

**CUSHIONED CENTRIFUGATION OF STALLION SEMEN: FACTORS  
IMPACTING EQUINE SPERM RECOVERY RATE AND QUALITY**

A Thesis

by

JESSICA ARLENE WAITE

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

December 2007

Major Subject: Veterinary Medical Sciences

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Approved by:

Chair of Committee,	Dickson D. Varner
Committee Members,	Charles C. Love
	Steven P. Brinsko
	David Forrest
Head of Department,	William Moyer

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## ABSTRACT

Cushioned Centrifugation of Stallion Semen: Factors Impacting Equine

Sperm Recovery Rate and Quality. (December 2007)

Jessica Arlene Waite, B.S., Texas Tech University

Chair of Advisory Committee: Dr. Dickson D. Varner

Centrifugation of stallion semen is an integral part of the cryopreservation procedure, primarily allowing for the concentration of sperm and removal of seminal plasma. In addition, centrifugation is required for maximizing spermatozoal quality in semen from some stallions subjected to cooled transport, because of the detrimental effects of long-term exposure to high levels of seminal plasma. The centrifugation process, however, has potential deleterious effects, including reduction in sperm quality as well as loss of sperm numbers. Since centrifugation plays such a crucial role in semen processing, two experiments were designed to evaluate more efficient centrifugation methods to meet the demands of the equine industry. In Experiment 1, semen was centrifuged in two different tube types (nipple- or conical-bottom), using a cushioned technique (Eqcellsire® Component B) with two different extenders (opaque-INRA96 or clear-HGLL). For Experiment 2, nipple-tube centrifugation was conducted at two different *g* forces (400 or 600) for 20 min, using three different iodixanol cushion media, Eqcellsire® Component B, OptiPrep™, or Cushion Fluid™.

Regardless of tube or extender types, centrifugation of semen resulted in sperm recovery rates  $\geq 90\%$ ; however, centrifugation in INRA 96 extender yielded higher sperm motility values than did centrifugation in HGLL extender ( $P < 0.05$ ). Cushion type or  $g$  force did not impact post-centrifugation semen quality, based on the laboratory values measured ( $P > 0.05$ ).

These results indicate that cushioned centrifugation of stallion semen in either conical-bottom or nipple-bottom tubes can yield a high sperm harvest, while maintaining sperm function. An optically opaque extender, as is typically used in the equine breeding industry, can be used to achieve this goal. The fertility rate (94%; 131/140) following cushioned semen centrifugation in a commercial program this past year indicates that these laboratory results are transferable to the clinical setting.

## DEDICATION

I dedicate this work to:

My parents: Shane and Kristy Waite,

Dr. Glenn Blodgett,

&

My family and friends.

These loved ones have guided and supported me, and without their direction and encouragement I would not be who I am today. Doc, thank you for your perpetual guidance and constant compassion; your generous counsel propelled me to this point. Mom and Dad, thank you for providing for me, inspiring me, and instilling in me the work ethic that has shaped my persona.

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## INTRODUCTION

As the horse breeding industry continues to grow to meet the needs of owners, breeders, and enthusiasts, better utilization of the breeding stallion and his semen are required. Transport and insemination of cooled and frozen semen are accepted by many horse breed registries, but require appropriate semen processing prior to insemination for maximum success [1]. For sperm to survive the cooling and transportation process, many factors are crucial, such as quality of semen extender, sufficient dilution ratio, and correct cooling rate and storage temperature [2-17]. Some stallions routinely have a relatively low ejaculate sperm concentration, ejaculate sperm with a low tolerance to seminal plasma, or have seminal plasma which contains factors toxic to sperm. In such instances, it is necessary to centrifuge semen in extended form, with resuspension of sperm in extender. This protocol aids in removing seminal plasma or reducing the seminal plasma concentration of extended semen, while maintaining sperm concentration above a tolerable threshold for normal fertility. Decreased semen quality (i.e., reduced sperm motility and chromatin quality) occurs when higher levels of seminal plasma are included in cooled-shipped semen or cryopreserved semen. Reduction of seminal plasma to 5-10% (v/v) in extended semen is beneficial to increasing the tolerance of sperm to cooling and freezing [5-8,13,18-20].

As centrifugation of semen is oftentimes applied in equine breeding programs for reasons addressed above, the value of improving centrifugation procedures to maximize

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This thesis follows the style of Theriogenology.

sperm harvest, without associated sperm injury, becomes apparent. Development of a more efficient centrifugation method, i.e., one that increases post-centrifugation recovery rate while maintaining semen quality would optimize the number of sperm in an ejaculate that are available for breeding purposes. There are two options for increasing sperm concentration with the removal of seminal fluids—collecting only the sperm-rich portion of the ejaculate or centrifuging the entire gel-free portion of ejaculate. Both methods help reduce the potentially detrimental contribution of excessive seminal plasma to an ejaculate that is subjected to various preservation methods [11,19]. Centrifugation is a more logical approach for accomplishing this task because collection of the sperm-rich portion of the ejaculate through fractionation of semen during collection can result in a significant loss (>20%) of ejaculated sperm [15,21].

One of the main concerns when attempting to maximize sperm recovery through centrifugation is the adverse effect that centrifugation can have on the integrity of sperm [22]. Typically, an increase in centrifugation time or gravitational ( $g$ ) force results in an increased sperm recovery, but can also lead to decreased sperm motility or quality due to the mechanical forces associated with centrifugation and excessive packing of the sperm [23,24]. Ideally, centrifugation should result in a 100% sperm recovery rate with no resulting damage in sperm quality. Previous studies have used a variety of centrifugation forces and times to accomplish this task [7,13,18,19,22,24-32]. Macpherson et al. reported that centrifugation at 500 x  $g$  for 10 min in standard 50-mL conical tubes optimized sperm recovery without a negative impact on sperm motility

[24]. However, such a protocol can lead to a 15-25% loss of sperm that could otherwise be used for breeding purposes.

A non-ionic iodinated compound, iodixanol, was first reported for density-gradient cell fractionation [33-35], and has since been used as either a density gradient or as a cushion for centrifugation of sperm [36-43]. Investigations regarding cushioned centrifugation of stallion semen with this product demonstrated excellent yields of sperm that were undamaged by the centrifugation process, but an optically clear centrifugation medium was required to reduce sperm losses [36,38,41,42].

## **OBJECTIVES**

The objective of this thesis project was to investigate potential modifications in the centrifugation procedure for stallion semen. Specifically, experiments were designed to determine if alterations in centrifugation-tube type, centrifugation medium, centrifugation force, or source of iodixanol would impact sperm recovery rate and semen quality. The purpose of Experiment 1 was to evaluate the effects of centrifugation-tube type and centrifugation extender on resulting sperm recovery rate, sperm motion characteristics, and sperm chromatin quality. Experiment 2 addressed the effects of centrifugation-cushion type and nipple-tube centrifugation force on sperm recovery rate, sperm motion characteristics, and sperm chromatin quality. The overall goal of the study was to develop methods to maximize sperm recovery following centrifugation without an attendant reduction in sperm quality, thereby allowing more sperm per ejaculate to be available for insemination of mares following the centrifugation procedure.

## LITERATURE REVIEW

### Artificial Insemination

Artificial insemination, i.e., the deposition of semen collected from a stallion into a mare's uterus by a human delivery system, provides many benefits for the breeding industry. These benefits include the possibility of breeding multiple mares with sperm from a single ejaculate, increased safety for mare and stallion, the ability to breed mares that are in different locations than a stallion, and the ability to modify semen prior to insemination. Artificial insemination has progressed from using raw fresh semen to using semen diluted with extender that can also be preserved by cooling or freezing.

Although artificial insemination was not accepted by most equine breed registries in the United States until after 1940, it was being performed successfully prior to that time in a number of other countries. In 1939, Saltzman et al. inseminated 29 mares after ovulation and 38 mares before ovulation, resulting in pregnancy rates of 72.4% and 63.1%, respectively [44]. Over the next two years, insemination prior to ovulation in over seven hundred mares yielded pregnancy rates of 85.6 to 87.6% [45]. Zivotkov et al. also reported pregnancy rates of 72.7 to 76.7% for insemination of 63 mares immediately preceding ovulation and pregnancy rates of 27.3 to 34.7% for 44 mares inseminated two to 10 h after ovulation [45].

Subsequent studies focused on development of semen extenders to enhance longevity of sperm quality. In 1957, one investigator used a glucose diluent to extend semen prior to artificial insemination [46]. Arhipov et al. found that insemination with 50 mL of extended semen at a 1:6 (semen:glucose extender) "more closely resembled"

the results of natural mating and was better than insemination with a 1:4 dilution ratio or with no dilution prior to insemination [46]. At the same time, two studies reported the use of egg yolk-glycerol-glucose-containing extenders for frozen semen preservation [47,48]. Chinese workers observed “satisfactory viability” post-thaw for semen diluted 1:3 in extender [47]. After thawing, Iljinskaja et al. observed an average of 50% or greater “survival rate” [48]. These investigators were not taking sperm numbers into account.

Even though these reports offered promising results for cryopreserved semen, only one group of researchers reportedly performed artificial insemination with frozen-thawed semen. Barker et al. reported the first mare pregnancy and resulting live foal from frozen-thawed stallion semen in 1957 [49]. Three years later, Žmurin et al. described a method to freeze semen using an egg yolk-glycerol-glucose extender which resulted in a 41.2% pregnancy rate [50]. Although sperm motility of the extended semen was examined in that study, it was not reported [50]. Workers in China reported pregnancy rates ranging from 59 to 100% from semen that was deep frozen (-79 °C), but the details of the extender and the methods used were not described [51]. Krause and Grove cryopreserved jackass and stallion semen diluted with glucose, lactose, or raffinose and insemination with this extended semen resulted in a 50% pregnancy rate (2/4 from stallion semen and 1/2 from jackass semen) [52].

In an attempt to improve the fertility of frozen-thawed sperm, Rombe et al. introduced centrifugation so that seminal plasma could be removed and replaced with extender [53]. Semen was centrifuged for 10 min: one min at 1000 revolutions per min



(rpm) followed by nine min at 1500 rpm [53]. Supernatant was removed, leaving 20 to 30% seminal plasma, and then diluted with a lactose-glycerol-egg yolk-antibiotic-containing extender [53]. Insemination with this semen apparently resulted in an 80% pregnancy rate but the number of mares bred in this study was not reported [53].

Because the centrifugation speed was reported in rpm and not gravitational force, which is a uniform unit that changes with the radial arm length of the centrifuge, duplication of this method was not possible.

At about the same time that Rombe et al. began work with centrifugation, Japanese investigators centrifuged extended semen at 1300 x g for 20 min [54,55]. Insemination with frozen-thawed semen containing 30-40% progressively motile sperm resulted in pregnancy rates of 33.3% (14/42) [56] to 46.7% (43/93) [55]. Despite some of the early success using centrifuged semen prior to freezing [53], subsequent research focused on cooling semen and improvement in extenders as ways to enhance semen preservation [5,8,10,16,20,57].

### **Extender (medium and antibiotics)**

As artificial insemination gained popularity within the equine industry, breeders looked to the cattle breeding industry, which was more highly advanced in this area, in hopes of finding a proper extender to preserve and protect semen. Researchers had shown semen extenders to provide nutrients, protection, and survival for bovine sperm [58-60]. Furthermore, semen diluents had been used successfully with dairy bull semen long before (1953, 1954, and 1959) they were used routinely with stallion semen.

