EFFECT OF CRYOPRESERVATION PROTOCOL ON POST-THAW CHARACTERISTICS OF STALLION SPERMATOZOA

A Thesis

by

JOSE L. SALAZAR

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

August 2009

Major Subject: Veterinary Medicine

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Approved by:

Chair of Committee, Dickson D. Varner
Committee Members, Charles C. Love
Steve P. Brinsko
David W. Forrest
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ABSTRACT

Effect of Cryopreservation Protocol on Post-Thaw Characteristics of Stallion Spermatozoa. (August 2009)

Jose L. Salazar, B.S., Texas A&M University

Chair of Advisory Committee: Dr. Dickson D. Varner

Three ejaculates from each of eight stallions were initially centrifuged in INRA 96 extender and spermatozoal pellets were resuspended in a milk/egg yolk-based freezing extender or an egg yolk-based freezing extender. Extended semen was exposed to a fast pre-freeze cooling rate (FAST - semen immediately subjected to cryopreservation) or a slow pre-freeze cooling rate (SLOW - semen pre-cooled at a controlled rate for 80 minutes prior to cryopreservation). After thawing, semen was diluted in initial freezing medium (FM) or INRA 96 prior to analysis of 9 experimental endpoints: total motility (MOT; %), progressive motility (PMOT; %), curvilinear velocity (VCL; μm/sec), average-path velocity (VAP; μm/sec), straight-line velocity (VSL; μm/sec), linearity (LIN; %), intact acrosomal and plasma membranes (AIVIAB; %), intact acrosomal membranes (AI; %), and intact plasma membranes (VIAB; %). Eight of nine experimental endpoints (MOT, PMOT, VAP, VSL, LIN AIVIAB, AI, and VIAB) were affected by extender type, with LE extender yielding higher values than MF extender for these variables ($P<0.05$). Exposure of extended semen to a slow pre-freeze cooling period resulted in increased values for seven of nine endpoints, as compared to a

fast pre-freeze cooling period ($P<0.05$). Mean VAP and VSL were unaffected by pre-freeze cooling rate ($P>0.05$). As a post-thaw diluent, INRA 96 yielded higher mean values than FM for MOT, PMOT, VCL, VAP, and VSL ($P<0.05$). Treatment group FM yielded slightly higher values than INRA 96 for LIN and VIAB ($P<0.05$). Extender x rate interactions ($P<0.05$) were detected for the variables MOT, AIVIAB, AI and VIAB. Mean values for these endpoints were higher following spermatozoal exposure to a slow pre-freeze cooling period, regardless of freezing extender type ($P<0.05$). The effects of pre-freeze cooling rate on MOT, AIVIAB, AI, and VIAB were more pronounced in spermatozoa cryopreserved in MF extender, as compared to LE extender. Within treatment groups SLOW and FAST, mean MOT, AIVIAB, AI, and VIAB were higher ($P<0.05$) for spermatozoa cryopreserved in LE extender, as compared to MF extender. Extender x diluent interactions ($P<0.05$) were detected for MOT, PMOT, VCL, VAP, VSL, and LIN. Within Group MF, mean MOT, PMOT, VCL, VAP, and VSL were higher in INRA diluent, as compared to FM diluent ($P<0.05$). Within Group LE, FM diluent yielded slightly higher values than INRA diluent for PMOT, VAP, VSL, and LIN ($P<0.05$). In conclusion, a slow pre-freeze cooling rate was superior to a fast pre-freeze cooling rate, regardless of freezing extender used, and INRA 96 served as a satisfactory post-thaw diluent prior to semen analysis.

DEDICATION

I dedicate this work to:

My parents: Olga and Luis Salazar,

Dr. Clint Kainer

&

My family and friends.

These loved ones provided me with a stable foundation that has allowed me to live my dreams and accomplish lifelong goals that would have been impossible otherwise. Without them I would not be the man I am today nor the one I continue to mold into. Mom and Dad, thank you for always affording me with everything I needed to succeed. Clint, thanks for giving me the opportunity to learn and gain priceless experiences by shadowing you over the years. You have all had many appreciated and respectable impacts throughout my life and career. Thank You!

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INTRODUCTION

With the rapid growth of the equine breeding industry, the use of frozen semen has become more common in order to preserve superior genetics as well as sustain a successful breeding program. Some of the benefits of using cryopreserved semen include access to semen from stallions standing abroad and from those involved in competition. Perhaps one of the major advantages with the availability of frozen semen is that breeders can more easily inseminate a mare at the optimal breeding time instead of having to rely on the availability of stallion semen. Unfortunately, the success of cryopreservation with stallion spermatozoa is generally considered to be lower than that of some other domestic species, especially that of dairy cattle. The overall reduced fertility of cryopreserved stallion semen, as compared to cooled or fresh semen, is disadvantageous to both stallion and mare owners. The reason for this reduction in fertility is likely due to the fact that stallions are most commonly selected as sires based on performance record, pedigree, and conformation, as opposed to fertility. Researchers have developed and tested many extenders and freezing techniques over the years in an effort to improve the post-thaw quality of stallion spermatozoa. Nonetheless, there has not been a major break through with the cryopreservation of stallion spermatozoa that has drastically enhanced post-thaw semen quality of stallions.

This thesis follows the style of Theriogenology.

The purpose of this experiment was to identify which of two commonly used commercial cryopreservation extenders for stallion semen produces the best post-thaw semen quality following two different pre-freeze cooling techniques. The two extenders used were the E-Z Freezin® "MFR5" extender (MFR5) and E-Z Freezin® "LE" extender (LE). Semen in either extender was exposed to a fast or a slow pre-freeze cooling process. The effect of post-thaw diluent on a variety of laboratory endpoints was also studied.

OBJECTIVES

The objectives of this study were to determine the effects of cryopreservation extender type and pre-freeze cooling period on post-thaw semen quality in stallions, based on an array of laboratory measures. The effect of post-thaw semen diluent on semen quality was also tested. Determining an overall superior treatment may lessen the need for testing various freezing extenders and pre-freeze cooling rates among individual stallions. With this study, we hope to discover a treatment providing superior results that may be suggested as an optimal cryopreservation protocol across a range of stallions.

LITERATURE REVIEW

Freezing Extenders

Over the years researchers have tested and proposed numerous freezing extenders of various compositions in attempt to improve the quality and use of frozen stallion semen. Comparisons among stallion freezing extenders are documented poorly [1]. It has also been reported that the most effective semen extender for one stallion is not necessarily the most effective for another [2]. In 1984, Palmer indicated that freezing horse semen could use the same milk diluents (used for diluting semen immediately after collection) with the addition of 2% egg yolk and 2.5% glycerol [3]. In this study, he concluded that the milk + sugar + citrate freezing media proved to be significantly higher in regards to post-thaw motility compared to milk alone and HF20 extenders [3]. The composition of this extender resulted in the formation of the milk based Modified French formula freezing extender (MFR5) that is used commercially today for freezing equine spermatozoa. The Lactose EDTA (LE) freezing extender is another commercially used equine freezing extender that is egg-yolk based. The composition of this extender developed by Martin et al. in 1979 was an experimentally developed extender formulated by combining two extenders; lactose and EDTA. Lactose EDTA resulted in higher post-thaw progressive motility (53.4%) when compared to the control (42.3%) which was composed of lactose, egg yolk, and glycerine [4]. In a study conducted by Ecot et al. in 2000, a variety of extenders along

with cooling treatments were tested in attempt to determine the effects of different cooling treatments and semen extenders on spermatozoal samples from individual stallions. The extenders used in this study were INRA82, Kenney, and Gent. The INRA82 and Kenney extenders are similar and all three contain milk. Different concentrations of egg yolk (2 or 4%) and glycerol (2.5 or 3.5%) were applied to the INRA and Kenney extenders in experiment 2. Ecot et al. concluded that modification of egg yolk and glycerol concentrations resulted in higher spermatozoal motility in samples frozen in Kenney extender than in INRA82 extender [5]. As a result of experiment one where all three extenders were compared, several stallions had the same motility across all three, whereas some stallions had better motility when their semen was frozen in Kenney extender [5]. It was indicated that the experiments did not clearly establish differences in tolerance to semen extender among stallions.

Only a few comparisons have been made among freezing extenders for freezing stallion spermatozoa [1,5-9]. Results concluded that, although Kenney FE extender yielded higher motility compared to Lactose-Glucose EDTA and INRA82 FE extenders, it also produced a higher percentage of acrosome-reacted and capacitated spermatozoa thus resulting in lower per cycle pregnancy rate [10].

One of the important components of freezing extenders is egg yolk. The concentration of egg yolk varies in different extenders used and has been studied as well. Vidament et al. found that increasing egg yolk from 2 to 4% did not improve post-thaw

motility [11]. The results of their particular experiment were consistent with similar results of frozen semen by Ecot et al. in 2000 [5,11]. Ecot et al. suggested that the concentration of egg yolk likely depends upon the extender used and the level of glycerol and milk [5].

Cooling Rates

The major damage to spermatozoa as a result of cold shock is dependent upon the final temperature before freezing as well as the cooling speed until that temperature is reached. The results of cold shock are characterized by abnormal motility patterns, rapid loss of motility, and membrane damage [12]. Kayser et al. reported that spermatozoa could be rapidly cooled from 37 to 20°C but required a linear cooling rate of -0.05 to -0.1°C/min between 20 and 5°C to maximize spermatozoal motility [13]. In a study conducted by Moran et al. the temperature range demonstrating when stallion spermatozoa were most susceptible to cold shock was between 19 and 8°C [12]. As a result the cooling rates recommended for extended semen are as follows 1) rapid from 37 to 19°C (-0.7 to -2°C/min) 2) slow from 19 to 8°C (-.05 to -.1°C/min) and 3) rapid from 8 to 4°C (-.7°C/min) [12]. Vidament et al. performed a study which incorporated a freezing rate of 37°C to 4°C in 1 hour [14]. The cooling rates ranged from -4 to -2°C/min between 37 and 22°C, -0.4°C/min between 20 and 10°C, then -0.2°C/min between 10 and 8°C [14]. After cooling, semen was maintained at 4°C for 1 hour before freezing [14]. Among all of the treatments, this delayed cooling treatment resulted in the

highest percentage of post thaw-motility. Vidament et al. recommended a procedure for freezing and thawing in INRA82 that involved cooling extended semen to 4°C in 1 h 20 min, then filling straws at 4°C, and freezing [11].

