Pregnancy rates of mares inseminated with semen cooled for 18 hours and then frozen[1]

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ABSTRACT: The ability to ship cooled stallion sperm for subsequent freezing at a facility specializing in cryopreservation would be beneficial to the equine industry. Stallion sperm has been centrifuged, cooled to 5°C for 12 h, and frozen without a detrimental effect on motility in a previous study; however, no fertility data were available. Experiment 1 compared the post-thaw motility of sperm cooled for 18 h at 15 or 5°C at either 400 or 200×10^6 sperm/mL and then frozen. Storage temperature, sperm concentration, or the interaction of temperature and concentration had no effect on total (TM) and progressive motility (PM) after cooling. Post-thaw TM and PM were higher for control than ($P < 0.05$) for treated samples. There was no difference in post-thaw TM and PM due to temperature or concentration. Experiment 2 further evaluated procedures for cooling before freezing. Ejaculates were either cooled to 5°C for 18 h and centrifuged, centrifuged at room temperature and then cooled to 5°C for 18 h before freezing, or centrifuged and frozen immediately (control). There was no difference among treatments on post-thaw TM or PM. In Exp. 3, mares were inseminated with semen that had been extended in skim milk-egg yolk without glycerol, centrifuged, resuspended at 200×10^6 sperm/mL, cooled to 5°C for 18 h, and then frozen or not cooled for 18 h before freezing (control). Pregnancy rates did not differ for mares receiving semen cooled and then frozen (21 of 30, 70%) or semen frozen directly without prior cooling (16 of 30, 53%). In summary, a procedure was developed for cooling stallion sperm for 18 h before freezing without a resultant decrease in fertility.

Key Words: Cooling, Fertility, Freezing, Stallion Sperm, Storage

 J. Anim. Sci. 2004. 82:690–694

Introduction

Cryopreservation of equine spermatozoa is not a service that all breeding farms offer. If stallion owners want semen cryopreserved, they must transport the stallion to a facility that has this expertise and equipment.

Equine spermatozoa used in a cooled-semen breeding program are commonly cooled to 5°C for 24 to 48 h (Varner et al., 1989; Moran et al., 1992; Squires et al., 1999). However, there has been some debate about whether 5°C or 15°C is better for the storage of equine spermatozoa (Province et al., 1985). Batellier et al. (2001) hypothesized that storage at 15°C may protect sperm plasma membranes from cold shock.

Several studies (Cochran et al., 1984; Vidament et al., 2000; Crockett et al., 2001) have reported higher post-thaw motility and fertility when stallion semen is centrifuged at 22 to 25°C and then cooled to 5°C compared to semen centrifuged at 5°C. Crockett et al. (2001) reported similar motility for sperm stored for 0 and 12, but not 24 h, at 5°C before freezing.

Spermatozoa can be frozen in a skim milk-egg yolk (**SMEY**) extender at concentrations of 20, 200, or 400×10^6/mL with acceptable post-thaw motilities, but semen frozen at concentrations of 800×10^6/mL or higher had lower post-thaw motilities (Heitland et al., 1996). The recommended concentration for cooling semen is 50×10^6/mL (Varner et al., 1988). Crockett et al. (2001) reported that stallion spermatozoa, if centrifuged, could be cooled to 5°C for 12 h at 250×10^6 spermatozoa/mL before cryopreservation without detriment to motility. No fertility data was collected on equine sperm cooled for an extended period before freezing.

Our objectives were to determine the effects of 1) temperature and concentration during cooling and storage of equine spermatozoa before cryopreservation, 2) time of storage before centrifugation, and 3) cooling before freezing on the fertility of frozen-then-thawed sperm.

[1]Supported by the benefactors of the Preservation for Equine Genetics Program.

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Received June 17, 2003.

Accepted October 28, 2003.