Thacker et al., Flipse et al., and Almquist et al. reported that semen extended with previously boiled milk resulted in improved motility and fertility of bovine sperm [58-60]. Flipse et al. showed that this was due to the inactivation of lactenin, a protein known to be toxic to bovine sperm [58].

One of the first reports on diluting stallion semen in extender was in 1957 when Arhipov et al. used a glucose-based diluent to extend semen at a 1:6 (semen:diluent) ratio prior to insemination [46]. The first reported pregnancy from insemination with frozen equine semen was diluted with a milk-based extender [49]. This extender was prepared by heating pasteurized, homogenized, whole milk to 97°C for 20 min and cooled to 30° to 35°C, followed by the addition of antibiotics and 10% glycerol [49]. Later, Householder et al. reported the effects on progressive sperm motility following the addition of previously heated (10 min at 92° to 95°C) skim milk to either fresh or cooled equine semen [61]. Progressive sperm motility was higher ( $P < 0.05$ ) for samples diluted 1:20 in the heated skim milk than in the non-heated skim milk, regardless of incubation temperature (5°C, 15°C, and 38°C) or h of incubation (0-24 h) [61]. Progressive sperm motility ranged from 35% when stored at 5°C to 10% when stored at 38°C and from 27% after 0 h incubation to 10% after 24 h incubation [61]. One-cycle pregnancy rates following the insemination of fresh semen added to one of three heated extenders (skim milk, skim milk-gel, and cream-gel) were 62.5% (10/16), 62.5% (10/16), and 43.8% (7/16), respectively ( $P > 0.10$ ) [61].

Prior to the research by Householder et al., Hughes and Loy first utilized the cream-gelatin extender for stallion semen [62]. This extender was prepared by heating

half-and-half cream to 95°C for two to four min. After removal of the foam from the heated cream, 1.3 g of Knox gelatin, which had been autoclaved with 10 mL of distilled water, was added and brought to a volume of 100 mL. After the extender was cooled, penicillin, dihydrostreptomycin, and polymyxin B sulfate were added. Hughes and Loy suggested that addition of antibiotics to the extender aided in reproductive management of stallions that harbored potentially pathogenic organisms in their reproductive tracts. Insemination of mares using semen diluted with cream-gelatin extender and stored for 48-96 h at 0°-5°C yielded a 73.9% (17/23) pregnancy rate [62].

Following the introduction of the cream-gel extender, Pickett et al. reported that insemination of fresh semen diluted with a cream-gel extender had a higher ( $P < 0.01$ ) pregnancy rate than did semen diluted in a Tris extender [63]. The Tris extender, which contained Tris-hydroxymethyl aminomethane, glucose, citric acid, egg yolk, and glycerol, resulted in a 75.0% (18/24) overall pregnancy rate compared to 95.8% (23/24) with semen extended in the cream-gel extender [63]. Upon storage at 5 °C, pregnancy rates with semen diluted in the cream-gel extender dropped from 83.3% (15/18) after two h of cooling to 55.6% (10/18) after 24 h cooling ( $P > 0.05$ ) [63]. However, this pregnancy rate (55.6%) obtained with the insemination of cream-gel extended semen cooled for 24 h was significantly lower ( $P < 0.05$ ) than the pregnancy rate achieved by insemination with fresh extended semen (94.4%; 17/18) [63].

While Pickett et al. were experimenting with cream-gel and Tris extenders, Nishikawa introduced and formulated three more extenders to counter the deleterious effects of seminal plasma on equine sperm and demonstrated that cooling semen in

extender improved sperm motility [64]. He compared three different extenders, and determined that one, CGH-27, was superior to the other two, Baken-II solution and 5% glucose [64]. The CGH-27 components included gelatin, sodium phosphate, potassium chloride, potassium sodium tartrate, glycine, glucose, casein, and egg yolk. Sperm diluted with this extender maintained high motility—97% at 0 h and 62% at 72 h—when cooled at 4°C [64]. After cooled-storage for two to eight h, insemination of this extended semen yielded a 67.3% (274/407) pregnancy rate [64]. Nishikawa believed that large amounts of sodium chloride present in seminal plasma were harmful to sperm motility and caused “salt injury” during semen freezing. To help sperm maintain motility after thawing, seminal plasma was removed after centrifugation for 10 min at 2000 to 2200 rpm (*g* force and model of centrifuge not reported) and replaced with an extender, HF-20, prior to cryopreservation [64]. This extender contained glucose, lactose, raffinose, sodium citrate, sodium phosphate, potassium sodium tartrate, egg yolk, penicillin, and streptomycin. After thawing, insemination resulted in a 56.3% (14/24) pregnancy rate [64].

In an effort to address contamination of semen with bacteria following collection, Kenney et al. described the use of antibiotic containing extenders [65]. When comparing bacterial growth in raw semen versus semen extended 1:1 with instant, non-fat, dry milk-glucose containing either penicillin and streptomycin or 7.5% sodium bicarbonate and gentamicin sulfate, no growth appeared on the treated samples but heavy growth appeared on all the raw samples [65]. Furthermore, the extender with penicillin allowed much less growth of *Pseudomonas aeruginosa* than raw semen and no growth of

*Klebsiella pneumoniae*, while the extender containing gentamicin prevented growth of both of these bacteria [65]. In this study, Kenney et al. also diluted the sperm with warm extenders, followed by centrifugation. After removal of the supernatant, the resulting sperm pellet was re-suspended in fresh, warm extender. Centrifugation allowed removal of the supernatant (seminal plasma) and, with the addition of antibiotic-containing extender, drastically reduced the number of recovered microorganisms following bacterial isolation procedures [65]. In 1989, Varner et al. used Kenney-type extenders to inseminate 45 mares [66]. The percentages of total motile and progressively motile sperm were 89% and 84%, respectively, for fresh semen; 57% and 41%, respectively, for semen cooled for 24 h at 20°C; and 80% and 72%, respectively, for semen cooled for 24 h at 5°C [66]. A per-cycle pregnancy rate of 73% (11/15) was attained with all three treatments [66].

More recently, certain antibiotics have been shown to be more effective than others for preventing bacterial growth in semen, and some antibiotics can be harmful to sperm motion characteristics [67]. Varner et al. compared various antibiotics in semen extended with a milk-glucose extender in which, after 12 h at 5 °C, total motility and progressive motility were both lower ( $P < 0.05$ ) when polymixin B was used compared to the other six antibiotic treatments. Furthermore, gentamicin and other antibiotics including amikacin, streptomycin, and potassium penicillin, were more effective ( $P < 0.05$ ) at eliminating bacterial growth than polymixin B [67]. The reduced sperm motility caused by treatment with polymixin B was in agreement with a previous study [68]. However, Jasko et al. reported that getamicin resulted in reduced ( $P < 0.05$ ) total motility

and progressive motility after 24 h at 4 °C [68]. Varner et al. also reported that semen samples treated with the combination of potassium penicillin-amikacin resulted in higher sperm velocities than either antibiotic used alone ( $P < 0.05$ ) [67]. No difference in sperm motility was detected ( $P > 0.05$ ) among three antibiotic treatments: potassium penicillin G-amikacin, ceftiofur, and a combination of ticarcillin and clavulanic acid (Timentin) [67]. These studies suggest that the use of polymixin B as an antibiotic in semen extenders should be avoided if semen is to be stored for prolonged periods because of its suppressive effect on spermatozoal motility [67,68].

With the control of bacterial contamination [65,67,68] and advanced cooling techniques, as described in 1984 by Douglas-Hamilton et al. in which a 91% (42/46) pregnancy rate was achieved [69], investigators began to examine the effect of different dilution ratios of seminal plasma. In 1984, Palmer et al. showed that a dilution ratio of 1/20, 1/10, and 1/5 yielded higher ( $P < 0.05$ ) sperm survival rate (% motility recovered/ % initial motility) than sperm diluted 1/2, when semen was stored for 24 h at 4 °C in a milk-based extender [70]. Sperm survival rates were 56.4%, 57.2%, 54.9%, and 41.2%, respectively [70]. Following centrifugation at 600 x g for 10 min, supernatant removal, and addition of 0%, 10%, 20%, or 50% seminal plasma, extended semen was stored for 24 h at 4 °C. Sperm survival rates were 55.9%, 50.8%, 44.2%, 27.3%, respectively [70].

Palmer also evaluated semen diluted in different extenders and stored 72 h at 4 °C. Sperm motility was highest ( $P < 0.05$ ) in a 1.5% fat, milk solution (51.6%) compared to Baken I (19.1%) and HF-20 (16.5%) [70]. After 36 h of storage at 4 °C, sperm motility was higher ( $P < 0.05$ ) with INRA 82 than with a milk diluent or a milk-

glucose diluent—46.0%, 30.0%, and 39.6%, respectively [70]. Skim milk plus 2.5% glycerol and 2.5% egg yolk freezing extender was then compared to INRA 82 plus 2.5% glycerol and 2.5% egg yolk. Post-thaw motility was higher ( $P < 0.05$ ) in the INRA 82 than in the skim milk—48.1% versus 40.1% [70].

In 1987, the effect of seminal plasma was evaluated by collection of the sperm-rich portion of the ejaculates [15]. After 12 to 24 h of storage at 25°C, total motility and progressive motility of semen extended in skim milk-glucose extender were slightly higher ( $P < 0.05$ ) in the sperm-rich ejaculates than in whole ejaculates—28-38% total motility and 6-10% progressive motility versus 22-35% total motility and 2-6% progressive motility, respectively [15]. Furthermore, dilution of the sperm-rich portion to 25 million/mL resulted in improved ( $P < 0.05$ ) longevity of motility—45% motility and 12% progressive motility—when compared to no dilution, dilution to 50 million/mL, and dilution to 100 million/mL—4% motility and 0% progressive motility, 31% motility and 3% progressive motility, 22% motility and 1% progressive motility, respectively [15].

The more common method to remove seminal plasma is by centrifugation. In 1991, Jasko et al. evaluated the effect of centrifugation at 500 x *g* for 18 min, with or without the removal of seminal plasma [7]. Removal of seminal plasma and resuspension with fresh extender resulted in an improved ( $P < 0.05$ ) motility of 33% and mean velocity of 117µm/s after 24 h cooled-storage compared to centrifugation with no removal of seminal plasma—28% and 110µm/s—after 24 h cooled-storage [7].

The same year, Padilla and Foote centrifuged semen diluted in a Kenney extender modified Tyrodes medium for 5 min at 350 g in 50-mL conical tubes to remove seminal plasma [11]. Using this method, longevity of motility was maintained for up to 72 h (0h,76%; 24h,75%; 48h,72%; 72h,64% motility) and was higher ( $P < 0.05$ ) than when Kenney extender without the high-potassium Tyrodes was used (0h,74%; 24h,47%; 48h,39%; 72h,24% motility) [11].

In 1992 Magistini et al. reported that a Hanks' salt solution (HGLL; supplemented with HEPES, Glucose, and Lactose) and INRA82 yielded a higher ( $P < 0.05$ ) percentage of rapid sperm (sperm with a velocity greater than  $30\mu\text{m/s}$ ) after 24 h than did HGLL supplemented with bovine serum albumin (BSA)—81% versus 50%, respectively [9].

In 1997, Batellier et al. also reported an increase ( $P < 0.05$ ) in percentage of rapidly motile sperm when using INRA 82 extender (66%) compared to buffered sugar-electrolyte extender (HGLL) supplemented with native phosphocaseinate (NPPC; 53%) following 48 h of storage at  $4^{\circ}\text{C}$  [2]. Furthermore, HGLL-BSA yielded a significantly lower ( $P < 0.05$ ) percentage of rapidly motile sperm than did HGLL-NPPC after 48 h storage at  $4^{\circ}\text{C}$  (13% versus 33%) and at  $15^{\circ}\text{C}$  (17% versus 29%) [2]. Insemination with extended semen stored at  $4^{\circ}\text{C}$  for 24 to 48 h yielded higher ( $P < 0.05$ ) pregnancy rates when semen was extended in HGLL-NPPC (26/43; 60%), as compared to semen extended in INRA 82 (15/41; 36%) [2].