Vidament et al. showed significant results in post-thaw motility among all treatments when centrifugation and addition of glycerol was added at 22°C instead of 4°C [14]. Direct freezing after centrifugation at 22°C resulted in lower post-thaw motility compared to cooling to 4°C in 1 hour before freezing [14]. This is also in agreement with reports by Heitland et al. [1,14]. Another study conducted by Crockett et al. reported that progressive motility (PMOT) for spermatozoa was higher when centrifuged before cooling (30%) vs. after cooling (19%) [15]. When centrifugation and addition of glycerol was performed at 4°C, post-thaw motility was also higher when cooling from 37°C to 4°C in 1 hour vs. 4 hours [14].

Heitland et al. reported that skim milk-egg yolk-glycerol extender yielded higher total and progressive spermatozoal motility, as compared to Lactose EDTA when spermatozoa were cooled to 5°C over a 2.5 hour period prior to freezing rather than freezing at 20°C [1]. In a latter study conducted by Crockett et al. to demonstrate the effects of cooling before freezing on post-thaw motility they found that samples frozen 2.5 hours after cooling to 5°C had higher percentages of progressive motility (27%) vs. samples frozen 24 hours after cooling (10%) [15]. Samples also frozen 2.5 hours after cooling in skim milk extenders containing egg yolk yielded a higher percentage of

progressively motile spermatozoa (avg. 32%) than skim milk alone (avg. 16%) [15]. Crockett et al. also stated that skim milk extenders generally require a relatively slow cooling of spermatozoa to 5°C over 2-2.5 hours prior to freezing [15].

Glycerol Concentration

Glycerol is an essential cryoprotectant in all conventional extenders at (at concentrations of 2.5-6%) used for freezing stallion spermatozoa [16]. In 2001, a study conducted by Vidament et al. revealed there was no overall effect of glycerol concentration on post-thaw motility, regardless of the freezing protocol, however there seemed to be differences among stallions [11]. For example, for four stallions motility tended to increase when glycerol was above 3%. However, for one, motility tended to decrease as glycerol increased. Vidament et al. concluded that different glycerol concentrations (range: 1.5-4.5%) had no significant effect on post-thaw motility although 2.4-2.8% resulted in slightly higher (nonsignificant) motility [11]. These results were similar with other studies within the same laboratory where glycerol concentrations from 0 to 5% were tested and the optimum values obtained were at 2 or 3%. Ecot et al. also reported similar post-thaw motilities for spermatozoa frozen in INRA82 containing 2.5 and 3.5% glycerol [5]. Although increasing the glycerol concentration from 2.5 to 3.5% did not significantly increase spermatozoal motility in extenders containing 2% egg yolk, increasing the glycerol concentration in 4% egg yolk extenders did increase spermatozoal motility [5]. Cochran et al. and Cristanelli et al.

also observed increased spermatozoal motility in Martin's extender containing 4% glycerol versus 2 or 3% glycerol [17,18]. These results may indicate an interaction between egg yolk and glycerol in semen extenders. Burns and Reasner reported that the lowest concentration of glycerol in a Kenney extender that provided maximal cryoprotective effects was 2% [19]. Vidament et al. concluded based on their studies, that 2.5% glycerol in freezing extender routinely provides satisfactory per-cycle fertility [11]. In 2005, Vidament et al. recommended a glycerol concentration range from 2.5-3.5% when considering both motility and fertility [10].

The optimal glycerol concentration for maximal post-thaw motility using INRA82 extender is around 2-3% [5,11,20], 4% for the commonly used Lactose-Glucose EDTA [17-18], and between 0 and 2% for Kenney freezing extender [19].

Spermatozoal Freezability and Fertility

In 2005, Vidament demonstrated a relationship between motility and fertility. The study showed a 43% pregnancy rate per cycle when motility was less than 45% and a pregnancy rate of 52% when the motility was above that value [10]. A similar relationship was found between average path velocity (VAP) and fertility as well. Pregnancy rate per cycle was 45% when VAP was <66 μm/s and 54% when VAP was over this value [10]. However, no relationship was found between amplitude of lateral head displacement (ALH) and fertility. This study also yielded a high rate of selected ejaculates. They reported that 64% of stallions had more than 90% of their ejaculates

selected after freezing for use on mares [10]. Others have obtained high freezability results as well [21-22].

Vidament et al. reported significant improvement on per cycle fertility with semen subjected to centrifugation and addition of glycerol at 22°C 10 min after collection [5].

There have been numerous attempts to find different post-thaw criteria to predict the fertility of frozen semen however, there are only few with a sufficient number of stallions and mares [23-24]. Nevertheless, all data support the conclusion that there is a relationship between post-thaw motility and fertility of frozen semen, at least among ejaculates frozen by the same technique and used in a similar manner [10]. However, when applying different protocols, the relationship may no longer exist because of possible variable reactions of spermatozoa with different extenders and cooling rates [10].

Vidament et al. reported that there was a strong relationship between fertility of fresh semen and semen freezability [25]. However, the relationship between fertility of frozen semen and freezability was not as marked [25].

A study by Brinsko et al. concluded that commonly used methods for assessing spermatozoal function do not appear to be useful in predicting stallion semen freezability [26]. Their results only indicated that as the percentage of progressively motile morphologically normal spermatozoa in fresh semen decreases, the percentages will be

even lower in cooled and frozen-thawed samples. Therefore, high percentages of progressively motile spermatozoa in fresh semen samples, is not indicative of similar motility patterns in cooled and frozen-thawed samples [26]. However, the better a fresh semen sample was in terms of progressive spermatozoal motility, the better a frozen-thawed semen sample would be in terms of total spermatozoal motility [26].

Post-thaw Diluent

There are limited reports on the effects of different media used as post-thaw diluents for analyzing spermatozoa. Palmer reported a significant decrease in fertility per cycle when glycerol was in the post-thaw diluent (INRA82) [3]. Some other reports of post-thaw diluents used for post-thaw motility analysis; Vidament et.al (2001) and Ecot et al. (2000) used INRA82, Cristanelli et al. (1984) used lactose EDTA egg yolk extender without glycerol, Burns and Reasner (1995) used BF extender without egg yolk or glycerol, Heitland et al. (1996) used E-Z Mixin, and Backman et al. (2004) used SMEY (skim milk egg-yolk) extender without glycerol however, no data are available [1,5,11,18,19,27].

MATERIALS AND METHODS

Semen Collection and General Processing

Twenty-four ejaculates (three from each of eight mature sexually active light-breed stallions) were collected using an artificial vagina (Missouri-model; Nasco, Ft. Atkinson, WI, USA) equipped with an in-line nylon micromesh filter (Animal Reproduction Systems, Chino, CA, USA) to permit collection of gel-free semen. An ovariectomized mare was used for sexual stimulation and as mount source. Total spermatozoal number in gel-free semen was obtained by measuring semen volume with a graduated cylinder and measuring spermatozoal concentration photometrically (SpermaCue; Minitube of America, Inc., Verona, WI, USA). One-ml aliquots of raw (neat) semen were immediately snap frozen on dry ice in 1-ml polypropylene tubes (Cryogenic vials [1.2-ml]; Corning Life Sciences, Lowell, MA, USA) then stored at -80 ^{o}C until analyzed for spermatozoal chromatin susceptibility to denaturation (*ie*, Sperm Chromatin Structure Assay; SCSA).

An aliquot of gel-free semen was immediately diluted with a warmed (37 ^{o}C) milk extract-based extender (INRA96; IMV, Maple Grove, MN, USA) to a final spermatozoal concentration of 25 million spermatozoa/ml for evaluation of spermatozoal motility (Time 0; T0), using a computerized spermatozoal motion analyzer (IVOS Version 12.2L, Hamilton Thorne Biosciences, Beverly, MA, USA). Aliquots of semen were also diluted with INRA 96 to a final spermatozoal concentration of 20 x 10^{6}

spermatozoa/ml, and then packaged with minimal air space in capped 5-ml polypropylene tubes (Cryogenic vials [5.0-ml]; Corning Life Sciences, Lowell, MA, USA). Prepared vials were than packaged as recommended by the manufacturer in a commercial semen transport container (Equitainer™ II; Hamilton Research, Inc., South Hamilton, MA, USA) for 24 hours of cooled storage (Time 24 h; T24). Following this storage period, aliquots of semen were subjected to frozen storage, as described above, for SCSA. Remaining semen was warmed for 15 min in a water bath set at 37 ^{o}C and subjected to computerized motility analysis, as described above. The percent change in values for dependent variables (MOT, PMOT, VCL, COMP) from T0 to T24 (DIFF) were also determined.

Spermatozoal Cryopreservation Procedures

Gel-free semen was diluted in INRA 96 extender to obtain a final spermatozoal concentration of approximately 50 x 10^{6}/ml. Thirty-ml aliquots of extended semen were loaded into glass nipple-bottom centrifuge tubes (Pesce Lab Sales, Kennett Square, PA, USA). Thirty microliters of iodixanol (OptiPrep,™ Axis-Shield, Oslo, Norway) was added beneath extended semen to provide a cushion for spermatozoa during centrifugation. Extended semen was centrifuged at 400 x *g* for 20 min, followed by aspiration of supernate. The remaining spermatozoal pellet (with approximately 1 ml of overlying supernate) was resuspended in one of two extender types; 1) E-Z Freezin™ - "LE" semen extender; LE (Animal Reproduction Systems, Chino, CA, USA) or 2) E-Z

Freezin™ - "MFR5" semen extender; MFR5 (Animal Reproduction Systems, Chino, CA, USA) with the final spermatozoal concentration adjusted to approximately 200 x 10^6 spermatozoa/ml. The LE extender contained approximately 21.5% egg yolk (v/v) and no milk products. The MFR5 extender was a milk-based product, containing approximately 3% egg yolk (v/v). The glycerol concentration was adjusted to 2.5% (v/v) in each extender. Freezing extenders were stored frozen at -80^{o}C until used. Prior to use, thawed extenders were centrifuged at 1500 x g for 15 min. The supernate was harvested aseptically and passed through 5-µm and 1.2-µm pore size nylon filters (GE Osmonics, Minnentonka, MN, USA) in tandom. These procedures were performed to eliminate particulate matter in the freezing extenders that might otherwise interfere with computerized spermatozoal motion analysis.

