Cryopreservation of Frog (*Rana Pipiens*) Sperm Cells Collected By Non-lethal Methods

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ABSTRACT

There are few reported studies of cryopreservation of frog spermatozoa. None have used non-lethal methods for collection, which involves a simple IP injection, followed by collection of sperm from the urine. Our study compared post-thaw survival of spermatozoa frozen using three different diluents: Salamon's ram semen diluent (SSD); our modification of Salamon's diluent (MSSD), and a diluent reported to be currently the most successful for freezing frog sperm: Browne's frog diluent (BFD). Sperm cells were divided and combined with diluents in 1.5-mL microcentrifuge tubes. Tubes were suspended in an ice bath to allow slow cooling to 2 °C over 45 min. Aliquots of 100 µL were pipetted into indentations on dry ice and left to freeze for 3 min. Frozen pellets were plunged into liquid N_2. Upon thawing, pellets were removed from LN_2 and dropped into 200 µL of thawing solution at 21 °C while gently vortexing. Spermatozoa were assessed using live/dead cell stain and fluorescent microscopy. Percentages of live spermatozoa from the three treatments were 58.5%, 70.5%, and 44.1% in SSD, MSSD, and BFD, respectively. When considering the number of sperm cells lysed along with live/dead cell stain data, 40%, 55.2%, and 34.4% in SSD, MSSD, and BFD, respectively, survived post-thaw. All values were significantly different ($P < 0.001$). Superior results were observed with MSSD; use of SSD also resulted in better results than BFD. The most prevalent method for collection of frog sperm is to pith the frog and then macerate the testis. With frog populations dwindling worldwide, use of a technique that is virtually harmless such as used here in conjunction with cryopreservation of specimens could be useful for preserving genetic variation in endangered frog species.

INTRODUCTION

The purpose of this project is to test a method for cryopreservation of spermatozoa collected by non-lethal methods. We used a commercially viable method of cryopreservation for ram semen against the most current methods successful for preservation of frog spermatozoa. Salamon's ram semen diluent (Evans and Maxwell 1987) was chosen because cryobiologists have found that preserving ram semen has proven relatively difficult compared to other commercial mammalian species. We reasoned that by using a diluent that works well for fragile semen samples such as ram semen we would have a better chance of achieving success in other species. The steep decline in amphibian populations around the world has created the need for greater efforts in preserving species genetics (Stuart et al. 2004). In recent years there has been more work done in cryopreservation of a number of different amphibian species (Michael and Jones 2004; Browne et al. 2002; Sargent and Mohun 2005). However, each of these studies has involved pithing the animal followed by maceration of the testis for spermatozoa collection. For cryopreservation of endangered species spermatozoa to be a viable tool for the preservation of species genetics, we need a method that does not involve the destruction of valuable individuals in the process.

METHODS

Animals

Rana pipiens purchased from the Carolina Biological Supply were housed in 100 gal tanks tilted to create a habitat that is half aquatic and half terrestrial. Lighting schedule was maintained at 12 hours light and 12 hours dark. Frogs were fed a diet of crickets twice a week and tanks were cleaned daily.

Cryopreservation Diluents

Three cryoprotective solutions were compared in this study: Salamon's semen diluent (SSD), our modification of Salamon's diluent (MSSD), and Browne's frog diluent (BFD). SSD contains components from Table 1. MSSD includes all the components from Table 1 with the addition of 11.84 mL of glycerol and 13.16 g of sucrose. For both SSD and MSSD egg yolk is added fresh immediately before mixing with sperm and freezing. BFD is an aqueous diluent based on what is most successful today containing 20% (w/v) sucrose and 30% (v/v) dimethyl sulfoxide (DMSO) (Browne et al. 2002). All diluents formulae are twice final concentration.

METHODS CONTINUED

Tris (hydroxymethyl)aminomethane (g)	5.814
Glucose (g)	0.800
Citric acid (monohydrate) (g)	3.184
Egg yolk (mL)	24
Glycerol (mL)	9
Penicillin (IU)	100,000
Streptomycin (mg)	100
Distilled water to	100 mL

Table 1: The composition of freezing diluents for one-step dilution of semen

Sperm collection

Frogs were injected with 200 IU of hCG (Chorulon; Intravet, Millsboro, DE) into the peritoneal cavity. Urine samples were taken at 30 min, 1 hour and 2 hours following injections. All samples were kept in 1.5 mL microcentrifuge tubes in a cold room at 8 °C until final collection. Urine samples containing sperm were pooled into one sample (650 µL). Sperm counts were performed to find the concentration of the pooled sample. Counts were performed by creating a 1/10 dilution of a small sample of pooled sperm so that sperm could be counted in 2 µL drops by observation under a microscope.

Figure 1: Collection of urine by cloacal lavage

Cryopreservation

Pooled sperm sample was floated in an ice water bath while final preparations of diluents and dry ice were prepared (45 min). A block of dry ice was placed in the bottom of a styrofoam container with a flat surface face up. Small indentations were pressed into the surface of the block. Aliquots of pooled sperm (200 µL) were added to microcentrifuge tubes containing 200 µL of cryopreservation diluent. After mixing, samples remained at room temperature for 10 minutes. Then, 100 µL samples of sperm/diluent were pipetted into the indentations on the dry ice. Semen pellets were left on the block for 3 min., then removed with pre-cooled plastic forceps and plunged into liquid nitrogen. Each pellet was stored separately in a microcentrifuge tube.