## Centrifugation

Historically, semen centrifugation has been a common step in the cryopreservation of stallion semen, functioning primarily to concentrate sperm and remove the majority of the seminal plasma. Studies have varied in the rotational force and time used to centrifuge semen, and the results, with respect to sperm recovery rate and quality, have been inconsistent and, in many cases, not reported. Therefore, comparisons amongst studies are difficult to make.

In an early study, Rombe et al. centrifuged semen for one minute at 1000 rpm ( $g$  force was not recorded) then for 9-10 min at 1500 rpm to form a sperm pellet so that 70-80% of the supernatant could be “poured off” and replaced with extender (lactose-glycerol-yolk-antibiotics) prior to freezing [53]. Using the same protocol, an 80% pregnancy rate (number of mares inseminated were not reported) was achieved [53]. Japanese workers were not as successful when centrifuging semen at 1300 x  $g$  for 20 min prior to freezing, as demonstrated by the lower pregnancy rates, 33.3 % and 46.7 % [55,71]. It is possible that the higher  $g$  force applied led to sperm damage and contributed to the lower pregnancy rates.

Even though Rombe et al. were successful with centrifugation [53], it wasn't until during the mid-nineteen seventies and the nineteen eighties that centrifugation was commonly utilized in semen preservation methods [7,11,13,22,25,27]. Pickett et al., centrifuged semen at 370 x  $g$  or 829 x  $g$  with no decrease in motility before and after cryopreservation in the presence of 10% seminal plasma. [13]

Martin et al. compared post-thaw sperm motility in extended semen or raw semen subjected to centrifugation at 1000 x *g* for 5 min [22]. Both samples were diluted with a glycerol containing extender, frozen, and further diluted with milk prior to analysis. The resulting percentages of progressively motile sperm were 42% and 33%, respectively [22]. In another experiment, 19 mares were inseminated with frozen-thawed semen that was extended prior to centrifugation, as described above, resulting in a 63% (12/19) pregnancy rate from a single inseminations within six h after ovulation [22].

In 1983, Colorado State University workers completed fertility trials comparing one-cycle pregnancy rates using frozen semen versus fresh semen. The results with frozen semen were half (49% and 54%) of that with fresh semen and, therefore, were considered unacceptable [18,26]. Based on these results, Cochran et al. designed experiments to improve processing, freezing, and thawing of stallion semen [27]. One experiment was designed to increase sperm recovery rate and minimize damage following centrifugation. Cochran et al. layered 0.25 mL of a more dense glucose-EDTA solution beneath the extended semen prior to centrifugation at 400 x *g* for 15 min [27]. This provided a lightly packed sperm pellet, resulting in a 92% recovery of sperm [27] and laid the ground work for a cushioned centrifugation technique. Following Cochran's research, Cristanelli and co-workers conducted a fertility trial using Cochran's techniques, resulting in a 56% pregnancy rate using frozen semen, only slightly lower than the 65% pregnancy rate attained using fresh semen from the same stallions [28]. However, these investigators were unable to determine which factor (such

as the addition of a cushion medium) or combination of factors lead to the increase in pregnancy rates when compared to previous studies at the same institution [18,26].

### **Cushioned Centrifugation**

Recently, several types of centrifugation-cushion media have become commercially available, including, OptiPrep™ (Greiner Bio-One, Axis-Shield, Oslo, Norway), Eqcellsire® B (IMV Technologies; Maple Grove, MN), and Cushion Fluid™ (Minitüb; Tiefenbach, Germany). OptiPrep™ is a 60% (wt/vol) iodixanol solution, originally designed for in-vitro isolation of biological particles by density gradient centrifugation. This product has been determined to be both metabolically inert and non toxic to cells [72]. The high density ( $1.320 \pm .001$  g/mL) of this medium prevents interaction with mammalian cells [72] when appropriately layered in a centrifugation tube. This medium is hypotonic, with a reported osmolarity of  $170 \pm 15$  mOsm [72]. Two other high-density cushion fluids have recently become available commercially and were developed to be isotonic ( $\sim 300$  mOsm) to protect sperm during high-speed centrifugation. These products, Eqcellsire® Component B (IMV Technologies; Maple Grove, MN) and Cushion Fluid™ (Minitüb; Tiefenbach, Germany), have both been used in other studies [36,41,42].

These iodixanol-based media are more dense than extender or semen and can therefore be layered on the bottom of the centrifugation tube to serve as a “sperm cushion” during the centrifugation process. This protocol may be especially important when faster centrifugation speeds and longer centrifugation times are used. These media

act to prevent sperm damage that occurs when sperm are packed tightly against the hard surface at the bottom of the centrifugation tube. The cushion provides a “pillow” effect, presumably allowing sperm to more gently settle against the dense fluid, but not allowing them to pass through to the bottom of the tube. These cushions are thought to allow for longer centrifugation times and faster centrifugation speeds, resulting in higher recovery rates of undamaged sperm, which is critical to efficient sperm processing.

The first reported use of iodixanol-based cushion fluid in equine centrifugation was by Revell et al. in 1997 [40]. Semen centrifuged (600 x g for 15 min) without cushion fluid had approximately 30% fewer ( $P < 0.05$ ) live/normal sperm after thawing than did semen centrifuged (1000 x g for 25 min) with OptiPrep™ [40]. OptiPrep™ has been used as a cushion fluid during centrifugation of stallion semen in our laboratory as well [17].

Ecot et al. reported a 20% increase in sperm recovery when 3.5 mL of Eqcellsire® B cushion and Eqcellsire® A or HGLL extender was used during centrifugation at 1000 x g for 20 min (99% and 103% sperm recovery, respectively) versus centrifugation at 600 x g for 10 min with INRA82 plus 2% egg yolk extender (77% sperm recovery) or centrifugation at 1000 x g for 20 min with Eqcellsire® B cushion and INRA82-egg yolk extender (81% sperm recovery). A total of 1.5 to 2.0 billion sperm per tube were centrifuged. Post-thaw rapid motility was similar between the two treatments and no differences were observed in fertility. However, the cushioned technique allowed more straws to be processed per ejaculate because of the

higher sperm recovery, indicating that a higher rotational force for a longer period of time maintained sperm quality while increasing sperm recovery rate [36].

Sieme et al. compared centrifugation at 1000 x g for 20 min over 5 mL of Cushion-Fluid® (Minitüb, Landshut, Germany), to be removed post-centrifugation, versus centrifugation at 600 x g for 10 min with no cushion. A total of 2.3 billion sperm were extended in INRA 82, then placed in 50-mL sterile plastic tubes and centrifuged. Post-thaw progressive motility did not differ between the treatments. However, sperm recovery was higher ( $P < 0.05$ ) when using the cushioned technique, as compared to the conventional method (83% and 75%, respectively) [41].

In a separate study, Sieme et al. ( $P < 0.05$ ) reported that sperm recovery rates were higher when one of two optically clear extenders [Hepes buffered saline (HBS; 93%) or Equcellsire A (93%)], were used, as compared to an opaque extender [INRA 82 (86%)], following centrifugation over 5 mL of cushion media. After cryopreservation, progressive sperm motility was higher ( $P < 0.05$ ) in INRA82 (42.4%) and HBS (39.5%), as compared to Eqcellsire® A (22.6%) or centrifugation without cushion media (22.4%) [42].

### **Tube Type**

In addition to presence of a cushion medium, the size, shape, and material of the centrifugation tube can also affect sperm quality and recovery rate. The most commonly used tubes are plastic, polypropylene 50-mL volume containers [5,11,13,22,25,27,30]. These tubes are convenient and disposable, with a cone-shaped bottom. The plastic 50-

mL conical tubes can be centrifuged at 1000 x g for 20 min when using 3.5 mL of Eqcellsire® B cushion or 5 mL of Cushion-Fluid®, as shown by Ecot et al. and Sieme et al., respectively [36,41]. However, removal of the cushion fluid is probably necessary in these tubes because of the high volume of cushion fluid required to achieve the desired effect [36,41,42].

At least two different types of glass centrifugation-tubes have been used, with the difference mainly in the shape of the bottom (round versus nipple). Glass centrifugation-tubes are generally too expensive to be considered disposable, but can be reused with proper cleaning and sterilization. To prevent sperm adherence to the glass surface during centrifugation, these tubes are generally siliconized prior to use [17]. Sieme et al. found no difference in post-thaw progressive sperm motility when “sterile, siliconized, conical glass” tubes with no cushion were compared to the same tube with 5 mL of Cushion-Fluid® (Minitüb, Landshut, Germany), but the sperm recovery was higher using the glass tubes without cushion fluid (92% and 83%, respectively) [41]. These motility results might be explained by the smooth bottom surface of the glass tubes compared to the corners of the cone shape plastic tubes. Because no cushion was used, it was also unnecessary to risk sperm wastage during aspiration of the cushion following centrifugation. In this study, 2.3 billion spermatozoa were centrifuged per tube [41]. A modified version of the siliconized glass tubes mentioned above has been specially designed with a 2-mL capacity cylindrical bottom or nipple (Pesce Lab Sales, Kennett Square, PA; 610-444-1440 in MM). When using this nipple-tube type, less cushion fluid (30µl) is probably required to protect sperm during centrifugation [17].

In all experiments described above utilizing cushion media during centrifugation for prolonged time and at a high speed, sperm recovery was higher than with a conventional method in conical tubes, but sperm motility varied among experiments [36,40-42]. Since these studies have shown that the use of a “cushion” is beneficial for improving sperm harvest following centrifugation, the next step in improving centrifugation and processing of semen might be to determine the cushion type, centrifugation-tube type and extender type that yields the highest sperm recovery while preserving post-centrifugation sperm quality.

## MATERIALS AND METHODS

Two experiments were conducted to evaluate the effects of centrifuge-tube type (conical-bottom versus nipple-bottom), centrifugation medium (clear versus opaque), centrifugation-cushion type (i.e., OptiPrep™, Eqcellsire® Component B, Cushion Fluid™), and centrifugation force (400 x *g* versus 600 x *g*) on sperm recovery rate and quality. Sperm recovery rates were determined in post-centrifuged semen and sperm motion characteristics and sperm chromatin integrity were evaluated in both fresh and cool-stored semen.

### Stallions and Semen Collection

For Experiment 1, three ejaculates from each of 8 stallions ( $n = 24$  ejaculates) were used. For Experiment 2, three ejaculates from each of three stallions ( $n = 9$  ejaculates) were used. All stallions were of light breed, sexually active, and mature (age range of 4 to 17 yrs.). Ejaculates were collected at 2- to 4-day intervals 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. Immediately prior to semen collection, artificial vaginas were lubricated with a small volume (approximately 5 mL) of sterile non-spermicidal lubricant (Priority Care; First Priority, Inc., Elgin, IL, USA). An insulated thermal jacket was also used to protect semen in the receptacle from light and temperature fluctuations during the semen-collection process (Animal Reproduction Systems, Chino, CA, USA). An ovariectomized mare was used for sexual stimulation and as a mount



source. After obtaining an erection, each stallion's penis was rinsed with warm tap water and dried thoroughly. Following semen collection, the gel-free semen was transported to an adjacent laboratory and placed in an incubator (37 °C) prior to processing.