Semen diluted in each freezing extender was loaded into appropriately labeled 0.5-ml capacity straws and subjected to one of two pre-freeze cooling periods: 1) fast pre-freeze cooling rate, whereby a static vapor freeze was implemented within 5 min. following straw loading, with the freeze cycle beginning at approximately 25 ^{o}C (FAST), and 2) slow pre-freeze cooling rate, whereby loaded straws were slowly cooled in the chamber of a programmable liquid nitrogen cell freezer (CBS Freezer 2100 Series; Custom Biogenics Systems, Shelby Township, MI, USA; SLOW). The chamber cooling ramps were -2.0 ^{o}C/min from approximately 25^{o}C to 22^{o}C; -0.3 ^{o}C/min from 22^{o}C to

10°C; and -0.2 °C/min from 10°C to 4°C. The total cooling time for Group SLOW was approximately 80 min. For Groups FAST and SLOW, semen-filled straws were frozen 4 cm above a liquid nitrogen bath for 15 min, and then plunged into liquid nitrogen for storage.

For analysis, straws were thawed by submersion for 30 s in a water bath set at 37 °C. Thawed semen was further diluted (semen:extender ratio of 1:9[v/v]) in one of two extenders: 1) INRA 96 extender (INRA96) or 2) the freezing extender used for cryopreservation (FM). Individual aliquots of prepared semen samples were subjected to computerized analysis of spermatozoal motion following incubation at 37 °C for 15 min; frozen in 1-ml vials for SCSA, and subjected to flow cytometric evaluation of spermatozoal acrosomal integrity and plasma membrane integrity.

Computer-Assisted Sperm Motion Analysis (CASMA)

Spermatozoa were analyzed by CASMA, in a manner similar to that previously described [28]. A 6-µl aliquot of extended semen was placed in a warmed (37 °C) counting chamber with a fixed height of 20 µm (Leja Standard Count 2 Chamber slides; Leja Products, B.V., Nieuw-Vennep, The Netherlands). The slide was then placed on a stage set at 37 °C and inserted into the IVOS computerized spermatozoal motion analyzer for evaluation. A total of 10 microscopic fields and a minimum of 500 spermatozoa were examined. Preset values for the IVOS system consisted of the

following: frames acquired – 45; frame rate – 60 Hz; minimum contract – 70; minimum cell size – 4 pixels; minimum static contrast – 30; straightness threshold for progressive motility – 50; average-path velocity (VAP) threshold for progressive motility - 30; VAP threshold for static cells - 15; cell intensity – 106; static head size – 0.60 to 2.00; static head intensity – 0.20 to 2.01; static elongation – 40 to 85; LED illumination intensity – 2200; Experimental endpoints included: 1) percentage of motile spermatozoa (MOT); percentage of progressively motile spermatozoa (PMOT); mean curvilinear velocity (VCL; μm/s); mean average-path velocity (VAP;μm/s); mean straight-line velocity (VSL; μm/s), and linearity ([VSL/VCL]x100; % ;LIN).

Sperm Chromatin Structure Assay

This assay was performed as previously described [29-31]. Individual semen samples were thawed in a water bath set at 35-37 ^{o}C. A five-μl aliquot of thawed semen was combined with 195 μl of a buffered solution which was then cobined with a low pH (~ 1.2) solution (400 μl) for 30 s. A solution of the heterochromatic dye, acridine orange, was added (1.2 ml at 4.0 μg/ml) to the sample and it was processed immediately on a flow cytometer (FACScan; Becton Dickinson, Mountain View, CA, USA). The sample was allowed to pass through the tubing for 2 min before evaluation of cells. A cell flow rate of 200 cells/s was used and a total of 5000 events were evaluated per sample. The flow cytometer was adjusted so that the mean green fluorescence was set at 500 channels (FL-1 @ 500) and mean red fluorescence at 150 channels (FL-3 @ 150)

fluorescence. Data were acquired in a list-mode and translated by WinList™ software (Verity Software House, Topsham, ME, USA) to PC-files, then analyzed using the same software. Quantification of DNA denaturation in each cell was determined by the term alpha-t, which is defined as the ratios of red/red + green fluorescence. The alpha-t designation is used to describe the relationship between the amounts of green (double-stranded DNA) and red (single-stranded DNA) fluorescence. The results were recorded as both scattergrams and frequency histographs. The endpoint, percent COMPαt (COMP; the percentage of cells outside the main population) was determined by selecting those cells to the right of the main population, and represents the number of cells outside the main population, as a percentage of the total number of cells evaluated.

Evaluation of Sperm Acrosomal and Plasma Membrane Integrity

The integrity of spermatozoal acrosomal and plasma membranes was evaluated using a modification of procedures described previously [32-35].

Fifty µl of thawed semen were added to 133 µl of Dulbecco's phosphate buffered saline (PBS; Invitrogen Gibco,® Carlsbad, CA, USA). Three µl of propidium iodide (Invitrogen Molecular Probes, Euigene, OR, USA; 2.4 mM working solution) and 10 µl Pisum sativum agglutinin (PSA)-FITC conjugate (Sigma-Aldrich, St. Louis, MO, USA; 0.05 mg/ml working solution) were added to the semen-buffer solution. The samples were incubated at room temperature (approximately 25 ^{o}C) in the dark for 10 minutes. Fifty µl of semen were then mixed with 1 ml PBS and processed immediately on a flow

cytometer (FACScan; Becton Dickinson, Mountain View, CA, USA). The sample was allowed to pass through the tubing for 30 s before evaluation of cells. A cell flow rate of 300 cells/s was used and a total of 5000 events were evaluated per sample. The voltage settings on the flow cytometer were as follows: SSC 240, FL1 798, FL2 657, and FL3 150. The compensation was set at FL1 1.9% of FL2, and FL2 18.8% of FL1. Data were acquired using a log scale and anylazed by WinList™ software, with scatterplots divided into quadrants: minimal green and red fluorescence (representing spermatozoa with intact plasma membrane and intact acrosomal membrane; AIVIAB); minimal green and enhanced red fluorescence (representing spermatozoa with damaged plasma membrane and intact acrosomal membrane; minimal red and enhanced green fluorescence (representing spermatozoa with intact plasma membrane and damaged acrosomal membrane); and enhanced red and greed fluorescence (representing spermatozoa with damaged plasma membrane and damaged acrosomal membrane). Data were also sorted by spermatozoa with intact plasma membrane, regardless of acrosomal status (VIAB) and spermatozoa with intact acrosomal membrane, regardless of plasma membrane status (AI).

Statistical Analysis

The effects of extender (LE, MF), pre-freeze cooling rate (SLOW, FAST), and post-thaw diluent (INRA96, FM) on 10 experimental endpoints (MOT, PMOT, VCL, VAP, VSL, LIN, AIVIAB, AI, VIAB, and COMP) were evaluated using a general linear

model [36]. Variables measured in percentages were transformed to angles corresponding to *arc sine of the square root of percentage* for variance analyses. The Student-Newman-Keuls multiple range test was used to separate main effect means when treatment F ratios were significant ($P<0.05$) and for mean separation where significant interactions occurred ($P<0.05$). The linear relationships among post-thaw dependent variables and among cooling and freezing effects on dependent variables were evaluated using a general correlations procedure [36].

RESULTS

The main effects of cryopreservation extender (LE vs MF), pre-freeze cooling rate (SLOW vs FAST) and post-thaw diluent (INRA96 vs FM) are presented in Table 1. Overall, eight of ten experimental endpoints (MOT, PMOT, VAP, VSL, LIN AIVIAB, AI, and VIAB) were affected by extender type, with LE extender yielding higher values than MF extender for all of these outcomes ($P<0.05$). Mean VCL and COMP were not impacted by extender type ($P>0.05$). Exposure of extended semen to a slow pre-freeze cooling period resulted in increased values for six of ten endpoints, as compared to a fast pre-freeze cooling period ($P<0.05$). Mean VAP, VSL, COMP were unaffected by pre-freeze cooling rate ($P>0.05$). Mean LIN was slightly greater for semen exposed to a fast pre-freeze cooling period ($P<0.05$). Post-thaw diluent resulted in significant differences in seven of 10 endpoints, with INRA96 yielding higher mean values than FM for MOT, PMOT, VCL, VAP, and VSL ($P<0.05$). Treatment group FM yielded slightly higher values than INRA96 for LIN and VIAB ($P<0.05$). A treatment effect was not detected for AIVIAB, AI, and COMP ($P>0.05$).

Significant extender x rate interactions ($P<0.05$) were detected for the variables MOT, AIVIAB, AI and VIAB. Mean values for these endpoints were higher following spermatozoal exposure to a SLOW pre-freeze cooling period, regardless of freezing extender type ($P<0.05$). The effect of pre-freezing cooling rate on MOT, AIVIAB, AI, and VIAB appeared to be more pronounced in spermatozoa cryopreserved in MF

extender, as compared to LE extender (Table 2). Within treatment groups SLOW and FAST, mean MOT, AIVIAB, AI, and VIAB were higher ($P<0.05$) for spermatozoa cryopreserved in LE extender, as opposed to MF extender (Table 3). The effect of extender type appeared to be more pronounced when spermatozoa were subjected to a FAST cooling rate.

Table 1: Main effects of extender, rate, and diluent on mean (±SD) value of spermatozoal motility, viability, acrosomal intactness, and chromatin quality.

