphibian (1, 11, 12) and fish (9) spermatozoa. Spermatozoa from *R. sylvatica* incubated in 1.5 M Me_2SO or glycerol tolerated freezing as well as spermatozoa treated with 3.0 M concentrations of these CPAs (Table 1). Perhaps concentrations <1.5 M may effectively cryopreserve cells while also alleviating the mildly cytotoxic effects of cryoprotectants.

Effect of long-term storage on sperm viability

Limited data suggest that *R. sylvatica* spermatozoa may tolerate extended periods of frozen storage. Suspensions prepared in 2.0 M glucose, 3.0 M glycerol, or 3.0 M Me_2SO from each of 4 animals had intact sperm counts that were statistically unchanged (paired *t*-tests:

Table 1. Intact sperm counts, viability rates, and counts of viable sperm from *R. sylvatica*, treated with 1.5 or 3.0 M concentrations of cryoprotectant, in chilled (unfrozen) samples and samples stored frozen at -80°C (frozen/thawed). Within each row, mean values (± 1 SE; $n = 5$ animals per group) identified by dissimilar superscripted letters differed significantly from control values (ANOVA, Fisher's PLSD).

	1.5 M		3.0 M		<i>P</i>
	Unfrozen	Frozen/thawed	Unfrozen	Frozen/thawed	
<i>Glycerol</i>					
Intact sperm count ($\times 10^4$ cells/ μL)	1.47 ± 0.25^a	1.29 ± 0.13^a	1.22 ± 0.19^a	1.28 ± 0.13^a	0.83
Viability (%)	54.8 ± 4.6^a	13.4 ± 4.3^b	50.0 ± 6.3^a	17.4 ± 4.1^b	<0.0001
Viable sperm count ($\times 10^4$ cells/ μL)*	0.84 ± 0.19^a	0.16 ± 0.04^b	0.64 ± 0.18^a	0.22 ± 0.56^b	0.006
<i>Me₂SO</i>					
Intact sperm count ($\times 10^4$ cells/ μL)	1.56 ± 0.20^a	1.42 ± 0.20^a	1.49 ± 0.18^a	1.33 ± 0.16^a	0.85
Viability (%)	59.8 ± 2.5^a	10.1 ± 2.7^b	59.8 ± 4.3^a	13.3 ± 5.3^b	<0.0001
Viable sperm count ($\times 10^4$ cells/ μL)*	0.92 ± 0.11^a	0.12 ± 0.02^b	0.88 ± 0.11^a	0.16 ± 0.05^b	<0.0001

*percent viability \times intact sperm count

Table 2. Counts and viability rates of spermatozoa from the freeze-tolerant wood frog frozen within excised, intact testes after pretreatment with 3.0 M glycerol or Me₂SO. Mean values (± 1 SE; $n = 4$ animals per group) were compared using Student's *t*-tests.

	Glycerol	Me ₂ SO	<i>P</i>
Intact sperm count ($\times 10^4$ cells/ μ L)	1.65 \pm 0.06	2.05 \pm 0.07	0.017
Viability (%)	5.0 \pm 2.9	13.8 \pm 3.8	0.119
Viable sperm count ($\times 10^4$ cells/ μ L)*	0.08 \pm 0.05	0.26 \pm 0.07	0.094

*percent viability \times intact sperm count

glucose, $t = 0.83$, $P = 0.47$; glycerol, $t = 2.3$, $P = 0.10$; Me₂SO, $t = 1.6$, $P = 0.21$) after 58 weeks of storage at -80°C compared to counts for corresponding suspensions held at -80°C for 30 h or less. Furthermore, in samples treated with glycerol or Me₂SO, both viability rates ($16.7 \pm 3.6\%$ and $14.4 \pm 1.8\%$, respectively) and counts of viable spermatozoa ($0.19 \pm 0.08 \times 10^4$ cells/ μ L and $0.19 \pm 0.07 \times 10^4$ cells/ μ L, respectively) were comparable to results obtained with shorter-term freezing (Table 1). These data indicate that prolonged storage at -80°C did not cause significant mortality from potential effects such as ice recrystallization, and that frozen storage at -80°C may be feasible for periods >1 year.

In vitro freezing of whole testes

Many intact spermatozoa were recovered from frozen/thawed testes, although most of these cells exhibited damaged plasma membranes as judged by vital dye assays (Table 2). Testes pretreated with Me₂SO yielded significantly higher intact sperm counts than organs pretreated with glycerol, and generally contained more viable spermatozoa. This trend suggests that the relatively poor results with spermatozoa frozen within testes may reflect a failure of the cryoprotectant, glycerol in particular, to adequately penetrate the tissues. Rostand, who provided early evidence that glycerol protects animal cells against cryoinjury (11), reported survival of spermatozoa from whole frog testes treated with glycerol and frozen at -4°C (12).

The results of our initial experiments demonstrate the feasibility of developing a cryopreservation protocol for amphibians. Future work should investigate fundamental permeability characteristics of spermatozoa plasma membranes so that optimal cryoprotectants, incubation time, and cooling/warming rates may be determined. Additionally, fertilization trials will be needed to ascertain whether spermatozoa deemed viable using dye assays are indeed functional, and whether a protocol can be adapted for use with freeze-intolerant frogs from both temperate and tropical climates.

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