### **General Semen Processing**

The total sperm number in gel-free semen was estimated by measuring semen volume with a graduated cylinder and measuring initial sperm concentration photometrically (SpermaCue; Minitube of America, Inc., Verona, WI, USA). One-mL aliquots of well-mixed 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°C until analyzed for the susceptibility of sperm chromatin to denaturation (i.e., Sperm Chromatin Structure Assay; SCSA) and for sperm concentration, using a fluorescence-based instrument (NucleoCounter SP-100; Chemometec A/S, Allerød, Denmark).

Aliquots of gel-free semen were immediately diluted with a warm (37 °C) milk-based extender (INRA 96; IMV, Maple Grove, MN, USA) to a final sperm concentration of approximately 20 million sperm/mL for evaluation of initial sperm motility measures, using computer-assisted sperm motion analysis (CASMA; IVOS Version 12.2L, Hamilton Thorne Biosciences, Beverly, MA, USA). The INRA 96 extender was selected because it is free of particulate debris that could otherwise be evaluated as sperm heads with the CASMA system. Similarly diluted samples were also packaged in

capped polypropylene tubes (Cryogenic vials [5.0-mL]; Corning Life Sciences, Lowell, MA, USA) with minimal air space. Prepared vials were then packaged, as recommended by the manufacturer with sufficient ballast for control of cooling rate, in a commercial semen-transport container (Equitainer™ II; Hamilton Research, Inc., South Hamilton, MA, USA) for 24 h of cooled storage. These treatments served as uncentrifuged controls (CONT) for both fresh and cooled-semen samples in Experiments 1 and 2, as described below.

Post-centrifugation spermatozoal pellets were resuspended in warm INRA 96 extender, supplemented with 5% seminal plasma from the same stallion, to a final volume of 12 or 14 mL (resulting in a sperm concentration of 50-100 x 10<sup>6</sup> sperm/mL). Aliquots of centrifuged and resuspended semen were further diluted with extender to obtain a final sperm concentration of approximately 20 x 10<sup>6</sup> sperm/mL, and then were subjected to CASMA. Aliquots of resuspended sperm were also packaged with minimal air space in capped 5-mL polypropylene tubes. Prepared vials were packaged in an Equitainer™ II and stored for 24 h.

### **Centrifugation Procedures**

All centrifugation tubes, centrifugation media, and cushion media were placed in an incubator (37°C) prior to processing semen. Specially designed, 40-mL capacity, glass nipple-bottom centrifugation tubes (Fig. 1a, 1b; Pesce Lab Sales, Kennett Square, PA, USA; NIPPLE) were washed, siliconized and sterilized by dry heat (120°C for 2 h) between uses. Polypropylene 50-mL capacity conical-bottom centrifuge tubes (Fig. 2a,

2b; Corning Life Sciences, Lowell, MA, USA; CONICAL tubes) were discarded following each use.

The nipple portion of NIPPLE tubes was filled with centrifugation media (approximately 1.5 mL), then 30  $\mu$ L of cushion media was layered beneath the centrifugation media, using a positive-displacement pipette (Rainin Instrument, Oakland, CA, USA). After careful addition (so as not to disrupt the cushion layer) of 35 mL of extended semen containing approximately one billion spermatozoa, NIPPLE tubes were placed in specially designed centrifugation adapters (Thermo Scientific, Waltham, MA, USA), then balanced by weight, and centrifuged (IEC Centra CL3; Thermo Scientific, Waltham, MA, USA) with a swinging rotor at either 400 x *g* (Experiment 1 and Experiment 2) or 600 x *g* (Experiment 2) for 20 min at ambient temperature.

Extended semen (35 mL containing approximately one billion spermatozoa) was first loaded into CONICAL tubes, then 3.5 mL of cushion media was layered beneath the extended semen, using a blunt-tipped 3.5-inch spinal needle (18 ga), attached to a 5-mL syringe. This volume filled the conical portion of the centrifuge tubes. The tubes were then centrifuged (IEC Centra CL2; Thermo Scientific, Waltham, MA, USA) at 1000 x *g* for 20 min at ambient temperature.

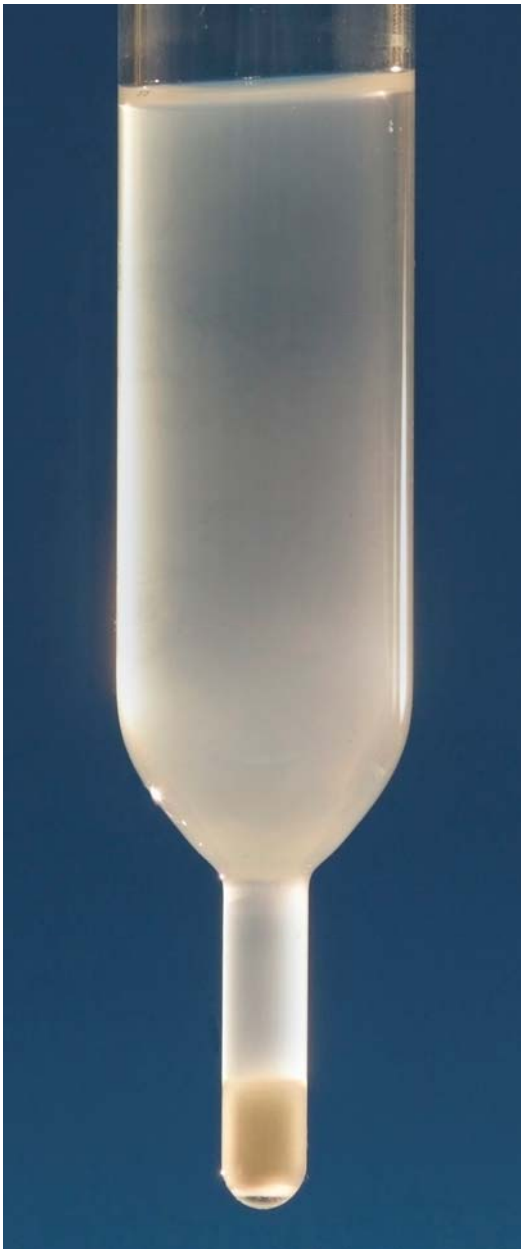


Fig. 1a

Photograph of semen centrifuged with clear extender in NIPPLE tube with cushion medium (30  $\mu$ L) added beneath semen



Fig. 1b

Photograph of semen centrifuged with opaque extender in NIPPLE tube with cushion medium (30  $\mu$ L) added beneath semen

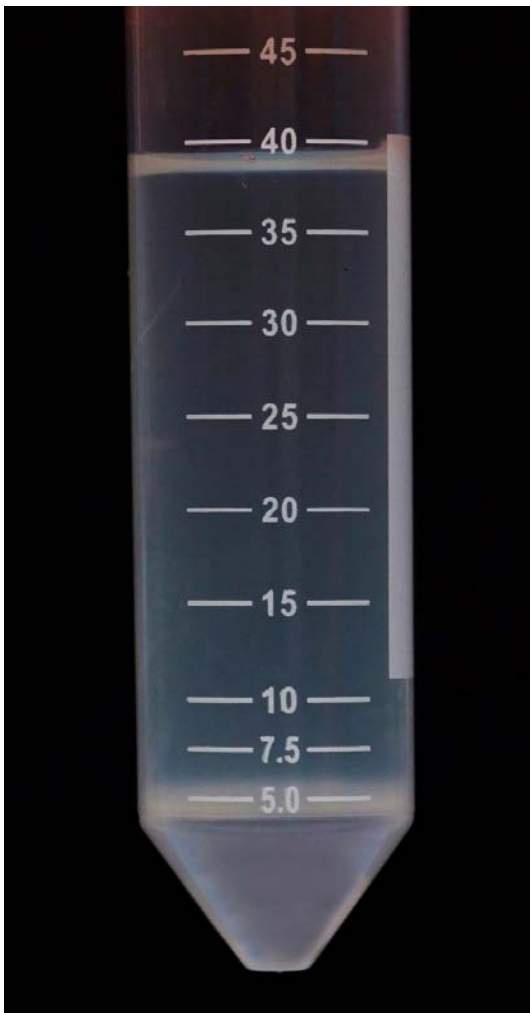


Fig. 2a

Photograph of semen centrifuged with clear extender in CONICAL tube with cushion medium (3.5 mL) added beneath semen

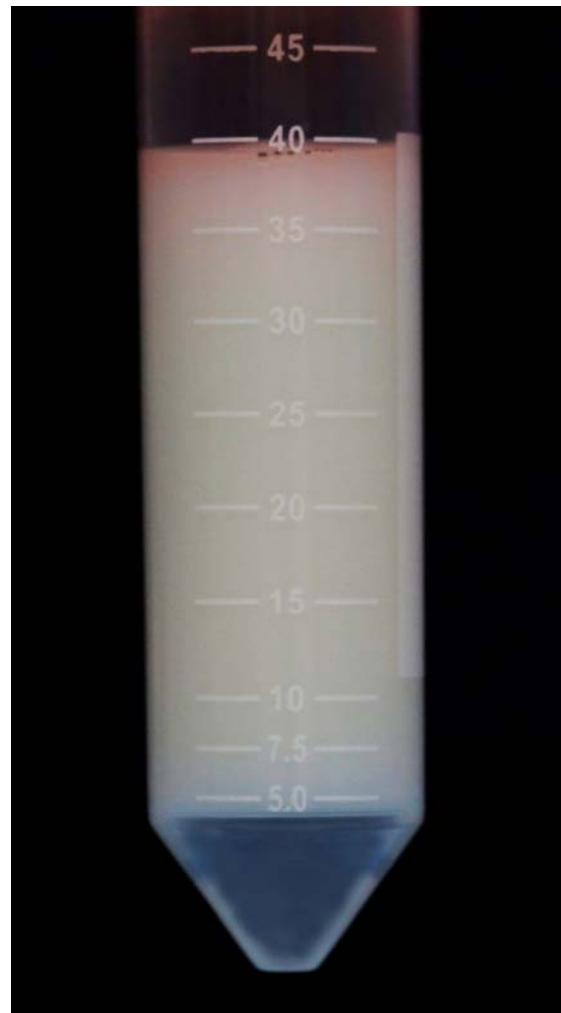


Fig. 2b

Photograph of semen centrifuged with opaque extender in CONICAL tube with cushion medium (3.5 mL) added beneath semen

## **Experimental Design**

### *Experiment 1*

To evaluate the effects of centrifugation-extender type and centrifugation-tube type on sperm recovery rates and semen quality, gel-free semen was diluted in one of two extender types; 1) INRA 96, an opaque, milk-based semen extender, or 2) an optically clear, balanced salt solution, “HGLL,” that was prepared in the laboratory as previously described [9,73]. Thirty-five-mL aliquots of extended semen were loaded into NIPPLE tubes or CONICAL tubes, as described above. The cushion medium, Eqcellsire® Component B (IMV, L’Aigle, France; EQB) was added beneath the extended semen, as described above, to provide a cushion for sperm during centrifugation. Following centrifugation, the supernatant in NIPPLE tubes was aspirated to the top of the nipple portion of the tube, and the cushion fluid (30- $\mu$ L volume) and sperm pellet were resuspended with INRA 96 extender, as described above. The centrifugation supernatant was removed to a preset volume mark in the CONICAL tubes (5-mL), and then the majority of the cushion medium was removed by aspiration. The resulting sperm pellet was resuspended with INRA 96 extender, as described above.