Laboratory parameter[§a]	Freezing extender		Pre-freeze cool rate		Post-thaw diluent	
	LE[b]	MF[c]	SLOW[d]	FAST[e]	INRA[f]	FM[g]
MOT	46a (1.1)	26b (1.5)	42a (1.5)	30b(1.6)	40a (1.5)	33b (1.8)
PMOT	20a (1.0)	7b (0.8)	17a (1.2)	10b (1.0)	14a (1.1)	13b (1.2)
VCL	131a (2.4)	131a (3.0)	133a (2.6)	128b (2.9)	137a (2.6)	125b (2.7)
VAP	68a (1.2)	64b (1.5)	67a (1.3)	66a (1.4)	69a (1.2)	64b (1.4)
VSL	57a (1.0)	51b (1.2)	54a (1.1)	54a (1.2)	55a (1.0)	52b (1.3)
LIN	45a (0.6)	41b (0.5)	42b (0.5)	44a (0.7)	42b (0.4)	44a (0.7)
AIVIAB	44a (1.0)	26b (1.4)	42a (1.1)	27b (1.5)	34a (1.5)	35a (1.6)
AI	75a (1.2)	55b (2.5)	77a (0.9)	52b (2.4)	65a (2.3)	65a (2.2)
VIAB	46a (1.0)	26b (1.4)	43a (1.1)	28b (1.6)	35b (1.6)	37a (1.7)
$COMP_{\alpha t}$	8a (0.3)	7a (0.3)	7a (0.3)	8a (0.4)	7a (0.3)	7a (0.3)

[§]Percentage data (MOT, PMOT, LIN, AIVIAB, AI, VIAB, $COMP_{\alpha t}$) were arc sine-root transformed for normalization prior to statistical analysis; untransformed data are presented for ease of interpretation. Within extender, rate, diluent, and within laboratory parameter, means with different letters (a and b) differ ($P < 0.05$).

[a] MOT: total spermatozoal motility (%); PMOT: progressive spermatozoal motility (%); VCL: curvilinear velocity (μm/s); VAP: average-path velocity (μm/s); VSL: straight-line velocity (μm/s); LIN: linearity ([VSL/VCL]100; %); AIVIAB: acrosome intact viable spermatozoa (%); AI: acrosome intact spermatozoa (%); VIAB: viable spermatozoa (%); $COMP_{\alpha t}$: percentage of spermatozoa with αt value outside the main population (%).

[b] LE = E-Z Freezin™ "LE" semen extender (n=84).

[c] MF = E-Z Freezin™ "MFR5" semen extender (n=84).

[d] SLOW = Slow pre-freeze cooling rate (n=88).

[e] FAST = Fast pre-freeze cooling rate (n=80).

[f] INRA = INRA 96 semen extender used as post-thaw diluent (n=84).

[g] FM = Freezing Media ("LE" or "MFR5" semen extender) used as post-thaw diluent (n=84).

Table 2: Effects of pre-freeze cooling rate on mean (±SD) value of spermatozoal motility, viability, acrosomal intactness, and chromatin quality as sorted by extender type.

Laboratory parameter[§a]	Freezing extender	Pre-freeze cool rate		Freezing extender	Pre-freeze cool rate	
	LE	SLOW[b]	FAST[c]	MF	SLOW[d]	FAST[e]
MOT	LE	51a (1.4)	42b (1.3)	MF	33a (2.0)	18b (1.5)
PMOT	LE	23a (1.5)	16a (1.2)	MF	11a (1.3)	4a (0.6)
VCL	LE	134a (3.4)	127a (3.4)	MF	133a (3.8)	129a (4.7)
VAP	LE	69a (1.6)	67a (1.7)	MF	64a (2.0)	65a (2.3)
VSL	LE	57a (1.4)	56a (1.5)	MF	51a (1.6)	51a (1.8)
LIN	LE	44a (0.7)	46a (0.8)	MF	40a (0.6)	42a (0.9)
AIVIAB	LE	48a (1.2)	39b (1.1)	MF	35a (1.2)	15b (1.1)
AI	LE	78a (1.5)	71b (1.6)	MF	75a (1.1)	34b (2.0)
VIAB	LE	51a (1.1)	41b (1.2)	MF	36a (1.2)	16b (1.1)
$COMP_{\alpha t}$	LE	7a (0.4)	8a (0.5)	MF	7a (0.4)	8a (0.6)

[§]Percentage data (MOT, PMOT, LIN, AIVIAB, AI, VIAB, $COMP_{\alpha t}$) were arc sine-root transformed for normalization prior to statistical analysis; untransformed data are presented for ease of interpretation. Within extender, rate, and within laboratory parameter, means with different letters (a and b) differ ($P < 0.05$).

[a] MOT: total spermatozoal motility (%); PMOT: progressive spermatozoal motility (%); VCL: curvilinear velocity (μm/s); VAP: average-path velocity (μm/s); VSL: straight-line velocity (μm/s); LIN: linearity ([VSL/VCL]100; %); AIVIAB: acrosome intact viable spermatozoa (%); AI: acrosome intact spermatozoa (%); VIAB: viable spermatozoa (%); $COMP_{\alpha t}$: percentage of spermatozoa with αt value outside the main population (%).

[b] LE SLOW = semen frozen in E-Z Freezin™ "LE" semen extender using the static vapor method with a slow pre-freeze cooling rate applied (n=44).

[c] LE FAST = semen frozen in E-Z Freezin™ "LE" semen extender using the static vapor method with no pre-freeze cooling rate applied (n=40).

[d] MF SLOW = semen frozen in E-Z Freezin™ "MFR5" semen extender using the static vapor method with a slow pre-freeze cooling rate applied (n=44).

[e] MF FAST = semen frozen in E-Z Freezin™ "MFR5" semen extender using the static vapor method with no pre-freeze cooling rate applied (n=40).

Table 3: Effects of extender type on mean (±SD) value of spermatozoal motility, viability, acrosomal intactness, and chromatin quality as sorted by pre-freeze cooling rate.

Laboratory parameter[§a]	Pre-freeze cool rate	Freezing extender		Pre-freeze cool rate	Freezing extender	
	FAST	LE[b]	MF[c]	SLOW	LE[d]	MF[e]
MOT	FAST	42a (1.3)	18b (1.5)	SLOW	51a (1.4)	33b (2.0)
PMOT	FAST	16a (1.2)	4a (0.6)	SLOW	23a (1.5)	11a (1.3)
VCL	FAST	127a (3.4)	129a (4.7)	SLOW	134a (3.4)	133a (3.8)
VAP	FAST	67a (1.7)	65a (2.3)	SLOW	69a (1.6)	64a (2.0)
VSL	FAST	56a (1.5)	51a (1.8)	SLOW	57a (1.4)	51a (1.6)
LIN	FAST	46a (0.8)	42a (0.9)	SLOW	44a (0.7)	40a (0.6)
AIVIAB	FAST	39a (1.1)	15b (1.1)	SLOW	48a (1.2)	35b (1.2)
AI	FAST	71a (1.6)	34b (2.0)	SLOW	78a (1.5)	75b (1.1)
VIAB	FAST	41a (1.2)	16b (1.1)	SLOW	51a (1.1)	36b (1.2)
$COMP_{\alpha t}$	FAST	8a (0.5)	8a (0.6)	SLOW	7a (0.4)	7a (0.4)

[§]Percentage data (MOT, PMOT, LIN, AIVIAB, AI, VIAB, $COMP_{\alpha t}$) were arc sine-root transformed for normalization prior to statistical analysis; untransformed data are presented for ease of interpretation. Within extender, rate, and within laboratory parameter, means with different letters (a and b) differ ($P < 0.05$).

[a] MOT: total spermatozoal motility (%); PMOT: progressive spermatozoal motility (%); VCL: curvilinear velocity (μm/s); VAP: average-path velocity (μm/s); VSL: straight-line velocity (μm/s); LIN: linearity ([VSL/VCL]100; %); AIVIAB: acrosome intact viable spermatozoa (%); AI: acrosome intact spermatozoa (%); VIAB: viable spermatozoa (%); $COMP_{\alpha t}$: percentage of spermatozoa with αt value outside the main population (%).

[b] LE FAST = semen frozen in E-Z Freezin™ "LE" semen extender using the static vapor method with no pre-freeze cooling rate applied (n=40).

[c] MF FAST = semen frozen in E-Z Freezin™ "MFR5" semen extender using the static vapor method with no pre-freeze cooling rate applied (n=40).

[d] LE SLOW = semen frozen in E-Z Freezin™ "LE" semen extender using the static vapor method with a slow pre-freeze cooling rate applied (n=44).

[e] MF SLOW = semen frozen in E-Z Freezin™ "MFR5" semen extender using the static vapor method with a slow pre-freeze cooling rate applied (n=44).

Significant extender x diluent interactions ($P<0.05$) were detected for MOT, PMOT, VCL, VAP, VSL, and LIN. Within Group MF, mean MOT, PMOT, VCL,

VAP, and VSL were higher in INRA96 diluent, as compared to FM diluent ($P<0.05$). Within Group LE, FM diluent yielded slightly higher values than INRA96 diluent for PMOT, VAP, VSL, and LIN ($P<0.05$; Table 4).

Table 4: Effects of post-thaw diluent on mean (±SD) value of spermatozoal motility, viability, acrosomal intactness, and chromatin quality as sorted by extender type.

Laboratory parameter[§a]	Freezing extender	Post-thaw diluent		Freezing extender	Post-thaw diluent	
	LE	INRA[b]	FM[c]	MF	INRA[d]	FM[e]
MOT	LE	47a (1.4)	46a (1.6)	MF	33a (2.2)	20b (1.4)
PMOT	LE	18b (1.4)	21a (1.4)	MF	11a (1.4)	4b (0.5)
VCL	LE	132a (3.3)	130a (3.6)	MF	143a (3.8)	120b (4.0)
VAP	LE	67b (1.6)	70a (1.7)	MF	71a (1.8)	58b (1.9)
VSL	LE	55b (1.4)	59a (1.4)	MF	56a (1.5)	46b (1.6)
LIN	LE	43b (0.6)	47a (0.8)	MF	41a (0.5)	40a (0.9)
AIVIAB	LE	43a (1.4)	44a (1.5)	MF	26a (1.9)	26a (1.9)
AI	LE	75a (1.6)	75a (1.7)	MF	55a (3.6)	56a (3.6)
VIAB	LE	44a (1.4)	48a (1.3)	MF	26a (1.9)	27a (1.9)
$COMP_{\alpha t}$	LE	8a (0.5)	8a (0.5)	MF	7a (0.5)	7a (0.5)

[§]Percentage data (MOT, PMOT, LIN, AIVIAB, AI, VIAB, $COMP_{\alpha t}$) were arc sine-root transformed for normalization prior to statistical analysis; untransformed data are presented for ease of interpretation. Within extender, diluent, and within laboratory parameter, means with different letters (a and b) differ ($P < 0.05$).