Materials and Methods

Experiment 1

This experiment was conducted to compare the post-thaw motility of spermatozoa cooled for 18 h to 15 or 5°C at either 400 or 200×10^6 sperm/mL and then frozen in a SMEY extender. Two ejaculates were collected from each of 13 light-horse stallions before the start of the project using the Colorado State University model artificial vagina (Animal Reproduction Systems, Chino, CA). Thirteen stallions were sexually rested for 1 d, after which a single ejaculate was collected from each and used in this experiment. This collection scheme was used to provide a partial depletion of sperm in the epididymis, but stallions were not depleted of daily sperm output. Each ejaculate was evaluated immediately after collection for total and progressive motility (visually) and concentration was determined using the Densimeter model 534B MOD-1 (Animal Reproduction Systems). Each ejaculate was then extended to 50×10^6/mL in skim milk-glucose extender (EZ Mixin BF, Animal Reproduction Systems), of which 40 mL was poured into 50-mL conical tubes and then centrifuged at $800 \times g$ for 9 min. The supernatant was removed by aspiration, leaving 0.5-mL pellets. Following centrifugation, a clear medium for stallion spermatozoa was added (**SMED**; 100 mL of nanopure water, NaCl 37 m*M*, KCl 10 m*M*, KH_2PO_4 0.07 m*M*, $NaHCO_3$ 35.7 mm, $MgSO_4$ 2.4 m*M*, HEPES 10 m*M*, $CaCl_2$ 1.7 m*M*, fructose 84.3 m*M*, and glucose 5.5 m*M* supplemented with 0.3 g of BSA at a pH of 7.2) (0.5-mL pellet). Each pellet was then gently resuspended and pooled for each stallion. A hemacytometer was used to determine sperm concentration after centrifugation. Equal aliquots were measured for each of five treatments. Treatment 1 was extended in SMEY without glycerol (154.8 m*M* glucose, 4.2 m*M* lactose, 0.5 m*M* raffinose, 0.85 m*M* sodium citrate dihydrate, 1.25 m*M* potassium citrate, 29.8 m*M* HEPES, 51.5 mg/mL skim milk powder, 1 mg/mL ticarcillin, 2% egg yolk wt/vol, adjusted to a pH of 7.2 with sodium bicarbonate or hydrochloric acid) to a concentration of 400×10^6/mL and 200×10^6/mL. Two or four 0.5-mL straws at 400×10^6/mL and 200×10^6/mL, respectively, were frozen as control treatments for each ejaculate. The control samples were frozen in a programmable cell freezer (Planer Freezer model K10-22, series III; TS Scientific, Perkasie, PA). The freezing program started at 20°C and cooled at 0.5°C/min to 4°C, 10°C/min to 15°C, and 15°C/min to –120°C. At –120°C, the straws were quickly removed and plunged into liquid nitrogen for storage. Treatments 2 and 3 were extended to 400×10^6/mL, and Treatments 4 and 5 were extended to 200×10^6/mL in SMEY without glycerol. Treatments 2 and 4 were cooled to 15°C for 18 h, and Treatments 3 and 5 cooled to 5°C for 18 h. Treatments cooled to 5°C were placed in 2-mL tubes in an Equitainer (Hamilton-Thorne Research, Danvers, MA) surrounded by ballast bags. Treatments cooled to 15°C were placed in 2-mL tubes in a 15°C water bath kept in a 5°C cold storage room. After 18 h of storage, the percentage of motile sperm in each treatment was assessed using computer-assisted analysis (**CASA**; Hamilton Thorne Motility Analyzer; Hamilton Thorne Research). At least three fields, or a minimum of 200 cells, were evaluated. Settings for these evaluations were 15 frames acquired at 30 frames per second; progressive cells 50 (m/s; magnification factor 1.95; minimum contrast 75, minimum size 4 pixels; lower and upper static size limits of 0.7 and 7.75, respectively; and lower and upper static head intensities of 0.43 and 1.99, respectively. Depending on the volume of the above-mentioned treatments, a 1:1 glycerol/SMED solution was added to bring the treatments to a 4% (vol/vol) glycerol solution (for example, for a 2.5-mL aliquot: (2.5)(0.04) = 0.1 of glycerol and 0.1 of SMED per 2.5-mL aliquot). Glycerol was used as the cryoprotectant and the SMED was used to mix the glycerol into the semen aliquot. The glycerol/SMED mixture was mixed into the aliquot using a pipette. The resulting sperm concentrations were approximately 192×10^6/mL and 386×10^6/mL. Each treatment held at 15°C was packaged at that temperature into 0.5-mL straws. These straws were frozen in a cell freezer with a starting temperature of 15°C and cooled at 10°C/min until –15°C, and then cooled at 15°C/min until it reached –120°C. The straws were quickly removed and plunged into liquid nitrogen for storage. Each treatment held at 5°C was packaged at that temperature into 0.5-mL straws. The straws were frozen in a cell freezer, with a starting temperature of 5°C, in which it cooled at 10°C/min until –15°C and then cooled at 15°C/min until it reached –120°C. The straws were quickly removed and plunged into liquid nitrogen for storage. Straws were thawed in a 37°C water bath for 30 s. The contents of each straw was diluted with SMEY without glycerol to achieve a concentration of 25×10^6/mL. Motility was estimated visually and using CASA.