Aliquots of resuspended semen samples were prepared immediately for CASMA or were frozen in 1-mL vials for later analysis of sperm concentration and sperm chromatin integrity (SCSA). Additional aliquots were subjected to 24 h of cooled storage, followed by preparation for CASMA or frozen storage for later analysis. The effects of tube type and extender type on sperm recovery rate, sperm motion characteristics, and integrity of sperm chromatin were examined.

## *Experiment 2*

To examine the effects of centrifugation-cushion type and NIPPLE-tube centrifugation force on sperm recovery rates and semen quality, gel-free semen was diluted in INRA 96 extender. Extended semen (containing approximately 1 billion spermatozoa) was added to NIPPLE tubes, as described above, with one of three different proprietary forms of iodixanol-based cushion medium (30- $\mu$ L volume) layered beneath the extended semen: 1) Eqcellsire® Component B (IMV, L' Aigle, France; EQB), 2) Cushion Fluid™ (Minitüb, Tiefenbach, Germany; CF), or 3) OptiPrep™ (Greiner Bio-One, Axis-Shield, Oslo, Norway; OP). Semen was then centrifuged at either 400 x g or 600 x g for 20 min. Following centrifugation, the supernatant was aspirated to the top of the nipple portion of the tubes, and the cushion medium (30- $\mu$ L volume) and sperm pellet were mixed with INRA 96 extender, as described above. Aliquots of resuspended semen samples were prepared immediately for CASMA or frozen in 1-mL vials for later analysis of sperm concentration and sperm chromatin integrity (SCSA). Additional aliquots were subjected to 24 h of cooled storage, followed by preparation for CASMA or frozen storage for later analysis. The effects of centrifuge-cushion type and centrifugation force on sperm recovery rate, sperm motion characteristics, and integrity of sperm chromatin were examined.

### **Computer Assisted Sperm Motion Analysis (CASMA)**

Sperm were analyzed by CASMA in a manner similar to that previously described [74]. Warmed (37 °C) analysis chambers (fixed height of 20  $\mu$ m) affixed to

microscope slides (Leja Standard Count 2 Chamber slides; Leja Products, B.V., Nieuw-Vennep, The Netherlands) were slowly loaded with a 6- $\mu$ L volume of extended semen. The slides were then placed on a stage (37 °C) and inserted into the CASMA instrument for evaluation. A total of 10 microscopic fields and a minimum of 500 sperm were examined per sample. Preset values for the IVOS system consisted of the following: frames acquired – 45; frame rate – 60 Hz; minimum contrast – 70; minimum cell size – 4 pixels; minimum static contrast – 30; straightness (STR) 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 sperm (MOT); percentage of progressively motile sperm (PMOT); mean curvilinear velocity (VCL;  $\mu$ m/s); mean average-path velocity (VAP;  $\mu$ m/s); mean straight-line velocity (VSL;  $\mu$ m/s), straightness ( $[\text{VSL}/\text{VAP}] \times 100$ ; % ;STR), and linearity ( $[\text{VSL}/\text{VCL}] \times 100$ ; % ;LIN).

### **Sperm Chromatin Structure Assay (SCSA)**

This assay was performed as previously described [75-77]. Individual semen samples were thawed in a water bath set at 37 °C. Approximately five- $\mu$ L aliquots of thawed semen were mixed with 195  $\mu$ L of a buffered solution which was then combined with a low pH (~ 1.2) detergent solution (400  $\mu$ L) for 30 sec. A solution of the heterochromatic dye, acridine orange, was added (1.2 mL at 4.0  $\mu$ g/mL) to the sample



and it was processed immediately (30 sec) on a flow cytometer (FACScan; Becton Dickinson, Mountain View, CA, USA). The sample was allowed to pass through the tubing for two min before evaluation of cells. Semen volume was adjusted so the flow rate approximated 200 cells/sec. 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). Data were acquired in a list-mode and Sperm Chromatin Structure Assay values were calculated using WinList™ software (Verity Software House, Topsham, ME, USA). Quantification of DNA denaturation in each cell was determined by the term alpha-t ( $\alpha$ ), which is defined as the ratio of red/(red + green fluorescence). The alpha-t ( $\alpha$ ) 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 histograms. The endpoint, Cells Outside the Main Population (COMPot), was determined by selecting those sperm cells to the right of the main population, and represents the number of sperm cells outside the main population, as a percentage of the total number of sperm cells evaluated.

### **Fluorescence-based Measurement of Sperm Concentration**

Sperm concentrations in aliquots of both frozen-thawed neat semen and frozen-thawed semen that had been previously subjected to centrifugation and resuspension in extender were subjected to sperm enumeration using a fluorescence-based instrument (NucleoCounter SP-100; Chemometec A/S, Allerød, Denmark). This instrument was

previously validated in our laboratory for measurement of sperm concentration in stallion semen (unpublished observations). Briefly, semen was thawed and subjected to vortex-mixing to evenly distribute the cells. An aliquot (10  $\mu$ L) of the semen sample was then mixed with a detergent-based solution at a volume ratio appropriate for the estimated sperm concentration (1:100 to 1:400). The dilution ratio was determined by modification of manufacturer recommendations for bull semen, based on preliminary experiments conducted in our laboratory with stallion semen (unpublished observations). The diluted semen was aspirated into a proprietary cassette laced with propidium iodide, and the loaded cassette was placed in the instrument chamber for analysis, using proprietary software. Three replicates were evaluated per sample and the mean value of the replicates was used for statistical comparisons.

### **Statistical Analysis**

A general linear model [78] was used to evaluate effects of centrifugation-tube type, centrifugation medium, and centrifugation-cushion type on 8 experimental endpoints (MOT, PMOT, VCL, VAP, VSL, STR, LIN, and COMPat) and the effects of centrifugation-tube type, centrifugation medium, centrifugation-cushion type, and centrifugation force on sperm recovery rate. Variables measured as percentages were normalized by transformation to angles corresponding to *arc sine of the square root of percentage* for variance analyses. Tabular data are presented as non-transformed values, for ease of interpretation. The Student-Newman-Keuls multiple range test was used to separate main-effect means when treatment F ratios were significant ( $P < 0.05$ ).

## RESULTS

### Experiment 1

The effects of centrifugation medium (INRA 96 versus HGLL) and tube type (CONICAL versus NIPPLE) on sperm quality values at two different time points are presented in Tables 1 and 2, respectively. At either time point, i.e., Time 0 h (T0; unstored extended semen) or Time 24 h (T24; extended semen stored cooled for 24 h), no extender by tube-type interaction was detected for any dependent variable ( $P > 0.05$ ). At T0, mean MOT was higher for semen centrifuged in INRA 96 as compared to HGLL ( $P < 0.05$ ), but was similar to CONT ( $P > 0.05$ ). Mean PMOT was higher for semen centrifuged in INRA 96 than the other treatment groups ( $P < 0.05$ ). Measures of sperm velocity (mean VCL, VAP, and VSL) were similar between semen centrifuged in INRA 96 and HGLL, but were lower for centrifuged semen than for CONT. One measure of sperm trajectory, mean STR, was similar for semen centrifuged in INRA 96 or HGLL ( $P > 0.05$ ), but was higher for semen centrifuged in INRA 96 than for CONT ( $P < 0.05$ ). Mean LIN of CONT was higher than for centrifuged semen ( $P < 0.05$ ); however, no difference was detected between treatment groups subjected to centrifugation ( $P > 0.05$ ). Mean COMP<sub>at</sub> was higher for semen centrifuged in INRA 96, as compared to HGLL ( $P < 0.05$ ), but mean COMP<sub>at</sub> for both treatment groups was similar to that of CONT ( $P > 0.05$ ).

At T0, mean MOT and PMOT were higher for semen centrifuged in NIPPLE tubes than that centrifuged in CONICAL tubes ( $P < 0.05$ ). Mean MOT was higher for CONT than for semen centrifuged in NIPPLE tubes and CONICAL tubes ( $P < 0.05$ );

however, mean PMOT was similar between CONT and NIPPLE ( $P > 0.05$ ). Velocity measures (mean VCL, VAP, and VSL) were similar between NIPPLE tubes and CONICAL tubes ( $P > 0.05$ ), but were lower than that of CONT ( $P < 0.05$ ). Mean STR was similar across treatment groups ( $P > 0.05$ ), but mean LIN was higher ( $P < 0.05$ ) in CONT than in centrifuged-semen groups. Mean COMPat was similar among treatment groups ( $P > 0.05$ ).

Table 1. Effect of centrifugation medium on measures (mean  $\pm$  SEM) of sperm motion characteristics and chromatin quality (COMPat) for fresh extended (Time 0 h) and cool-stored extended (Time 24 h) semen.

Laboratory Parameter*	Time 0 h			Time 24 h <sup>†</sup>		
	CONT <sup>‡</sup>	Centrifugation medium		CONT <sup>‡</sup>	Centrifugation medium	
		INRA 96	HGLL		INRA 96	HGLL
MOT	80 <sup>a</sup> $\pm$ 2.1	80 <sup>a</sup> $\pm$ 1.4	75 <sup>b</sup> $\pm$ 1.4	78 <sup>b</sup> $\pm$ 2.0	80 <sup>a</sup> $\pm$ 1.2	74 <sup>c</sup> $\pm$ 1.3
PMOT	55 <sup>b</sup> $\pm$ 3.1	57 <sup>a</sup> $\pm$ 2.0	49 <sup>c</sup> $\pm$ 1.9	54 <sup>a</sup> $\pm$ 3.2	54 <sup>a</sup> $\pm$ 2.1	46 <sup>b</sup> $\pm$ 1.9
VCL	221 <sup>a</sup> $\pm$ 7.8	188 <sup>b</sup> $\pm$ 3.8	192 <sup>b</sup> $\pm$ 3.9	182 <sup>b</sup> $\pm$ 5.4	204 <sup>a</sup> $\pm$ 3.7	202 <sup>a</sup> $\pm$ 3.3
VAP	121 <sup>a</sup> $\pm$ 3.0	91 <sup>b</sup> $\pm$ 2.1	94 <sup>b</sup> $\pm$ 2.2	91 <sup>b</sup> $\pm$ 3.0	99 <sup>a</sup> $\pm$ 1.8	98 <sup>a</sup> $\pm$ 1.5
VSL	87 <sup>a</sup> $\pm$ 2.2	65 <sup>b</sup> $\pm$ 1.3	67 <sup>b</sup> $\pm$ 1.3	66 <sup>a</sup> $\pm$ 1.9	67 <sup>a</sup> $\pm$ 1.1	66 <sup>a</sup> $\pm$ 1.1
STR	71 <sup>b</sup> $\pm$ 1.6	73 <sup>a</sup> $\pm$ 0.9	72 <sup>ab</sup> $\pm$ 0.9	73 <sup>a</sup> $\pm$ 1.4	69 <sup>b</sup> $\pm$ 1.3	68 <sup>c</sup> $\pm$ 1.1
LIN	42 <sup>a</sup> $\pm$ 1.6	36 <sup>b</sup> $\pm$ 0.4	36 <sup>b</sup> $\pm$ 0.5	37 <sup>a</sup> $\pm$ 0.8	34 <sup>b</sup> $\pm$ 0.7	34 <sup>b</sup> $\pm$ 0.7
COMPat	7 <sup>ab</sup> $\pm$ 0.6	7 <sup>a</sup> $\pm$ 0.4	6 <sup>b</sup> $\pm$ 0.4	9 <sup>a</sup> $\pm$ 1.0	8 <sup>b</sup> $\pm$ 0.4	8 <sup>b</sup> $\pm$ 0.5

\* MOT = total spermatozoal motility (%); PMOT = progressive sperm motility (%); VCL = curvilinear velocity ( $\mu\text{m/s}$ ); VAP = average-path velocity ( $\mu\text{m/s}$ ); VSL = straight-line velocity ( $\mu\text{m/s}$ ); STR = straightness ( $[\text{VAP}/\text{VCL}]100$ ; %); LIN = linearity ( $[\text{VSL}/\text{VCL}]100$ ; %); COMPat = percentage of sperm with at value outside the main population (%).