[a] MOT: total spermatozoal motility (%); PMOT: progressive spermatozoal motility (%); VCL: curvilinear velocity (μm/s); VAP: average-path velocity (μm/s); VSL: straight-line velocity (μm/s); LIN: linearity ([VSL/VCL]100; %); AIVIAB: acrosome intact viable spermatozoa (%); AI: acrosome intact spermatozoa (%); VIAB: viable spermatozoa (%); $COMP_{\alpha t}$: percentage of spermatozoa with αt value outside the main population (%).

[b] LE INRA = semen frozen in E-Z Freezin™ "LE" semen extender and diluted in INRA 96 semen extender for post-thaw analysis (n=42).

[c] LE FM = semen frozen in E-Z Freezin™ "LE" semen extender and diluted in E-Z Freezin™ "LE" semen extender for post-thaw analysis (n=42).

[d] MF INRA = semen frozen in E-Z Freezin™ "MFR5" semen extender and diluted in INRA 96 semen extender for post-thaw analysis (n=42).

[e] MF FM = semen frozen in E-Z Freezin™ "MFR5" semen extender and diluted in E-Z Freezin™ "MFR5" semen extender for post-thaw analysis (n=42).

Significant rate x diluent interactions (P<0.05) were detected for MOT, PMOT, and LIN.(P<0.05), as revealed in Table 5. Within Group SLOW, mean values for MOT and PMOT were higher for INRA96 than for FM (P<0.05). Within Group FAST, mean MOT was higher in INRA96 than FM (P<0.05), whereas mean LIN was slightly higher in FM than in INRA96 (P<0.05).

For post-thaw data, mean CASMA values for MOT and PMOT were highly correlated (P<0.05) to FC values for AIVIAB, AI, and VIAB (Table 6). The correlations among dependent variables were generally more pronounced when the dataset included all treatments (N=168). When data were sorted to provide the optimal semen treatment (*ie*, LE extender, SLOW cooling rate, and INRA96 diluent; N=22), the number of significant correlations between variables was reduced. This was attributed, to a large part, to the smaller sample size and reduced variability for individual endpoints (Table 7).

The linear relationships of T0 to T24 (*ie*, immediate to cool-stored) spermatozoal measures were as follows: MOT (r = 0.86, P<0.0001), PMOT (r = 0.88, P<0.0001), VCL (r = 0.79, P<0.0001), VAP (r = 0.74, P<0.0001), VSL (r = 0.82, P<0.0001), LIN (r = 0.87, P<0.0001), and COMP (r = 0.71, P<0.0001). The T0 MOT was also highly correlated with T0 PMOT and T24 PMOT (r = 0.80; P<0.0001 and r = 0.75, P<0.0001, respectively). The T0 COMP was not correlated with other T0 values (P>0.05), but was negatively correlated with T24 PMOT (r = -0.44; P=0.03).

Table 5: Effects of post-thaw diluent on mean (±SD) value of spermatozoal motility, viability, acrosomal intactness, and chromatin quality as sorted by pre-freeze cooling rate.

Laboratory parameter[§a]	Pre-freeze cool rate	Post-thaw diluent		Pre-freeze cool rate	Post-thaw diluent	
	FAST	INRA[b]	FM[c]	SLOW	INRA[d]	FM[e]
MOT	FAST	32a (2.3)	28b (2.3)	SLOW	47a (1.4)	37b (2.6)
PMOT	FAST	10a (1.3)	10a (1.5)	SLOW	19a (1.4)	15b (1.9)
VCL	FAST	135a (3.7)	122a (4.2)	SLOW	139a (3.5)	127a (3.5)
VAP	FAST	68a (1.7)	64a (2.2)	SLOW	70a (1.8)	64a (1.8)
VSL	FAST	54a (1.4)	53a (2.0)	SLOW	56a (1.5)	52a (1.7)
LIN	FAST	42b (0.7)	45a (1.1)	SLOW	42a (0.5)	42a (0.9)
AIVIAB	FAST	27a (2.2)	27a (2.1)	SLOW	41a (1.5)	42a (1.6)
AI	FAST	52a (3.6)	53a (3.4)	SLOW	77a (1.3)	77a (1.4)
VIAB	FAST	27a (2.2)	30a (2.4)	SLOW	42a (1.5)	44a (1.7)
$COMP_{\alpha t}$	FAST	8a (0.5)	8a (0.5)	SLOW	7a (0.4)	7a (0.4)

[§]Percentage data (MOT, PMOT, LIN, AIVIAB, AI, VIAB, $COMP_{\alpha t}$) were arc sine-root transformed for normalization prior to statistical analysis; untransformed data are presented for ease of interpretation. Within rate, diluent, and within laboratory parameter, means with different letters (a and b) differ ($P < 0.05$).

[a] MOT: total spermatozoal motility (%); PMOT: progressive spermatozoal motility (%); VCL: curvilinear velocity (μm/s); VAP: average-path velocity (μm/s); VSL: straight-line velocity (μm/s); LIN: linearity ([VSL/VCL]100; %); AIVIAB: acrosome intact viable spermatozoa (%); AI: acrosome intact spermatozoa (%); VIAB: viable spermatozoa (%); $COMP_{\alpha t}$: percentage of spermatozoa with αt value outside the main population (%).

[b] FAST INRA = semen frozen using static vapor method with no pre-freeze cooling rate applied and diluted in INRA 96 semen extender for post-thaw analysis (n=40).

[c] FAST FM = semen frozen using static vapor method with no pre-freeze cooling rate applied and diluted with the same freezing media ("LE" or "MFR5" semen extender) for post-thaw analysis (n=40).

[d] SLOW INRA = semen frozen using static vapor method with a slow pre-freeze cooling rate applied and diluted in INRA 96 semen extender for post-thaw analysis (n=44).

[e] SLOW FM = semen frozen using static vapor method with a slow pre-freeze cooling rate applied and diluted with the same freezing media ("LE" or "MFR5" semen extender) for post-thaw analysis (n=44).

Table 6
Correlation coefficients and p-values among 10 dependent variables over all treatments (all data; N=168) and for semen exposed to LE extender, using a slow pre-freeze cooling rate, and using INRA 96 as a post-thaw diluent (N=22).

LE extender / SLOW rate / INRA diluent (N=22)

	MOT	PMOT	VCL	VAP	VSL	LIN	AIVIAB	AI	VIAB	COMP	
MOT		0.90 0.0001	0.27 0.0004	0.39 0.0001	0.48 0.0001	0.28 0.0002	0.78 0.0001	0.69 0.0001	0.79 0.0001	-0.18 0.02	MOT
PMOT	0.94 0.0001		0.31 0.0001	0.47 0.0001	0.56 0.0001	0.36 0.0001	0.69 0.0001	0.59 0.0001	0.69 0.0001	-0.18 0.02	PMOT
VCL	NS	NS		0.93 0.0001	0.83 0.0001	-0.32 0.0001	NS	NS	NS	NS	VCL
VAP	NS	NS	0.96 0.0001		0.96 0.0001	NS	NS	NS	NS	-0.18 0.02	VAP
VSL	NS	NS	0.87 0.0001	0.97 0.0001		0.22 0.003	0.25 0.001	0.23 0.003	0.24 0.002	-0.21 0.005	VSL
LIN	0.51 0.01	0.54 0.01	NS	NS	NS		0.19 0.01	NS	0.20 0.008	0.19 0.01	LIN
AIVIAB	0.66 0.0008	0.56 0.007	NS	NS	NS	NS		0.81 0.0001	0.98 0.0001	NS	AIVIAB
AI	0.50 0.02	0.59 0.004	NS	NS	NS	NS	0.44 0.04		0.79 0.0001	NS	AI
VIAB	0.66 0.0008	0.55 0.009	NS	NS	NS	NS	0.99 0.0001	NS		NS	VIAB
COMP	-0.46 0.03	-0.49 0.02	NS	NS	NS	-0.46 0.03	NS	NS	NS		COMP
	MOT	PMOT	VCL	VAP	VSL	LIN	AIVIAB	AI	VIAB	COMP	

All data (N=168)

[§]LE = E-Z Freezin™ "LE" semen extender
INRA 96 = INRA 96 semen extender

Table 7
Comparison of values (Mean, S.D., Minimum, Maximum) for dependent variables for the complete post-thaw dataset which included all treatments (N=188) and the post-thaw dataset selected to optimize post-thaw spermatozoal quality (LE/ SLOW/ INRA post-thaw dataset; N=22).

	Complete post-thaw dataset (N=168)				LE / SLOW / INRA post-thaw dataset (N=22)			
	Mean	S.D.	Minimum	Maximum	Mean	S.D.	Minimum	Maximum
MOT	36	16	4	72	50	9	30	71
PMOT	14	11	0	49	21	10	6	49
VCL	131	25	71	190	135	21	98	181
VAP	66	12	39	96	67	11	48	91
VSL	54	11	30	78	56	10	40	77
LIN	43	5	31	62	42	4	36	49
AIVIAB	35	14	4	67	48	7	26	57
AI	65	21	13	94	79	9	59	94
VIAB	36	15	4	68	49	7	27	58
COMP	7	3	3	16	7	3	3	14

[§]LE/SLOW/INRA = treatment group where semen was frozen using E-Z Freezin™ "LE" semen extender, applied a slow pre-freeze cooling rate, and diluted in INRA 96 for post-thaw analysis.
[§]MOT: total spermatozoal motility (%); PMOT: progressive spermatozoal motility (%); VCL: curvilinear velocity (μm/s); VAP: average-path velocity (μm/s); VSL: straight-line velocity (μm/s); LIN: linearity ([VSL/VCL]100; %); AIVIAB: acrosome intact viable spermatozoa (%); AI: acrosome intact spermatozoa (%); VIAB: viable spermatozoa (%); $COMP_{\alpha t}$: percentage of spermatozoa with αt value outside the main population (%).