Experiment 2

Results from the first experiment indicated that cooling semen in SMEY without glycerol at 200×10^6/mL for 18 h before freezing provided the highest numerical post-thaw motility. However, because these sperm motilities were still low in relation to unstored samples, a second experiment was performed to evaluate further procedures for cooling before freezing. Single ejaculates from four stallions used in the previous study were diluted to 50×10^6/mL in SMEY without glycerol and split into four aliquots: 1) cooled to 5°C for 18 h and then warmed to room temperature before centrifugation; 2) centrifuged, resuspended to 200×10^6/mL in SMEY without glycerol, and cooled to 5°C for 18 h; 3) cooled to 5°C for 18 h, then centrifuged at 5°C, and suspended to 200×10^6/mL in SMEY; and 4) centrifuged, resuspended to 200×10^6/mL in SMEY without glycerol, and frozen. For all treatments, samples were centrifuged at $400 \times g$ for 20 min based on preliminary studies in our

laboratory. Supernatant was removed and the pellet resuspended to 200×10^6/mL. Semen was packaged in 0.5-mL straws and frozen in SMEY with glycerol in a cell freezer. The concentration of each treatment was determined postcentrifugation using a hemacytometer. Motility was analyzed both visually and using CASA after cooling and post-thaw.

Experiment 3

Based on the two previous studies, the centrifugation of semen upon collection and cooling it to 5°C at 200 $\times 10^6$/mL for 18 h before freezing yielded the highest numerical post-thaw motility. Therefore, four light-horse stallions were used in a fertility trial to determine the effect of cooling semen for 18 h before freezing. Approximately six ejaculates were collected from each stallion. Each ejaculate was extended to 50×10^6/mL in SMEY without glycerol and centrifuged at $400 \times g$ for 20 min. The supernatant was removed and the pellets combined. A hemacytometric count determined the postcentrifugation concentration. The combined pellets were separated into two equal aliquots: control and treated. The control sample was extended to 200×10^6/mL in SMEY with glycerol and frozen immediately. Treated semen was resuspended to 200×10^6/mL in SMEY without glycerol and cooled to 5°C in an Equitainer for 18 h. Following the 18 h of incubation, a 4% (vol/vol) glycerol solution was added to the treatment at 5°C, packaged into 0.5-mL straws at 5°C, and frozen in a cell freezer programmed to start at 5°C and to cool at the rates stated in Exp. 2. Post-thaw motility was evaluated visually at the time of insemination.