<sup>†</sup> Semen stored in an Equitainer<sup>TM</sup> for 24 h.

<sup>‡</sup> CONT samples were diluted in INRA 96 semen extender.

<sup>§</sup> Percentage data (MOT, PMOT, STR, LIN, COMPat) were arc sine-root transformed for normalization prior to statistical analysis. Original means and SEM values are presented in table to ease interpretation but statistical tests were conducted on transformed data.

<sup>a,b</sup> Within time period and within laboratory parameter, means with different superscripts differ ( $P < 0.05$ ).

Table 2. Effect of centrifugation-tube type on measures (mean  $\pm$  SEM) of sperm motion characteristics and chromatin quality (COMP<sub>at</sub>) for fresh extended (Time 0 h) and cool-stored extended (Time 24 h) semen.

Laboratory Parameter*	Time 0 h			Time 24 h <sup>†</sup>		
	CONT <sup>‡</sup>	Centrifugation-tube type		CONT <sup>‡</sup>	Centrifugation-tube type	
		NIPPLE	CONICAL		NIPPLE	CONICAL
MOT	80 <sup>a</sup> $\pm$ 2.1	78 <sup>b</sup> $\pm$ 1.4	76 <sup>c</sup> $\pm$ 1.5	78 <sup>a</sup> $\pm$ 2.0	78 <sup>a</sup> $\pm$ 1.2	75 <sup>b</sup> $\pm$ 1.3
PMOT	55 <sup>a</sup> $\pm$ 3.1	55 <sup>a</sup> $\pm$ 2.0	52 <sup>b</sup> $\pm$ 2.0	54 <sup>a</sup> $\pm$ 3.2	52 <sup>b</sup> $\pm$ 2.1	49 <sup>c</sup> $\pm$ 2.1
VCL	221 <sup>a</sup> $\pm$ 7.8	188 <sup>b</sup> $\pm$ 3.6	191 <sup>b</sup> $\pm$ 4.1	182 <sup>c</sup> $\pm$ 5.4	206 <sup>a</sup> $\pm$ 3.7	200 <sup>b</sup> $\pm$ 3.3
VAP	121 <sup>a</sup> $\pm$ 3.0	92 <sup>b</sup> $\pm$ 2.0	94 <sup>b</sup> $\pm$ 2.3	91 <sup>c</sup> $\pm$ 3.0	100 <sup>a</sup> $\pm$ 1.8	97 <sup>b</sup> $\pm$ 1.5
VSL	87 <sup>a</sup> $\pm$ 2.1	65 <sup>b</sup> $\pm$ 1.2	67 <sup>b</sup> $\pm$ 1.4	66 <sup>a</sup> $\pm$ 1.9	67 <sup>a</sup> $\pm$ 1.2	66 <sup>a</sup> $\pm$ 1.0
STR	71 <sup>a</sup> $\pm$ 1.6	72 <sup>a</sup> $\pm$ 0.9	72 <sup>a</sup> $\pm$ 0.9	73 <sup>a</sup> $\pm$ 1.4	68 <sup>b</sup> $\pm$ 1.2	69 <sup>b</sup> $\pm$ 1.1
LIN	42 <sup>a</sup> $\pm$ 1.6	36 <sup>b</sup> $\pm$ 0.4	37 <sup>b</sup> $\pm$ 0.5	37 <sup>a</sup> $\pm$ 0.8	34 <sup>b</sup> $\pm$ 0.7	35 <sup>b</sup> $\pm$ 0.7
COMP <sub>at</sub>	7 <sup>a</sup> $\pm$ 0.6	6 <sup>a</sup> $\pm$ 0.4	7 <sup>a</sup> $\pm$ 0.4	9 <sup>a</sup> $\pm$ 1.0	7 <sup>c</sup> $\pm$ 0.4	8 <sup>b</sup> $\pm$ 0.5

\* MOT = total spermatozoal motility (%); PMOT = progressive sperm motility (%); VCL = curvilinear velocity ( $\mu$ m/s); VAP = average-path velocity ( $\mu$ m/s); VSL = straight-line velocity ( $\mu$ m/s); STR = straightness ([VAP/VCL]100; %); LIN = linearity ([VSL/VCL]100; %); COMP<sub>at</sub> = percentage of sperm with *at* value outside the main population (%).

<sup>†</sup> Semen stored in an Equitainer™ for 24 h.

<sup>‡</sup> CONT samples were diluted in INRA 96 semen extender.

<sup>§</sup> Percentage data (MOT, PMOT, STR, LIN, COMP<sub>at</sub>) were arc sine-root transformed for normalization prior to statistical analysis. Original means and SEM values are presented in table to ease interpretation but statistical tests were conducted on transformed data.

<sup>a,b</sup> Within time period and within laboratory parameter, means with different superscripts differ ( $P < 0.05$ ).

At T24, mean MOT for semen centrifuged in INRA 96 was higher than that centrifuged in HGLL or for CONT ( $P < 0.05$ ). Mean PMOT was similar for semen centrifuged in INRA 96 and CONT ( $P > 0.05$ ), but was higher in these groups than for semen centrifuged in HGLL. Two measures of sperm velocity, mean VCL and VAP, were higher in centrifuged semen than in CONT ( $P < 0.05$ ), whereas mean VSL was similar across all treatment groups. Mean COMP<sub>at</sub> was higher for CONT than for either centrifuged-semen group ( $P < 0.05$ ).

At T24, mean MOT and PMOT were higher for sperm centrifuged in NIPPLE tubes than for sperm centrifuged in CONICAL tubes ( $P < 0.05$ ). While mean MOT was similar between the NIPPLE tube treatment and CONT ( $P > 0.05$ ), mean PMOT was higher in CONT ( $P < 0.05$ ). Both mean VCL and mean VAP were higher for semen centrifuged in NIPPLE tubes, as compared to CONICAL tubes or CONT ( $P < 0.05$ ). Mean VSL, however, was similar among treatment groups. Mean STR and LIN were similar between CONICAL and NIPPLE treatments ( $P > 0.05$ ); yet, values were lower than that of CONT ( $P < 0.05$ ). Mean COMPat was lower for semen centrifuged in NIPPLE tubes, as compared to CONICAL tubes ( $P < 0.05$ ), and was lower in either centrifuged-semen group, as compared to CONT ( $P < 0.05$ ).

Sperm recovery rate (%) was similar ( $P > 0.05$ ) between semen centrifuged in INRA 96 ( $99.6 \pm 1.7$ ) and that centrifuged in HGLL ( $99.6 \pm 1.4$ ); however, sperm recovery rate was higher following centrifugation in CONICAL tubes ( $102.8 \pm 1.4$ ), as compared to NIPPLE tubes ( $96.4 \pm 1.4$ ). An extender by tube-type interaction was not detected for recovery rate ( $P > 0.05$ ).

## **Experiment 2**

The effects of centrifugation-cushion type (OptiPrep™ (OP; Greiner Bio-One, Axis-Shield, Oslo, Norway), Eqcellsire® Component B (EQB; IMV Technologies; Maple Grove, MN), and Cushion Fluid™ (CF; Minitüb; Tiefenbach, Germany)) and centrifugation force (400 or 600 x *g*) on sperm motility and chromatin integrity at two different time points (T0 and T24) are presented in Tables 3 and 4, respectively. At

either time point, no cushion by  $g$  force interaction was detected ( $P > 0.05$ ) for any experimental endpoint. At T0, mean MOT and PMOT were similar among all cushion types and CONT ( $P > 0.05$ ) whereas velocity measures (mean VCL, VAP, and STR) were higher for CONT, as compared to semen centrifuged using any of the three cushion types ( $P < 0.05$ ). Mean LIN was similar among all treatment groups ( $P > 0.05$ ), but mean STR was higher for centrifuged semen than for CONT ( $P < 0.05$ ).

At T0, no differences were detected between semen centrifuged at 400 x  $g$  or 600 x  $g$  for any variable tested. Mean MOT was similar between centrifuged semen and CONT ( $P > 0.05$ ), but centrifuged semen yielded a higher PMOT ( $P < 0.05$ ). All velocity measures (mean VAP, VAP, and VSL) were higher for CONT than for centrifuged treatments. Mean LIN was similar across treatments ( $P > 0.05$ ), but mean STR was higher for centrifuged semen than for CONT ( $P < 0.05$ ). Mean COMP<sub>at</sub> was higher for CONT than for centrifuged semen ( $P < 0.05$ ).

At T24, mean MOT of centrifuged semen was higher than that of the CONT ( $P < 0.05$ ), but mean PMOT was similar among all treatment groups ( $P > 0.05$ ). Of the velocity measures, mean VCL and VAP were similar among all cushion types ( $P > 0.05$ ), and were higher in semen centrifuged with CF or OP than in CONT ( $P < 0.05$ ). Mean VSL was similar across all treatment groups ( $P > 0.05$ ). Mean STR and LIN were similar for semen centrifuged with CF, OP, or EQB as a centrifugation cushion ( $P > 0.05$ ), but mean values for CONT were higher than those for centrifuged semen ( $P < 0.05$ ). Mean COMP<sub>at</sub> was higher for CONT than for centrifuged semen ( $P < 0.05$ ).

At T24, no differences were detected between centrifugation forces tested for any dependent variable ( $P > 0.05$ ). Centrifuged semen yielded a higher mean MOT ( $P < 0.05$ ) than, but a similar mean PMOT ( $P > 0.05$ ) to CONT. Mean VCL and VAP were also higher for centrifuged semen, as compared to CONT ( $P < 0.05$ ); yet, mean VSL was similar across treatments ( $P > 0.05$ ). Group CONT yielded a higher mean STR and LIN than did centrifuged semen ( $P < 0.05$ ), but mean COMPat of CONT exceeded that of centrifuged semen ( $P < 0.05$ ).

Sperm recovery rate (%) was similar ( $P > 0.05$ ) for semen centrifuged at 400 x g ( $90.6 \pm 2.9$ ) or at 600 x g ( $89.6 \pm 1.7$ ).



Table 3. Effect of centrifugation-cushion medium on measures (mean  $\pm$  SEM) of sperm motion characteristics and chromatin quality (COMP<sub>at</sub>) for fresh extended (Time 0 h) and cool-stored extended (Time 24 h) semen.