Over all data, initial (T0) CASMA values, cool-stored (T24) CASMA values, and percent change in CASMA values following cooled storage (DIFF) did not provide

useful predictive information regarding tolerance of spermatozoa to cryopreservation, as determined by all post-thaw CASMA and FC endpoints measured (Table 8). The majority of correlations between pre-freeze and post-thaw values were not significant ($P>0.05$), and 26 of 47 significant ($P<0.05$) correlations were of lower magnitude (correlation coefficient less than 30). Data separation to provide optimal post-thaw semen quality (*ie*, LE extender / SLOW cooling rate / INRA diluent) yielded 42 significant correlations ($P<0.05$) between pre-freeze and post-thaw endpoints, and all significant correlations were moderate to high (*ie*, correlation coefficients ranging from 0.42 to 0.81; Table 9). In this data subset, the actual pre-freeze values for MOT, PMOT, VCL or COMP, either initially or following cooled storage of extended semen, provided better predictive information regarding post-thaw measures than did the percent change in these variables following cooled storage of extended semen. (Table 9).

Tables 10-21 represent correlation coefficients and p-values for fresh (T0, T24, DIFF) semen values and post-thaw endpoints for the individual treatment groups. The four fresh semen values used to show predictability on post-thaw endpoints were MOT, PMOT, VCL, and COMP. Overall, when evaluating the eight treatment groups there were only a few highly significant correlations (*ie*, correlation coefficients ranging from ~0.60 to ~0.80). For T0 fresh semen values, MOT and COMP showed the highest predictive value on post-thaw AI and COMP for the majority of treatment groups (Table 10 and Table 13). As for the T24 fresh semen values, MOT had a highly significant

correlation with post-thaw AI (Table 14). There was also a high predictability of T24 PMOT on post-thaw LIN and AI (Table 15). The final significant correlation that offered appreciable predictive value across the majority of treatment groups was seen between T24 VCL and post-thaw VCL (Table 16).

Table 8
Correlation coefficients and p-values to determine effect of pre-freeze motility and chromatin values (T0, T24, DIFF) on post-thaw measures of semen quality following spermatozoal exposure to LE or MF extender, a SLOW or FAST pre-freeze cooling rate, and INRA 96 or FM diluent (N=168).

	TO MOT	TO PMOT	TO VCL	TO COMP	T24 MOT	T24 PMOT	T24 VCL	T24 COMP	DIFF MOT	DIFF PMOT	DIFF VCL	DIFF COMP
MOT	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
PMOT	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	-0.19 0.01	NS
VCL	-0.17 0.03	-0.31 0.0001	0.29 0.0001	NS	-0.17 0.03	-0.33 0.0001	0.56 0.0001	-0.20 0.008	NS	NS	-0.39 0.0001	NS
VAP	NS	-0.20 0.009	NS	-0.18 0.02	NS	-0.19 NS	0.42 0.0001	-0.32 0.0001	NS	NS	-0.39 0.0001	NS
VSL	NS	NS	NS	-0.18 0.02	NS	NS	0.30 0.0001	-0.37 0.0001	NS	-0.18 0.02	-0.38 0.0001	NS
LIN	0.16 0.04	0.38 0.0001	-0.40 0.0001	-0.31 0.0001	0.25 0.0009	0.47 0.0001	-0.37 0.0001	-0.30 0.0001	NS	NS	NS	NS
AIVIAB	NS	NS	-0.18 0.01	NS	NS	NS	NS	-0.15 0.04	NS	-0.19 0.01	NS	NS
AI	0.19 0.02	NS	NS	NS	0.22 0.005	0.23 0.003	NS	-0.18 0.02	NS	-0.17 0.03	NS	NS
VIAB	NS	NS	NS	NS	NS	NS	NS	NS	NS	-0.16 0.04	NS	NS
COMP	-0.40 0.0001	-0.47 0.0001	NS	0.57 0.0001	-0.29 0.0002	-0.48 0.0001	NS	0.48 0.0001	-0.19 0.01	NS	0.24 0.005	NS

[§]LE = E-Z Freezin™ "LE" semen extender.
MF = E-Z Freezin™ "MFR5" semen extender.
INRA 96 = INRA 96 semen extender.
FM = Freezing Media ("LE" or "MFR5" semen extender) used as diluent.
[a] T0 = Fresh semen evaluated prior to cooling.
[b] T24 = Semen stored in an Equitainer® for 24 hours.
[c] DIFF = Difference between T0 and T24.

Table 9
Correlation coefficients and p-values to determine effect of pre-freeze motility and chromatin values (T0, T24, and DIFF) on post-thaw measures of semen quality following spermatozoal exposure to LE extender, a SLOW pre-freeze cooling rate, and INRA 96 diluent (N=22).

	TO[a] MOT	TO[a] PMOT	TO[a] VCL	TO[a] COMP	T24[b] MOT	T24[b] PMOT	T24[b] VCL	T24[b] COMP	DIFF[c] MOT	DIFF[c] PMOT	DIFF[c] VCL	DIFF[c] COMP
MOT	0.49 0.02	0.45 0.03	NS	-0.47 0.03	0.54 0.01	0.53 0.01	NS	-0.43 0.04	NS	NS	NS	NS
PMOT	0.51 0.01	0.44 0.04	NS	-0.42 0.04	0.58 0.005	0.56 0.007	NS	-0.47 0.03	NS	NS	NS	NS
VCL	NS	NS	0.43 0.04	NS	NS	-0.43 0.04	0.73 0.0001	NS	NS	NS	-0.43 0.04	NS
VAP	NS	NS	NS	NS	NS	NS	0.65 0.001	-0.46 0.03	NS	NS	-0.44 0.04	NS
VSL	NS	NS	NS	NS	NS	NS	0.54 0.009	-0.54 0.009	NS	NS	-0.45 0.03	NS
LIN	NS	0.48 0.02	-0.43 0.04	-0.52 0.01	NS	0.64 0.001	NS	-0.62 0.002	NS	NS	NS	NS
AIVIAB	NS	NS	NS	-0.44 0.04	NS	NS	NS	-0.44 0.04	NS	-0.50 0.02	NS	NS
AI	0.76 0.0001	0.52 0.01	NS	NS	0.81 0.0001	0.70 0.0003	NS	NS	NS	NS	NS	NS
VIAB	NS	NS	NS	-0.48 0.02	NS	NS	NS	-0.45 0.04	NS	-0.47 0.03	NS	NS
COMP	-0.50 0.02	-0.51 0.02	NS	0.77 0.0001	NS	-0.52 0.01	NS	0.54 0.009	NS	NS	NS	NS

[§]LE = E-Z Freezin™ "LE" semen extender.
SLOW = semen frozen using static vapor with a slow pre-freeze cooling rate applied.
INRA 96 = INRA 96 semen extender used as post-thaw diluent.
[a] T0 = Fresh semen evaluated prior to cooling.
[b] T24 = Semen stored in an Equitainer® for 24 hours.
[c] DIFF = Difference between T0 and T24.

Table 10. Correlation coefficients and p-values for fresh semen total motility (T0-MOT; %) and post-thaw semen measures for stallion spermatozoa.

Post-thaw endpoints	T0-MOT: LE SLOW LE	LE SLOW INRA	LE FAST LE	LE FAST INRA	MF SLOW MF	MF SLOW INRA	MF FAST MF	MF FAST INRA
MOT	0.56 (0.007)	0.50 (0.02)	NS	NS	NS	NS	NS	NS
PMOT	NS	0.51 (0.01)	NS	NS	NS	NS	NS	NS
VCL	NS	NS	NS	NS	NS	NS	NS	NS
VAP	NS	NS	NS	NS	NS	NS	NS	NS
VSL	NS	NS	NS	NS	NS	NS	NS	NS
LIN	NS	NS	NS	NS	NS	NS	NS	NS
AIVIAB	NS	NS	NS	NS	NS	NS	NS	NS
AI	0.59 (0.004)	0.76 (0.0001)	0.60 (0.005)	0.65 (0.002)	0.67 (0.0007)	0.70 (0.0003)	NS	NS
VIAB	0.44 (0.04)	NS	NS	NS	NS	NS	NS	NS
COMP	-0.50 (0.01)	-0.50 (0.02)	-0.50 (0.03)	-0.50 (0.03)	NS	NS	NS	NS

[§]LE/SLOW/LE = treatment group where semen was frozen using E-Z Freezin™ "LE" semen extender, applied a slow pre-freeze cooling rate, and diluted in E-Z Freezin™ "LE" semen extender for post-thaw analysis.
LE/SLOW/INRA = treatment group where semen was frozen using E-Z Freezin™ "LE" semen extender, applied a slow pre-freeze cooling rate, and diluted in INRA 96 semen extender for post-thaw analysis.
LE/FAST/LE = treatment group where semen was frozen using E-Z Freezin™ "LE" semen extender, applied a fast pre-freeze cooling rate, and diluted in E-Z Freezin™ "LE" semen extender for post-thaw analysis.
LE/FAST/INRA = treatment group where semen was frozen using E-Z Freezin™ "LE" semen extender, applied a fast pre-freeze cooling rate, and diluted in INRA 96 semen extender for post-thaw analysis.
MF/SLOW/MF = treatment group where semen was frozen using E-Z Freezin™ "MFR5" semen extender, applied a slow pre-freeze cooling rate, and diluted in E-Z Freezin™ "MFR5" semen extender for post-thaw analysis.
MF/SLOW/INRA = treatment group where semen was frozen using E-Z Freezin™ "MFR5" semen extender, applied a slow pre-freeze cooling rate, and diluted in INRA 96 semen extender for post-thaw analysis.
MF/FAST/MF = treatment group where semen was frozen using E-Z Freezin™ "MFR5" semen extender, applied a fast pre-freeze cooling rate, and diluted in E-Z Freezin™ "MFR5" semen extender for post-thaw analysis.
MF/FAST/INRA = treatment group where semen was frozen using E-Z Freezin™ "MFR5" semen extender, applied a fast pre-freeze cooling rate, and diluted in INRA 96 semen extender for post-thaw analysis.

Table 11. Correlation coefficients and p-values for fresh semen progressive motility (T0-PMOT; %) and post-thaw semen measures for stallion spermatozoa.