Forty-seven mares were used to obtain 60 cycles (30 control and 30 treated). Mares were examined using transrectal ultrasonography as needed to determine follicular activity. Ovuplant (deslorelin; Fort Dodge Animal Health, Fort Dodge, IA) was administered subcutaneously in the mare's vulva when the largest follicle reached ≥35 mm to induce ovulation.

Mares were bred with 800×10^6 control or treated sperm using timed insemination (30 h after Ovuplant administration [preovulation] and 52 h after Ovuplant administration [postovulation]). If a mare did not ovulate between the 30- and 52-h breeding times, a third breeding occurred at 60 h after Ovuplant administration, but only if the mare had ovulated by that time. No mare was inseminated more than three times. Pregnancy was determined 14 and 16 d after ovulation by ultrasound examination. Estrumate (1 mL i.m.; cloprostenol sodium; Schering-Plough Animal Health Corp., Union, NJ) was administered to terminate pregnancies (at 14 or 16 d).

Semen was thawed at 37°C for 30 s. A final volume of 4 mL (eight 0.5-mL straws at 200×10^6 sperm/mL) was deposited in the body of the mare's uterus.

Statistical Analysis

Motility variables in Exp. 1 and 2 were analyzed using ANOVA (SAS Inst., Inc., Cary, NC) with stallion as the block effect and treatment × stallion as the error term for testing the main effect of treatment. Only one straw from each ejaculate for each treatment was evaluated. Means were compared using Duncan's multirange test. Pregnancy data in Exp. 3 were analyzed using χ^2 procedures (SAS Inst., Inc.).

Results

Experiment 1

The motion characteristics of sperm cooled and stored at 15 or 5°C for 18 h before freezing are presented in Table 1. Storage temperature, sperm concentration (200 vs. 400×10^6/mL), or the interaction of temperature and concentration had no effect on total and progressive motility after cooling. Post-thaw total and progressive motility were higher ($P < 0.05$) for control than for treated samples. There was no difference in sperm motility post-thaw due to temperature or concentration.

Experiment 2

There was no effect of treatment on total motility or progressive sperm motility (Table 2).

Experiment 3

The pregnancy rates of mares inseminated with semen cooled for 18 h and then frozen (21 of 30, 70%) did not differ significantly from the rates for mares inseminated with control frozen/thawed sperm (16 of 30, 53%).

Discussion

Cooling spermatozoa to 5°C rather than 15°C before cryopreservation resulted in no significant difference in the fertility of semen. There is an advantage to cooling semen to 5°C because passive cooling devices that maintain equine sperm at 5°C are readily available and are used extensively in the equine industry for shipping sperm (Squires et al., 1999).

The centrifugation of equine spermatozoa can be detrimental unless low (370 to $829 \times g$) forces are used (Pickett et al., 1975). The centrifugation of spermatozoa at 5°C proved to be detrimental to spermatozoal motility after 6 to 12 h of storage (Crockett et al., 2001) followed by freezing and thawing. Warming the semen after cooling and before freezing also had detrimental effects on post-thaw motility. Therefore, the centrifugation of semen was done immediately following collection, before cooling, storage, and cryopreservation, to ensure higher percentages of motile spermatozoa.

Vidament et al. (2000) also reported higher motility and fertility if semen was centrifuged at 22 and cooled to 5°C vs. centrifuging at 4°C. In their study, glycerol was added after centrifugation compared to addition after cooling immediately before freezing in the present

Table 1. The percentages of total motile (TM) and progressively motile (PM) equine spermatozoa held at 15 or 5°C and at concentrations of 200 and 400 × 10^6/mL for either 0 (control) or 18 h (treatment) and then frozen (n = 13 ejaculates)

	Postcooling				Postthawing			
	200 × 10^6/mL		400 × 10^6/mL		200 × 10^6/mL		400 × 10^6/mL	
Temperature	TM, %	PM, %	TM, %	PM, %	TM, %	PM, %	TM, %	PM, %
Control	—	—	—	—	49[a]	27[a]	47[a]	24[a]
15°C	62	21	61	16	27[b]	13[b]	26[b]	7[b]
5°C	62	23	57	18	30[b]	12[b]	28[b]	11[b]
SEM	5	3	5	2	3	3	3	2

[a,b]Values within columns with different superscripts differ ($P < 0.05$).

study. They also reported lower post-thaw sperm motility for semen cooled from 37 to 4°C in 4 h compared to 1 h.