Thawing and Staining

All samples thawed using Salamon's thawing solution (Table 2) in a 1:3 ratio (volume of pellets to volume of thawing solution). Pellets are removed from liquid nitrogen and plunged into microcentrifuge tubes containing thawing solution. Vortexing while thawing the pellets prevented pellets from freezing the solution when it hits the surface of the liquid.

Tris (hydroxymethyl)aminomethane (g)	0.9085
Fructose (g)	0.25
Citric acid	0.4549
Distilled water to	25 mL

Table 2: The composition of thawing solution for pellet-frozen semen

Using the commercially available living:dead fluorescent cell stain (catalog #L7011, Molecular Probes, Eugene, OR), we evaluated the survival rates of the intact cells. (This is a well-established method for assessment of post-thaw sperm viability.) Prior to cryopreservation, pooled sperm were stained and 2 µL aliquots were counted using an inverted epifluorescent microscope. The number of sperm lysed as a result of each freeze-thaw treatment was assessed by comparing cell counts before and after the freezing and thawing of samples. Our analysis of the ratios of living:dead and lysed:intact cells is based on the large-sample statistical test for estimating differences between binomial parameters of two binomial populations (Mendenhall 1979).

RESULTS

The pooled sperm sample contained about 1500 sperm per 2 µl; staining revealed 100% of the cells were viable pre-freeze. Calculations for the number of cells that remained intact post-thaw, including both live and dead, were compared to this number. The MSSD and BFD diluents provided significantly better protection ($P<0.0001$) than SSD at preventing cells from lysing during cryopreservation. There was no significant difference between MSSD and BFD ($P=0.49$) in preventing lysis (Fig 2). The average number of intact cells per 2 µl following cryopreservation was 948, 1175, 1170 for SSD, MSSD and BFD, respectively.

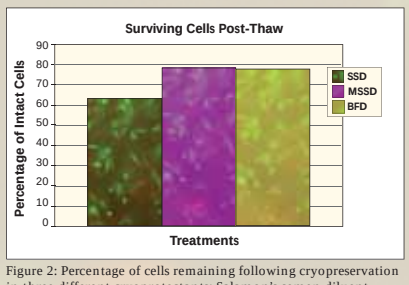

Figure 2: Percentage of cells remaining following cryopreservation in three different cryoprotectants: Salamon's semen diluent (SSD), modified Salamon's semen diluent (MSSD) and Browne's frog diluent (BFD)

Live/dead cell stains revealed that use of MSSD resulted in significantly higher numbers of live cells ($P<0.0001$) compared to either SSD or BFD. Sperm frozen in SSD resulted in significantly ($P<0.0001$) greater survival than BFD (Fig 3).

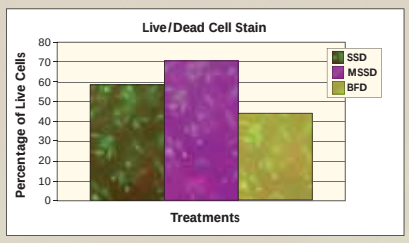

Figure 3: Percentage of cells with intact viable cell membranes following cryopreservation in three different cryoprotectants: Salamon's semen diluent (SSD), modified Salamon's semen diluent (MSSD) and Browne's frog diluent (BFD)

The estimate for post-thaw cell survival from starting numbers of intact viable sperm are:

SDD, 40% (58.5% live X 63.2% intact);

MSSD, 55.2% (70.5% live X 78.3% intact);

and, BFD, 34.4% (44.1% live X 78% intact).

DISCUSSION

Cryodiluents in this study contained both non-permeating (sucrose and egg-yolk) and permeating components (glycerol and DMSO). We discovered from this study the importance of a pre-freeze sperm count to determine amount of sperm lysing. Both solutions that contained sucrose had lower rates of cell lyses. However, this study did not directly compare the effects of sucrose, because problems with cell lyses has not been previously described. The components in the commercially developed diluents for the crypreservation of ram semen (SSD) seem to provide beneficial effects in preserving the integrity of the cells that ultimately survive the process of freezing and thawing than the currently used frog semen diluent. The addition of more glycerol and/or sucrose to Salamon's diluent significantly increased the cells ability to withstand the freezing and thawing as well as increase viability over both the original Salamon's and Browne's diluents. Most importantly, MSSD seems to be a promising new diluent of freezing spermatozoa. BFD was capable of preventing just as many cells from lysis as MSSD, but the percentage of viable cells that did not lyse were considerably lower. Although a significantly higher percentage of cells were intact in BFD when compared to SSD the notably lower percentage of actually viable cells in BFD when compared to SSD suggests that ultimately you get a greater number of viable cells by using SSD compared to BFD. Overall MSSD produces far greater percentage of viable cells following cryopreservation of *Rana pipiens* sperm.

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