Post-thaw endpoints \ T0-PMOT	LE SLOW LE	LE SLOW INRA	LE FAST LE	LE FAST INRA	MF SLOW MF	MF SLOW INRA	MF FAST MF	MF FAST INRA
MOT	0.57 (0.006)	0.45 (0.03)	NS	NS	NS	NS	NS	NS
PMOT	NS	0.44 (0.04)	NS	NS	NS	NS	NS	NS
VCL	NS	NS	NS	NS	NS	NS	NS	NS
VAP	NS	NS	NS	NS	NS	NS	NS	NS
VSL	NS	NS	NS	NS	NS	NS	NS	NS
LIN	0.53 (0.01)	0.48 (0.02)	NS	0.72 (0.0003)	0.61 (0.002)	NS	NS	NS
AIVIAB	NS	NS	NS	NS	NS	NS	-0.46 (0.04)	NS
AI	NS	0.52 (0.01)	NS	0.48 (0.03)	NS	0.46 (0.03)	NS	NS
VIAB	NS	NS	NS	NS	NS	NS	-0.46 (0.04)	NS
COMP	-0.51 (0.02)	-0.51 (0.02)	-0.63 (0.003)	-0.63 (0.003)	NS	NS	NS	NS

Table 12. Correlation coefficients and p-values for fresh semen curvilinear velocity (T0-VCL; µm/s) and post-thaw semen measures for stallion spermatozoa.

Post-thaw endpoints	T0-VCL: LE SLOW LE	LE SLOW INRA	LE FAST LE	LE FAST INRA	MF SLOW MF	MF SLOW INRA	MF FAST MF	MF FAST INRA
MOT	NS	NS	NS	NS	NS	NS	NS	NS
PMOT	NS	NS	NS	NS	NS	NS	NS	NS
VCL	NS	0.43 (0.045)	NS	NS	NS	0.47 (0.03)	NS	NS
VAP	NS	NS	NS	NS	NS	NS	NS	NS
VSL	NS	NS	NS	NS	NS	NS	NS	NS
LIN	-0.61 (0.003)	-0.43 (0.04)	-0.56 (0.01)	NS	-0.66 (0.0008)	-0.49 (0.02)	NS	-0.55 (0.01)
AIVIAB	NS	NS	-0.48 (0.03)	NS	NS	NS	NS	NS
AI	NS	NS	NS	NS	NS	NS	NS	NS
VIAB	NS	NS	NS	NS	NS	NS	NS	NS
COMP	NS	NS	NS	NS	NS	NS	NS	NS

Table 13. Correlation coefficients and p-values for fresh semen chromatin (T0-COMP; %) and post-thaw semen measures for stallion spermatozoa.

Post-thaw endpoints	T0-COMP: LE SLOW LE	LE SLOW INRA	LE FAST LE	LE FAST INRA	MF SLOW MF	MF SLOW INRA	MF FAST MF	MF FAST INRA
MOT	NS	-0.47 (0.03)	NS	NS	NS	NS	NS	NS
PMOT	NS	NS	NS	NS	NS	NS	NS	NS
VCL	NS	NS	NS	NS	NS	NS	NS	NS
VAP	NS	NS	NS	NS	NS	NS	NS	NS
VSL	NS	NS	NS	NS	NS	NS	NS	NS
LIN	NS	-0.52 (0.01)	NS	-0.53 (0.02)	NS	NS	NS	NS
AIVIAB	-0.45 (0.04)	-0.44 (0.04)	NS	NS	NS	NS	NS	NS
AI	NS	NS	NS	NS	NS	NS	NS	NS
VIAB	-0.55 (0.008)	-0.48 (0.02)	NS	NS	NS	NS	NS	NS
COMP	0.77 (0.0001)	0.77 (0.0001)	NS	NS	0.64 (0.001)	0.64 (0.001)	0.67 (0.001)	0.67 (0.001)

Table 14. Correlation coefficients and p-values for cooled semen total motility (T24-MOT; %) and post-thaw semen measures for stallion spermatozoa.

Post-thaw endpoints	T24-MOT: LE SLOW LE	LE SLOW INRA	LE FAST LE	LE FAST INRA	MF SLOW MF	MF SLOW INRA	MF FAST MF	MF FAST INRA
MOT	0.60 (0.003)	0.55 (0.008)	NS	NS	NS	NS	NS	-0.49 (0.03)
PMOT	NS	0.58 (0.005)	NS	NS	NS	NS	NS	NS
VCL	NS	NS	NS	NS	NS	NS	NS	NS
VAP	NS	NS	NS	NS	NS	NS	NS	NS
VSL	NS	NS	NS	NS	NS	NS	NS	NS
LIN	NS	NS	NS	0.57 (0.008)	NS	NS	0.53 (0.02)	NS
AIVIAB	NS	NS	NS	NS	NS	NS	NS	NS
AI	0.69 (0.0004)	0.81 (0.0001)	0.73 (0.0003)	0.75 (0.0001)	0.72 (0.0002)	0.75 (0.0001)	NS	NS
VIAB	NS	NS	NS	NS	NS	NS	NS	NS
COMP	NS	NS	NS	NS	NS	NS	NS	NS

Table 15. Correlation coefficients and p-values for cooled semen progressive motility (T24-PMOT; %) and post-thaw semen measures for stallion spermatozoa.

Post-thaw endpoints	T24-PMOT: LE SLOW LE	LE SLOW INRA	LE FAST LE	LE FAST INRA	MF SLOW MF	MF SLOW INRA	MF FAST MF	MF FAST INRA
MOT	0.62 (0.002)	0.53 (0.01)	NS	NS	NS	NS	NS	NS
PMOT	NS	0.56 (0.007)	NS	NS	NS	NS	NS	NS
VCL	NS	-0.43 (0.04)	-0.45 (0.04)	NS	NS	NS	NS	NS
VAP	NS	NS	NS	NS	NS	NS	NS	NS
VSL	NS	NS	NS	NS	NS	NS	NS	NS
LIN	0.63 (0.002)	0.64 (0.001)	0.50 (0.02)	0.80 (0.0001)	0.65 (0.001)	0.56 (0.006)	NS	0.52 (0.02)
AIVIAB	0.47 (0.03)	NS	NS	NS	NS	NS	NS	NS
AI	0.61 (0.003)	0.70 (0.0003)	0.65 (0.002)	0.70 (0.0006)	0.55 (0.007)	0.60 (0.003)	NS	NS
VIAB	0.52 (0.01)	NS	NS	NS	NS	NS	NS	NS
COMP	-0.52 (0.01)	-0.52 (0.01)	-0.54 (0.01)	-0.54 (0.01)	NS	NS	-0.46 (0.04)	-0.46 (0.04)

Table 16. Correlation coefficients and p-values for cooled semen curvilinear velocity (T24-VCL; μm/s) and post-thaw semen measures for stallion spermatozoa.

Post-thaw endpoints	T24-VCL: LE SLOW LE	LE SLOW INRA	LE FAST LE	LE FAST INRA	MF SLOW MF	MF SLOW INRA	MF FAST MF	MF FAST INRA
MOT	NS	NS	NS	NS	NS	NS	NS	NS
PMOT	NS	NS	NS	NS	NS	NS	NS	NS
VCL	0.60 (0.003)	0.74 (0.0001)	0.51 (0.02)	0.66 (0.002)	0.57 (0.005)	0.75 (0.0001)	NS	0.70 (0.0005)
VAP	0.44 (0.04)	0.65 (0.001)	NS	0.61 (0.004)	NS	0.66 (0.0009)	NS	0.63 (0.003)
VSL	NS	0.54 (0.009)	NS	0.51 (0.02)	NS	0.56 (0.007)	NS	NS
LIN	-0.64 (0.001)	NS	-0.58 (0.008)	NS	-0.45 (0.03)	-0.57 (0.006)	NS	-0.68 (0.001)
AIVIAB	NS	NS	NS	NS	NS	NS	NS	NS
AI	NS	NS	NS	NS	NS	NS	NS	NS
VIAB	NS	NS	NS	NS	NS	NS	NS	NS
COMP	NS	NS	NS	NS	NS	NS	NS	NS

Table 17. Correlation coefficients and p-values for cooled semen chromatin (T24-COMP; %) and post-thaw semen measures for stallion spermatozoa.

Post-thaw endpoints	T24-COMP							
	LE SLOW LE	LE SLOW INRA	LE FAST LE	LE FAST INRA	MF SLOW MF	MF SLOW INRA	MF FAST MF	MF FAST INRA
MOT	NS	NS	NS	NS	NS	NS	NS	NS
PMOT	NS	-0.47 (0.03)	NS	NS	NS	NS	NS	NS
VCL	NS	NS	NS	NS	NS	NS	NS	NS
VAP	NS	-0.46 (0.03)	NS	NS	NS	NS	-0.50 (0.02)	NS
VSL	NS	-0.54 (0.009)	NS	NS	NS	NS	-0.55 (0.01)	NS
LIN	NS	-0.62 (0.002)	NS	NS	NS	NS	NS	NS
AIVIAB	-0.48 (0.02)	-0.44 (0.04)	NS	NS	NS	NS	NS	NS
AI	NS	NS	NS	NS	NS	NS	NS	NS
VIAB	-0.54 (0.0089)	-0.45 (0.04)	NS	NS	NS	NS	NS	NS
COMP	0.54 (0.0093)	0.54 (0.009)	NS	NS	0.70 (0.0003)	0.70 (0.0003)	0.50 (0.02)	0.50 (0.02)

Table 18. Correlation coefficients and p-values for fresh/cooled semen total motility % change (DIFF-MOT; %) and post-thaw semen measures for stallion spermatozoa.

Post-thaw endpoints	DIFF-MOT							
	LE SLOW LE	LE SLOW INRA	LE FAST LE	LE FAST INRA	MF SLOW MF	MF SLOW INRA	MF FAST MF	MF FAST INRA
MOT	NS	NS	NS	NS	NS	NS	NS	NS
PMOT	NS	NS	NS	NS	NS	NS	NS	NS
VCL	NS	NS	NS	NS	NS	NS	NS	NS
VAP	NS	NS	NS	NS	NS	NS	NS	NS
VSL	NS	NS	NS	NS	NS	NS	NS	NS
LIN	NS	NS	NS	NS	NS	NS	NS	NS
AIVIAB	NS	NS	NS	NS	NS	NS	NS	NS
AI	NS	NS	NS	NS	NS	NS	NS	NS
VIAB	NS	NS	NS	NS	NS	NS	NS	NS
COMP	NS	NS	NS	NS	NS	NS	NS	NS

Table 19. Correlation coefficients and p-values for fresh/cooled semen progressive motility % change (DIFF-MOT; %) and post-thaw semen measures for stallion spermatozoa.