Laboratory Parameter*	Time 0 h				Time 24 h <sup>†</sup>			
	CONT <sup>‡</sup>	Centrifugation-cushion medium			CONT <sup>‡</sup>	Centrifugation-cushion medium		
		CF <sup>≈</sup>	EQB <sup>¶</sup>	OP <sup>#</sup>		CF	EQB	OP
MOT	89 <sup>a</sup> $\pm$ 0.7	89 <sup>a</sup> $\pm$ 0.6	89 <sup>a</sup> $\pm$ 0.6	89 <sup>a</sup> $\pm$ 0.6	85 <sup>b</sup> $\pm$ 1.4	88 <sup>a</sup> $\pm$ 0.7	88 <sup>a</sup> $\pm$ 0.9	87 <sup>a</sup> $\pm$ 0.9
PMOT	66 <sup>a</sup> $\pm$ 1.6	69 <sup>a</sup> $\pm$ 1.0	68 <sup>a</sup> $\pm$ 1.3	69 <sup>a</sup> $\pm$ 1.1	63 <sup>a</sup> $\pm$ 2.4	64 <sup>a</sup> $\pm$ 1.3	64 <sup>a</sup> $\pm$ 1.6	62 <sup>a</sup> $\pm$ 1.5
VCL	259 <sup>a</sup> $\pm$ 7.4	214 <sup>b</sup> $\pm$ 3.9	212 <sup>b</sup> $\pm$ 4.3	212 <sup>b</sup> $\pm$ 4.0	205 <sup>b</sup> $\pm$ 8.6	216 <sup>a</sup> $\pm$ 3.6	212 <sup>ab</sup> $\pm$ 3.5	215 <sup>a</sup> $\pm$ 3.8
VAP	132 <sup>a</sup> $\pm$ 2.7	106 <sup>b</sup> $\pm$ 2.7	105 <sup>b</sup> $\pm$ 2.9	106 <sup>b</sup> $\pm$ 2.8	101 <sup>b</sup> $\pm$ 4.7	106 <sup>a</sup> $\pm$ 2.5	104 <sup>ab</sup> $\pm$ 2.4	106 <sup>a</sup> $\pm$ 2.7
VSL	91 <sup>a</sup> $\pm$ 2.0	76 <sup>b</sup> $\pm$ 1.5	76 <sup>b</sup> $\pm$ 1.5	77 <sup>b</sup> $\pm$ 1.6	72 <sup>a</sup> $\pm$ 2.4	73 <sup>a</sup> $\pm$ 0.8	72 <sup>a</sup> $\pm$ 0.9	73 <sup>a</sup> $\pm$ 1.0
STR	68 <sup>b</sup> $\pm$ 0.7	72 <sup>a</sup> $\pm$ 1.4	72 <sup>a</sup> $\pm$ 1.4	73 <sup>a</sup> $\pm$ 1.3	72 <sup>a</sup> $\pm$ 2.0	69 <sup>b</sup> $\pm$ 1.6	70 <sup>b</sup> $\pm$ 1.6	70 <sup>b</sup> $\pm$ 1.5
LIN	36 <sup>a</sup> $\pm$ 0.6	37 <sup>a</sup> $\pm$ 0.4	37 <sup>a</sup> $\pm$ 0.5	37 <sup>a</sup> $\pm$ 0.5	36 <sup>a</sup> $\pm$ 0.7	35 <sup>b</sup> $\pm$ 0.5	35 <sup>b</sup> $\pm$ 0.5	35 <sup>b</sup> $\pm$ 0.4
COMP <sub>at</sub>	5 <sup>a</sup> $\pm$ 0.6	4 <sup>b</sup> $\pm$ 0.3	4 <sup>ab</sup> $\pm$ 0.4	5 <sup>ab</sup> $\pm$ 0.4	7 <sup>a</sup> $\pm$ 0.9	5 <sup>b</sup> $\pm$ 0.4	6 <sup>b</sup> $\pm$ 0.6	5 <sup>b</sup> $\pm$ 0.5

\* MOT = total spermatozoal motility (%); PMOT = progressive sperm motility (%);  
VCL = curvilinear velocity ( $\mu\text{m/s}$ ); VAP = average-path velocity ( $\mu\text{m/s}$ );  
VSL = straight-line velocity ( $\mu\text{m/s}$ ); STR = straightness ( $[\text{VAP}/\text{VCL}]100$ ; %);  
LIN = linearity ( $[\text{VSL}/\text{VCL}]100$ ; %); COMP<sub>at</sub> = percentage of sperm with at value outside the main population (%).

<sup>†</sup> Semen stored in an Equitainer™ for 24 h.

<sup>‡</sup> CONT samples were diluted in INRA 96 semen extender.

<sup>≈</sup> Cushion Fluid™; <sup>¶</sup> Eqcellsire® Component B; <sup>#</sup> OptiPrep™

<sup>§</sup> Percentage data (MOT, PMOT, STR, LIN, COMP<sub>at</sub>) were arc sine-root transformed for normalization prior to statistical analysis. Original means and SEM values are presented in table to ease interpretation but statistical tests were conducted on transformed data.

<sup>a,b</sup> Within time period and within laboratory parameter, means with different superscripts differ ( $P < 0.05$ ).

Table 4. Effect of centrifugation *g* force on measures (mean  $\pm$  SEM) of sperm motion characteristics and chromatin quality (COMP<sub>at</sub>) for fresh extended (Time 0 h) and cool-stored extended (Time 24 h) semen.

Laboratory Parameter*	Time 0 h				Time 24 h <sup>†</sup>	
	CONT <sup>‡</sup>	Centrifugation force		CONT <sup>‡</sup>	Centrifugation force	
		400 x <i>g</i>	600 x <i>g</i>		400 x <i>g</i>	600 x <i>g</i>
MOT	89 <sup>a</sup> $\pm$ 0.7	89 <sup>a</sup> $\pm$ 0.6	89 <sup>a</sup> $\pm$ 0.4	85 <sup>b</sup> $\pm$ 1.4	88 <sup>a</sup> $\pm$ 0.8	88 <sup>a</sup> $\pm$ 0.6
PMOT	66 <sup>b</sup> $\pm$ 1.6	69 <sup>a</sup> $\pm$ 0.9	68 <sup>a</sup> $\pm$ 0.8	63 <sup>a</sup> $\pm$ 2.4	63 <sup>a</sup> $\pm$ 1.4	64 <sup>a</sup> $\pm$ 1.1
VCL	259 <sup>a</sup> $\pm$ 7.4	212 <sup>b</sup> $\pm$ 3.3	213 <sup>b</sup> $\pm$ 3.2	205 <sup>b</sup> $\pm$ 8.6	214 <sup>a</sup> $\pm$ 3.0	214 <sup>a</sup> $\pm$ 2.9
VAP	132 <sup>a</sup> $\pm$ 2.7	106 <sup>b</sup> $\pm$ 2.3	106 <sup>b</sup> $\pm$ 2.2	101 <sup>b</sup> $\pm$ 4.8	105 <sup>a</sup> $\pm$ 2.0	105 <sup>a</sup> $\pm$ 2.0
VSL	91 <sup>a</sup> $\pm$ 2.0	76 <sup>b</sup> $\pm$ 1.3	76 <sup>b</sup> $\pm$ 1.2	72 <sup>a</sup> $\pm$ 2.4	73 <sup>a</sup> $\pm$ 0.8	73 <sup>a</sup> $\pm$ 0.7
STR	68 <sup>b</sup> $\pm$ 0.7	72 <sup>a</sup> $\pm$ 1.1	72 <sup>a</sup> $\pm$ 1.1	72 <sup>a</sup> $\pm$ 2.0	70 <sup>b</sup> $\pm$ 1.3	70 <sup>b</sup> $\pm$ 1.2
LIN	36 <sup>a</sup> $\pm$ 0.6	37 <sup>a</sup> $\pm$ 0.4	37 <sup>a</sup> $\pm$ 0.4	36 <sup>a</sup> $\pm$ 0.7	35 <sup>b</sup> $\pm$ 0.4	35 <sup>b</sup> $\pm$ 0.3
COMP <sub>at</sub>	5 <sup>a</sup> $\pm$ 0.6	4 <sup>b</sup> $\pm$ 0.3	4 <sup>b</sup> $\pm$ 0.3	7 <sup>a</sup> $\pm$ 0.9	6 <sup>b</sup> $\pm$ 0.4	5 <sup>b</sup> $\pm$ 0.4

\* MOT = total spermatozoal motility (%); PMOT = progressive sperm motility (%);  
VCL = curvilinear velocity ( $\mu\text{m/s}$ ); VAP = average-path velocity ( $\mu\text{m/s}$ );  
VSL = straight-line velocity ( $\mu\text{m/s}$ ); STR = straightness ( $[\text{VAP}/\text{VCL}]100$ ; %);  
LIN = linearity ( $[\text{VSL}/\text{VCL}]100$ ; %); COMP<sub>at</sub> = percentage of sperm with at value outside the main population (%).

<sup>†</sup> Semen stored in an Equitainer™ for 24 h.

<sup>‡</sup> CONT samples were diluted in INRA 96 semen extender.

<sup>§</sup> Percentage data (MOT, PMOT, STR, LIN, COMP<sub>at</sub>) were arc sine-root transformed for normalization prior to statistical analysis. Original means and SEM values are presented in table to ease interpretation but statistical tests were conducted on transformed data.

<sup>a,b</sup> Within time period and within laboratory parameter, means with different superscripts differ ( $P < 0.05$ ).

## DISCUSSION AND SUMMARY

Milk-based semen extenders have gained widespread acceptance in the equine breeding industry for use with both fresh and cooled semen [61,65]. Incorporation of these extenders for equine artificial insemination likely stems from successful results when used with dairy cattle semen [59,60]. Such extenders aid in preserving sperm viability following cooled storage, as evidenced by retention of both spermatozoal motility [7,7,16,16,60] and fertilizing capacity [7,16,66,69,70,79]. Some components of milk are known to be detrimental to sperm [58] and this may be overcome, at least in part, by subjecting milk to heat treatment prior to use [58,59,61]. A more recent study revealed that some milk fractions impart a detrimental effect on equine sperm whereas other milk fractions, namely  $\beta$ -lactoglobulin and native phosphocaseinate (NPPC), are protective [2]. Subsequent experimentation demonstrated that insemination of mares with cool-stored semen yielded higher pregnancy rates when the extender contained only beneficial milk fractions, as compared to standard milk products [80]. Such an extender is now available commercially as INRA 96 (IMV, Maple Grove, MN, USA). We selected this product as a milk-based extender for our study because it has been reported to optimize the quality of cool-stored equine semen [80]. Furthermore, it is devoid of particulate debris that can otherwise interfere with CASMA systems, where size and luminosity of debris can impede accurate analysis of sperm motility.

We anticipated that sperm motility would be superior following centrifugation in INRA 96, as compared to HGLL, in Experiment 1 because the HGLL was not supplemented with proteins which might maximize protection of sperm from thermal

injury [2]. We elected to use HGLL to represent an optically clear centrifugation extender because preliminary trials revealed that this solution yielded better sperm motility than the other clear extenders tested: Eqcellsire® A (IMV Technologies; Maple Grove, MN), Eqcellsire® A with 2% (v/v) egg yolk, modified Tyrodes medium [11], and phosphate-buffered saline (data not presented). In line with our studies, Magistrini et al. reported that sperm motility was better preserved following cooled storage when semen was diluted in a milk-based extender (INRA 82), as compared to HGLL supplemented with bovine serum albumin (BSA; 1% wt/vol) [9]. Interestingly, Batellier et al. (1997, 1998) reported that semen stored in HGLL supplemented with NPPC, i.e., INRA 96, at 15 °C under aerobic conditions for 24 h yielded a higher pregnancy rate than semen stored in INRA 82 extender and stored at 4 °C under anaerobic conditions, even though sperm motility was similar between these two treatments [2,80]. In addition supplementation of HGLL with NPPC resulted in better sperm motility following in-vitro storage than did HGLL supplemented with BSA [2].

We used HGLL without supplemental BSA, as albumin is a sterol acceptor that can induce efflux of cholesterol from the sperm plasma membrane [81-85], leading to increased permeability of the membrane to bicarbonate and calcium ions [86], and membrane destabilization characteristic of capacitation [81,81,87-90]. In addition, preliminary studies in our laboratory revealed that incorporation of albumin into clear media increased aggregation/agglutination of sperm heads, thereby disrupting CASMA analysis. A commercial source of purified NPPC was available for our study in the form of INRA 96. The NPPC yields an opaque solution, thereby negating its use as a

supplement to the clear centrifugation media in our study.

