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# Influence of Semen Storage and Cryoprotectant on Post-thaw Viability and Fertility of Stallion Spermatozoa

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## ABSTRACT

The aim of the current study was to verify that stallion spermatozoa could be cooled for 24 hours and then frozen. In experiment I, one ejaculate from each of 13 stallions was used. Semen was collected and split into two parts; one part immediately frozen using standard cryopreservation techniques and the other diluted, stored in an Equitainer for 24 hours, and then frozen. In experiment II, one ejaculate from each of 12 stallions was collected, diluted with Botu-Semen, and split into two parts: one cooled in an Equitainer and the other in Max-Semen Express without prior centrifugation. After 24 hours of cooling, the samples were centrifuged to remove seminal plasma and concentrate the sperm, and resuspended in Botu-Crio® extender containing one of three cryoprotectant treatments (1% glycerol + 4% dimethylformamide, 1% glycerol + 4% dimethylacetamide and 1% glycerol + 4% methylformamide), maintained at 5°C for 20 minutes, then frozen in nitrogen vapor. No difference was observed between the two cooling systems. The association of 1% glycerol and 4% methylformamide provided the best post-thaw progressive motility. For experiment III, two stallions were used for a fertility trial. Forty-three inseminations were performed using 22 mares. No differences were seen in semen parameters and pregnancy rates when comparing the two freezing protocols (conventional and cooled/frozen). Pregnancy rates for conventional and cooled/frozen semen were, respectively, 72.7% and 82.3% (stallion A), and 40.0% and 50.0% (stallion B). We concluded that cooling equine semen for 24 hours before freezing, while maintaining sperm viability and fertility, is possible.

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## INTRODUCTION

The use of modern technologies has contributed to reproductive advances in a variety of animal species. Semen preservation and storage represents one of the major steps in this advancement and provides a better use of animals with high genetic potential. In addition, this technique makes it possible to preserve genetic material from animals that are permanently or temporarily unable to reproduce and also allows long-distance semen shipment, which is considered the safest biological insurance for stallions with high genetic value, because the semen can be used after the death of the stallion.

Many horse breed associations in the last decade have accepted the use of frozen semen. The acceptance of this technology by the Quarter Horse and Paint Horse associations has renewed and stimulated research on protocols to freeze stallion semen.

Aiming to avoid transporting stallions to specialized centers, some studies developed protocols for freezing semen that cool the semen for a longer period before freezing.[1,2] These authors observed that cryopreservation after 24 hours of cooling reduced progressive motility,[1] but cooling for 18 hours before freezing did not reduce fertility.[2]

The efficiency of cooled semen depends on an adequate shipment system. If insemination takes place within 12 hours after semen collection, then storage can be performed at either 20°C or 5°C. If semen storage exceeds 12 hours, slow cooling to 5°C is required. Semen storage at 4°C and 5°C resulted in higher sperm cell viability than storage at either 0°C or 2°C.[3]

Equine semen is generally stored and shipped inside a passive cooling device. The most commonly used passive cooling device is the Equitainer (Hamilton Thorne Research, Danvers, MA), which cools the semen sample slowly at an initial rate of −0.03°C per minute to 4-8°C.[4,5] This temperature is maintained over 48 hours while shipping the semen to the mare's location for insemination. The use of this shipment system can provide pregnancy rates that are similar to those obtained with inseminations using fresh-diluted semen if semen from

fertile stallions is correctly processed and manipulated.[6] However, not all stallions produce semen that withstands storage.[7]

The use of alternative cryoprotectants in relation to the use of glycerol can reduce and protect equine spermatozoa against freeze/thawing effects, leading to better sperm motility and viability followed by improved fertility rates.[8-10] Zahn et al[11] obtained excellent pregnancy rates (9/12 mares, 75%) using MP50[12] containing 3% glycerol and 2% dimethylformamide.

Backman et al[2] cooled equine semen for 18 hours before freezing and achieved pregnancy rates that were not different between mares inseminated with standard frozen semen (56%) and mares inseminated with semen previously cooled and then frozen (70%). Thus, the aim of the current study was to determine the effect of cooling in two different passive cooling devices for 24 hours before freezing, and to evaluate the use of various cryoprotectant combinations.