Post-thaw endpoints	DIFF-PMOT: LE SLOW LE	LE SLOW INRA	LE FAST LE	LE FAST INRA	MF SLOW MF	MF SLOW INRA	MF FAST MF	MF FAST INRA
MOT	NS	NS	NS	NS	NS	NS	NS	NS
PMOT	NS	NS	NS	NS	NS	NS	NS	NS
VCL	NS	NS	NS	NS	NS	NS	NS	NS
VAP	NS	NS	NS	NS	NS	NS	NS	NS
VSL	NS	NS	NS	NS	NS	NS	NS	NS
LIN	NS	NS	NS	NS	NS	NS	NS	NS
AIVIAB	0.52 (0.01)	0.50 (0.02)	NS	NS	NS	NS	NS	NS
AI	0.45 (0.03)	NS	NS	NS	NS	NS	NS	NS
VIAB	NS	0.47 (0.03)	NS	NS	NS	NS	NS	NS
COMP	NS	NS	NS	NS	NS	NS	NS	NS

Table 20. Correlation coefficients and p-values for fresh/cooled semen curvilinear velocity % change (DIFF-VCL; µm/s) and post-thaw semen measures for stallion spermatozoa.

Post-thaw endpoints	DIFF-VCL							
	LE SLOW LE	LE SLOW INRA	LE FAST LE	LE FAST INRA	MF SLOW MF	MF SLOW INRA	MF FAST MF	MF FAST INRA
MOT	NS	NS	NS	NS	NS	NS	NS	NS
PMOT	NS	NS	NS	NS	NS	NS	NS	NS
VCL	0.46 (0.03)	0.43 (0.04)	NS	NS	NS	0.48 (0.03)	NS	0.51 (0.02)
VAP	0.48 (0.02)	0.44 (0.04)	NS	NS	0.48 (0.02)	0.47 (0.03)	NS	0.53 (0.02)
VSL	0.48 (0.02)	0.45 (0.03)	NS	NS	0.49 (0.02)	0.47 (0.03)	NS	NS
LIN	NS	NS	NS	NS	NS	NS	NS	NS
AIVIAB	NS	NS	NS	NS	NS	NS	NS	NS
AI	NS	NS	NS	NS	NS	NS	NS	NS
VIAB	NS	NS	NS	NS	NS	NS	NS	NS
COMP	NS	NS	NS	NS	-0.45 (0.03)	-0.45 (0.03)	NS	NS

Table 21. Correlation coefficients and p-values for fresh/cooled semen chromatin % change (DIFF-COMP; %) and post-thaw semen measures for stallion spermatozoa.

Post-thaw endpoints	DIFF-COMP							
	LE SLOW LE	LE SLOW INRA	LE FAST LE	LE FAST INRA	MF SLOW MF	MF SLOW INRA	MF FAST MF	MF FAST INRA
MOT	NS	NS	NS	NS	NS	NS	NS	NS
PMOT	NS	NS	NS	NS	NS	NS	NS	NS
VCL	NS	NS	NS	NS	NS	NS	NS	NS
VAP	NS	NS	NS	NS	NS	NS	NS	NS
VSL	NS	NS	NS	NS	NS	NS	NS	NS
LIN	NS	NS	NS	NS	NS	NS	NS	NS
AIVIAB	NS	NS	NS	NS	NS	NS	NS	NS
AI	NS	NS	NS	NS	NS	NS	NS	NS
VIAB	NS	NS	NS	NS	NS	NS	NS	NS
COMP	NS	NS	NS	NS	NS	NS	NS	NS

DISCUSSION AND SUMMARY

Several groups have reported optimal cooling rates and storage temperatures for maximizing the viability and fertilizing capacity of cool-stored spermatozoa [3,37-50]. Only limited information, however, is available regarding the effects of a pre-freeze cooling rate on equine semen subjected to cryopreservation [1,14]. Data evolving from cooled-semen trials indicate that equine spermatozoa are tolerant of rapid cooling rates to approximately 20 ^{o}C, but become susceptible to injury when rapid cooling rates are applied to temperatures between 20 ^{o}C and 4 ^{o}C [44]. Such injury is attributable to disruption in membrane functional states resulting from phase separation events in lipid bilayers [51-53] and alterations in water transport properties [54]. This study was conducted to evaluate the effect of two freezing extender types and two pre-freeze cooling rates on post-thaw spermatozoal function.

Our experimental data indicate that LE extender provided superior results to MFR5 extender when used for cryopreservation of semen from eight experimental stallions. In addition, a slower pre-freeze cooling rate also yielded better results than a fast pre-freeze cooling rate, based on several post-thaw spermatozoal characteristics with the LE-SLOW treatment ranking highest, when compared to the other three treatments. This finding contradicts unpublished claims that milk-based freezing extender provides better results when a slow pre-freeze cooling rate is applied, but that post-thaw viability of spermatozoa in egg yolk-based extender might be optimized when using a fast pre-

freeze cooling rate [15]. Standardization and reduction of the glycerol concentration to 2.5% (v/v) in both LE and MFR5 may have led to the similar response of spermatozoa in these two extender types of pre-freeze cooling conditions. Although this glycerol concentration is consistent with recent recommendations in the literature [5,11,19], previous studies have been conducted with glycerol concentration in the range of 4-5% [1,18], and MFR5 and LE extenders are currently available commercially with glycerol concentrations usually set at 4% and 4.75%, respectively (Animal Reproduction Systems, personal communication). The slow pre-freeze cooling rate allows for a gradual decrease in storage temperature to approximately 4 ^{o}C, thus reducing the potentially detrimental effects of cold shock on spermatozoal membranous structures [37,40]. Application of a slow cooling rate before freezing, combined with an increased concentration of egg yolk in LE extender (20%), as opposed to MFR5 extender (3%) may have increased the resistance of the outer acrosomal membrane and overlying plasma membrane to cooling and cryoinjury. Others have reported that the anterior segment of the stallion spermatozoal acrosome is the most susceptible to membranous damage following cold shock [55]. Additional reports reveal that acrosomal damage following cryopreservation is common in other mammalian species [56, 57], and that acrosomes may be more susceptible to cryoinjury when spermatozoa are exposed to increasingly higher glycerol concentrations [58]. In addition, a surfactant was incorporated into the LE extender but not the MRR5 extender. This may have improved

emulsification and dispersion of egg yolk lipids, rendering them more readily available for interaction with the spermatozoal membranes [59].

Overall, when diluting the semen for post-thaw analysis, spermatozoal motility endpoints tended to be higher in semen diluted in a non-glycerol containing extender (ie, INRA 96), as compared to freezing medium (either LE or MFR5). The effects were more pronounced for semen cryopreserved in MF extender. Flow-cytometer endpoints, i.e., VIAB and AI, were not affected by post-thaw diluent to the same extent as were the CASMA variables. We had hypothesized that dilution of post-thaw semen in a glycerol-free isotonic medium would have a deleterious effect on measures of spermatozoal quality, because of a sudden change in the osmotic environment. Such exposure does not appear to be detrimental, based on the laboratory endpoints tested. Conversely, overall semen quality appeared to be improved when frozen-thawed semen was diluted in a glycerol-free medium. This data is supported by fertility trials conducted by Palmer [3].

As expected, T0 endpoints were highly correlated with the same experimental endpoints at T24 for cooled semen. Likewise, the CASMA values, MOT and PMOT for post-thaw data were highly correlated with the FC endpoints, AI, VIAB, and AIVIAB. Of interest, the correlations of PMOT and MOT to VIAB were higher than that for acrosomal integrity. Fiser and Fairfull (1990) reported that post-thaw motility and acrosomal integrity of boar spermatozoa, as measured by phase-contrast microscopy,

were affected differently by alterations in cryopreservation technique. The differential response in these variables was most profound when glycerol concentration exceeded 3%. In the present study, glycerol concentration was set at 2.5% for all experimental treatments; thereby reducing the potentially detrimental impact that glycerol could have on acrosomal integrity.

Cooled semen (T24) values for MOT, PMOT, VCL, and COMP were better predictors of spermatozoal resistance to cryoinjury than were initial (T0) values, based on the post-thaw variables tested. The percent change in the pre-freeze endpoints following cooled storage (DIFF) proved to be an ineffective predictor of spermatozoal "freezability". Interestingly, pre-freeze COMP yielded stronger linear correlations to post-thaw endpoints than did pre-freeze values for MOT, PMOT, or VCL.

In summary, these data indicate that spermatozoa of the eight stallions used in this study survived the freeze-thaw cycle better in egg-based extender than in milk-based extender when glycerol concentration was adjusted to 2.5%. Although a stallion effect was detected, semen from all stallions performed better when mixed with LE extender, as opposed to MF extender, prior to cryopreservation. Regardless of freezing extender type, spermatozoa were more resistant to cryoinjury when subjected to a slow pre-freeze cooling rate, as opposed to immediate exposure to low cryopreservation temperatures. A non-glycerol-containing extender appears adequate for dilution of frozen-thawed semen prior to in-vitro analysis. Lastly, some pre-freeze measures of semen quality in our

study had limited, but distinct, relevance to spermatozoal resistance to cryoinjury. More investigation is required to determine why the egg-based extender used in this study produced uniformly better results than the milk-based extender.

FUTURE AIMS

While EZ Freezin "LE" semen extender appears to be superior to EZ Freezin "MFR5" the question remains as to exactly what components contribute to the improved post-thaw spermatozoal characteristics we observed. Perhaps removal of the surfactant that is incorporated in the "LE" extender and not the "MFR5" could explain the post-thaw quality we observed. Additionally, the non-detrimental effects of using an isotonic medium (*ie*, INRA 96) as a post-thaw diluent were not expected. Although, in vitro analysis of post-thaw spermatozoal quality indicated "LE/SLOW/INRA" to be the best treatment group regarding freezability the ultimate test would be to subject spermatozoa to fertility trials and observe pregnancy rates.

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