After centrifugation, semen can be frozen at concentrations of between 200 × 10^6/mL and 1,600 × 10^6/mL without detriment to motility (Squires et al., 1999). Similarly, Leipold et al. (1998) reported that semen frozen at concentrations of 400 and 1,600 × 10^6/mL had similar post-thaw motilities. Commercially, semen is commonly frozen between 200 × 10^6/mL and 400 × 10^6/mL. Crockett et al. (2001) stated that semen held at 5°C at a concentration of 250 × 10^6/mL before freezing resulted in better post-thaw motilities than sperm at a concentration of 500 × 10^6/mL. Based on our results, there was no difference in the post-thaw motility when freezing at a concentration of 200 or 400 × 10^6/mL. Thus, a concentration of 200 × 10^6/mL was chosen because this concentration is commonly used in the industry when freezing stallion semen.

Egg yolk-containing extenders appeared to maintain spermatozoal motility after freezing (Crockett et al., 2001) and after centrifugation during cooled storage, especially in stallions considered to be "poor coolers" (Brinsko et al., 2000). Egg yolk also is a component of freezing extenders (SMEY) and—along with sugars, salts, and HEPES—is thought to be beneficial to spermatozoal survival during cryopreservation (Jasko et al., 1992). It also has been shown that removal of seminal plasma before cryopreservation is essential (Martin et al., 1979). Based on this evidence, we felt that using SMEY would be beneficial to the centrifuged spermatozoa during cooled storage and during the cryopreservation process, once the cryoprotectant (glycerol) was added. Several different cryopreservation protocols have been used (Martin et al., 1979; Cristanelli et al., 1984; Palmer, 1984), but, because the procedures differed in many ways, a comparison of their results is inappropriate.

The percentages of total motile sperm in treatments for Exp. 2 were not significantly different. This is in contrast to the results of Exp. 1. This is likely due to the small number of ejaculates used in Exp. 2 and the relatively low progressive motility.

There have been no fertility data collected on mares bred with frozen semen that was previously cooled and stored for 18 h. Loomis (2001) collected data from 876 mares bred with frozen semen from 106 stallions during the 1999 and 2000 breeding seasons and achieved pregnancy rates of 51.3 and 75.6% on the first cycle and after the season, respectively. Pregnancy rates in our study were similar to those reported by Loomis (2001). Therefore, cooling spermatozoa for 18 h or less before cryopreservation resulted in pregnancy rates equal to those of spermatozoa collected and frozen immediately.

Table 2. Percentage of post-thaw total (TM) and progressive (PM) motile spermatozoa held for 18 h at 5°C before freezing

Treatment[a]	TM, %[b]	PM, %[b]
Trt. 1, cooled-stored-warmed/centrifuged	19	6
Trt. 2, centrifuged-cooled-stored	44	14
Trt. 3, cooled-stored-centrifuged	37	7
Control, centrifuged	33	9
SEM	8	3

[a]Treatments were centrifuged either after cooling but allowed to warm to room temperature before centrifugation (Trt. 1), before cooling (Trt. 2), or after cooling while still at 5°C (Trt. 3). The control was the portion of the ejaculate frozen without first being cooled (n = 4 ejaculates).

[b]Means within columns did not differ significantly ($P > 0.05$).

Implications

The use of frozen semen in horses has increased as a result of the recent acceptance by the major breed registries. Semen that is collected on the farm can be centrifuged, diluted in freezing extender with glycerol, and shipped to a centralized facility for subsequent freezing, storing, and distribution.

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