## MATERIALS AND METHODS

### Experiment I: Freezing Semen Immediately after Collection and at 24 Hours after Cooling in an Equitainer

The study used one ejaculate from 13 stallions, aged between 4 and 18 years, from different breeds: Quarter Horse, Westphalian, Arabian, Hanoverian, Paint Horse, and Holsteiner. The stallions were located at the following centers: Equine Reproduction Technology Center (CERBEQ—Department of Animal Reproduction and Veterinary Radiology//FMVZ-UNESP, Botucatu) and Agromen (Private Stud Farm, Orlândia, SP, Brazil). The semen was processed in the same way, by the same technician, despite using stallions from two different locations. All stallions were fertile and considered "good freezers." After collection, the ejaculates were divided into two aliquots: one submitted to freezing[12] using Botu-Crio (Biotech Botucatu/ME LTDA, Brazil) and the other diluted to a concentration of $50 \times 10^6$ motile sperm/ml, using Botu-Semen® extender (Biotech Botucatu/ME LTDA, Brazil), then stored in an Equitainer® (Hamilton Thorne Research, Beverly, MA) for 24 hours. Afterward, cooled semen was directly centrifuged at room temperature (20°C) at 600*g* for 10 minutes. The supernatant was removed and Botu-Crio® freezing extender was added at 20°C. Semen was packed with $100 \times 10^6$ per 0.5-ml straw, stored at 5°C for 20 minutes, frozen at 6 cm above liquid nitrogen for 20 minutes and then immersed into nitrogen.[12] After thawing at 46°C for 20 seconds,[13] the samples were transferred to a 1.5-ml plastic tube and maintained in a dry bath at 37°C during the sperm evaluation. The samples were analyzed by CASA (HTM–IVOS 12; Hamilton Thorne Research, Beverly, MA), selecting five fields per sample, and plasma membrane integrity evaluated by fluorescent stains,[14] carboxyfluorescein diacetate, and propidium iodide, using microscopic examination as modified by Zúccari.[15] System parameters for CASA were 30 frames acquired at 60 frames per second; minimum contrast, 30; minimum cell size, 5 pixels; path velocity (VAP) cut-off, 30 μm/s; and VAP cut-off for progressive cells, 70 μm/s and straightness 80%; straight line velocity (VSL) cutoff, 0 μm/s. The slow cells were considered static. A 10-μl drop of each sample was placed on a preheated (37°C) Makler counting chamber (10 μm depth).

### Experiment II: Comparison of Cryoprotectants for Freezing after 24 Hours of Cooling Using Two Storage Systems

This study used one ejaculate from 12 stallions aged between 4 and 18 years, from different breeds: Westphalian, Arabian, Hanoverian, Paint Horse, and Holsteiner. The semen samples were diluted to a concentration of $50 \times 10^6$ sperm/ml with Botu-Semen® and divided into two aliquots: one cooled using an Equitainer and the other using Max-Semen Express (MSE) (Agrofarma, SP, Brazil); then they were stored for 24 hours. After this period, the samples were centrifuged at 600*g* for 10 minutes, the supernatant was removed, and the pellet resuspended to a concentration of $100 \times 10^6$ sperm/ml with Botu-Crio® using three different cryoprotectant combinations: (1) 1% glycerol + 4% dimethylacetamide (G+DA); (2) 1% glycerol + 4% dimethylformamide (G+DF); and (3) 1% glycerol + 4% methylformamide (G+MF). The samples were placed into 0.5-ml straws, frozen as described in experiment I, and thawed at 46°C for 20 seconds.[13] After thawing, the motion characteristic was evaluated by CASA and the plasma membrane integrity was verified by fluorescent probes,[14] modified by Zúccari,[15] as in experiment I.

### Experiment III: Fertility Trial

Fertility trial was carried out using frozen semen from two fertile stallions, aged 4 (stallion A) and 18 years (stallion B), using 42 cycles from 22 mares. The mares were randomly assigned to stallion and treatment. Mare's estrus was detected by rectal palpation and reproductive tract ultrasonography. Ten milligrams equine pituitary extract (EPE) was given intravenously to induce ovulation when a preovulatory follicle of at least 35 mm was detected. According to our previously unpublished study, ovulation occurs $34.75 \pm 6.72$ hours from the EPE injection. Thus, inseminations were performed twice: 30 hours from the EPE injection and after ovulation (within 6 hours of ovulation) with 2 ml of semen containing approximately $400 \times 10^6$ spermatozoa, toward the tip of the uterine horn using a flexible pipette (MiniTüb®, Minitüb do Brasil, Porto Alegre-RS, Brazil). The combination of 1% glycerol and 4% methylformamide was chosen because it provided the best results in experiment II. The pregnancy diagnosis was performed 15 days after ovulation using ultrasonography.

### Statistical Analysis

Semen data were analyzed by ANOVA (SAS, Institute, Inc., Cary, NC) followed by Tukey's test to identify

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