Others have reported that sperm recovery rate is enhanced when semen is centrifuged by a cushion technique with a clear centrifugation extender, i.e., HGLL, Eqcellsire® A, or a HEPES buffered saline, as compared to opaque extenders, i.e., INRA 82 or INRA 82 with 2% egg yolk [36,41,42]. These workers indicated that the post-centrifugation supernatant and cushion were removed, followed by resuspension of the sperm pellet in a freezing extender. It is logical that the sperm recovery rate would be reduced if an opaque extender were used and the supernatant were aspirated to the level of the sperm pellet, as it would be quite difficult to detect the supernatant-sperm interface when using an opaque extender. In the present study, all supernatant was aspirated to a standardized volume level within the centrifugation tubes following centrifugation (5-mL), based on preliminary trials using a clear extender (HGLL) to determine the supernatant-sperm interface. A follow-up trial in our laboratory indicated that when 1, 2, 3, 4, or 5 billion sperm were centrifuged in 50-mL conical tubes (Corning Life Sciences, Lowell, MA, USA) at 1000 x g for 20 min, using a clear centrifugation medium and a 3.5-mL cushion, a resulting dense sperm band was easily detected between the cushion fluid and the supernatant, with a supernatant-sperm interface at tube-volume marks of approximately 4.6, 4.7, 4.8, 4.9, and 5.0 mL, respectively. This information allows one to extrapolate to the mixing of semen with an opaque centrifugation extender that is designed to provide maximum protection to sperm against thermal shock, without the attendant drawback of aspirating excessive supernatant following centrifugation. Based on these values, it appears that equine sperm pellets

following centrifugation have a volume of approximately 100  $\mu\text{L}$  per billion sperm in the upper portions of the sperm pellet. In the present study, the cushion fluid was aspirated following centrifugation to a point where no clear medium was detectable underneath the sperm pellet. It appeared as though the sperm in the lower portion of the pellet were less compact than those in the upper portion of the pellet. This could be a result of some intermixing of sperm with the cushion fluid at the sperm-cushion interface. This likely resulted in some residual cushion media in the resuspended sperm suspension following aspiration of the cushion.

Two types of centrifugation-tube were compared in this study: a plastic disposable conical-bottom centrifugation tube (CONICAL) that is considered a standard tube type for centrifugation of stallion semen, and a reusable hand-blown glass centrifuge tube (NIPPLE) that was designed to allow easier separation of the centrifuged sperm pellet from the overlying supernatant (J. Crump, personal communication). The centrifugation times were the same for these two tube types, but the centrifugation forces were different. A centrifugation force of 1000  $\times g$  was selected for the conical-bottom tubes, as this force was reported in previous trials for cushioned centrifugation of equine sperm [36,41,42]. Preliminary studies in our laboratory revealed that the glass NIPPLE tubes could not be subjected to a similar  $g$  force without a high breakage rate. We have routinely used a centrifugation force of 400  $\times g$  for clinical purposes, so elected to use this  $g$  force for Experiment 1 and to compare centrifugation forces of 400 and 600  $\times g$  for Experiment 2.

Overall sperm recovery rate in CONICAL tubes (including both HGLL and

INRA 96 centrifugation media - mean of 103%) was similar to that reported by Ecot et al. (2005) [36] when HGLL was used for cushioned centrifugation (mean of 103%). Interestingly, Sieme et al. (2005) reported a mean sperm recovery rate of 83% when using a milk-based centrifugation extender (INRA 82) with cushioned centrifugation at 1000 x g for 20 min. By comparison, the present study yielded a mean sperm recovery rate of 106% when using a similar centrifugation extender (INRA 96) and cushioned CONICAL-tube centrifugation, and a sperm recovery rate of 100% when using HGLL as the centrifugation medium. The recovery rate in excess of 100% is probably attributable to an artifactually reduced sperm concentration determined for semen introduced to tubes for centrifugation. The approximate number of sperm per tube subjected to centrifugation was determined initially by photometric means. In an attempt to secure a more exact measure of sperm numbers subjected to centrifugation and recovered in sperm pellets, cells were enumerated in both frozen-thawed neat semen and in frozen-thawed INRA 96-resuspended sperm pellets, using the NucleoCounter technique described above. We found that sperm in frozen-thawed neat semen had a tendency to clump, whereas this feature was not detected in sperm pellets that were resuspended in INRA 96 prior to freezing. While most frozen-thawed sperm were successfully dispersed by vortex mixing, a small percentage of the sperm remained clumped. This would result in a slight underestimation of initial sperm concentration which alters the amount of sperm introduced into the tubes prior to centrifugation, thereby yielding a corresponding slight overestimation of sperm recovery rate. Analysis of sperm recovery rates in our laboratory, based on hemacytometer counts when using the same

centrifugation methods and fresh sperm only (i.e., sperm not exposed to a freeze-thaw cycles), has revealed sperm recovery rates approaching 100% (unpublished observations). As such, we expect that the error with the methods applied for sperm enumeration in this study is slight, but minor.

Cochran et al. first introduced the concept of cushioned centrifugation of stallion semen, using a small volume (0.25 mL) of hypertonic solution (409 mOsm/kg) layered beneath semen diluted in an isotonic solution (290 mOsm/kg) [27]. Centrifugation of semen at 400 x g for 15 min yielded a 92% sperm recovery rate. A subsequent study suggested that this cushion type was unnecessary for protecting sperm against damage associated with close packing [91]. Revell and coworkers refined the technique by incorporating an iodixanol cushion to allow increased centrifugation force and time without attendant sperm damage [40]. These workers harvested the sperm by aspirating the band of sperm following centrifugation to resuspend in freezing extender. Since that time, others have described aspiration of the cushion fluid following centrifugation, allowing the sperm pellet to descend to the bottom of the centrifuge tube for further processing [36,41,42]. Direct aspiration of the sperm band, as reported by Revell and coworkers [40], resulted in sperm recovery rates averaging 81%; a value that is considerably lower than that reported by others [36,41,42] and the present study for sperm recovery rate following cushioned centrifugation.

While the iodixanol solution is considered to be inert and nontoxic [37], the OP cushion media is hypotonic (170 mOsm/L). As we were concerned about the potential adverse effects of sperm exposure to the hypotonic fluid, we compared sperm



centrifugation in OP with two other iodixanol solutions (EQB and CF) made available commercially in recent years, in which the product osmolality was adjusted by the manufacturers to an isotonic range (285-300 mOsm/kg). To determine the actual volume of iodixanol to which post-centrifugation sperm were exposed, a set volume (30  $\mu$ L) was added beneath extended semen in NIPPLE centrifugation-tubes and the cushion fluid was resuspended with the sperm pellet following removal of the majority of supernatant. The experiment revealed no detrimental effect of OP on sperm motility or chromatin quality, as compared to EQB or CF. We did not expect to observe a detrimental effect on the sperm because sperm processed in a similar manner with OP when using one stallion in a commercial breeding program in 1996 yielded normal fertility, i.e., a seasonal pregnancy rate of 94% (131/140) in a 5-month breeding season (D. Hartman and C. Rose, personal communication).

In the present study, we measured eight laboratory endpoints as indicators of sperm quality. Because samples were evaluated soon after centrifugation and again following 24 h of cooled storage, we also addressed the longevity of sperm viability (longer-term effects of processing on sperm quality). Our data support the notion that sperm can be subjected to cushioned centrifugation in an opaque centrifugation extender with excellent sperm recovery rates, so long as the supernatant is removed to a logical pre-determined volume. The experimental results indicate that sperm exposure to INRA 96 (i.e., HGLL with supplemented NPPC) also improved some features of sperm motility, namely MOT and PMOT, as compared to HGLL alone as a centrifugation extender. Based on longevity of sperm quality endpoints, sperm centrifugation in INRA

96 yielded superior results to uncentrifuged controls. While sperm recovery rates were better when using the described centrifugation method for CONICAL tubes, as compared to that for NIPPLE tubes, several measures of sperm quality in cool-stored semen were improved following centrifugation in NIPPLE tubes, namely MOT, PMOT, VCL, VAP, and COMPat. While some of the differences may not represent a difference of physiological importance, the endpoints studied do indicate an advantage of centrifugation in NIPPLE tubes, as compared to CONICAL tubes. Possibly, increased contamination of resuspended sperm in CONICAL tubes with iodixanol may have led to the changes detected. Additional studies are needed to address this consideration.

Of clinical importance, centrifugation of semen with either technique did not appear to result in any longstanding damage to sperm, when compared to uncentrifuged controls. In general, centrifugation of semen led to reduced velocity parameters immediately following centrifugation, but these values increased to that of uncentrifuged controls, or even exceeded that of uncentrifuged controls, following cooled storage. The experimental results also suggest that any of the three commercially available iodixanol solutions described above can be used for cushioned centrifugation with similar outcomes expected despite the hypotonicity of the OP cushion medium.

In summary, cushioned centrifugation of stallion semen appears to be a useful means for maximizing sperm harvest, while maintaining sperm function, and an opaque extender can be used to achieve this goal. No advantage was gained by increasing centrifugation force in NIPPLE tubes by 50%. The fertility rate following cushioned

semen centrifugation in a commercial program this past year indicates that our laboratory results are transferable to the clinical setting.

## **FUTURE AIMS**

While iodixanol solution appears to be satisfactory as a cushion for centrifuged semen, the effects of residual contamination of resuspended semen remain largely unstudied. To address this issue, future studies could be directed at evaluating the level of iodixanol solution that can be tolerated, but still maintain sperm quality. The relationship between laboratory measures of semen quality and fertility should also be addressed. In the present study, cushioned centrifugation of semen in the NIPPLE tubes yielded better semen quality than that in the CONICAL tubes. This difference could be attributable to increased contamination of sperm pellets in CONICAL tubes with iodixanol solution following resuspension of sperm pellets in extender, but could also be attributed to the higher centrifugation force applied.

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## APPENDIX

### Composition of HGLL Extender

Prepared as follows:

1. Dilute in distilled water the following:

FW	Ingredient	g/L
147	CaCl <sub>2</sub>	0.1852
74.56	KCL	0.3989
136.1	KH <sub>2</sub> PO <sub>4</sub>	0.0599
120.4	MgSO <sub>4</sub> * 7H <sub>2</sub> O	0.0975
55.44	NaCl	1.1853
142	Na <sub>2</sub> HPO <sub>4</sub> * 12H <sub>2</sub> O	0.0469
84.01	NaHCO <sub>3</sub>	0.3495
180.01	Glucose (anhydrous)	1.00
238.31	Hepes	4.7662
	Distilled water	250 mL
2. 180.01	Glucose (anhydrous)	12.0734
	Distilled water	250 mL
3. 360.3	Lactose (H <sub>2</sub> O)	45.3978
	Distilled water	500 mL
4.	Timentin	1.00
5.	Separately stir and heat 2.Glucose solution and 3.Lactose solution, then combine all three solutions and 4.Timentin, stir and heat	
6.	Final pH should be between 6.75 and 7.25	

**VITA**

Name: Jessica Arlene Waite

University Address: Department of Large Animal Clinical Sciences  
College of Veterinary Medicine and Biomedical Sciences  
Texas A&M University  
College Station, Texas 77843-4475

Email Address: [jwaite@tamu.edu](mailto:jwaite@tamu.edu)

Educational Background: B.S., Animal Science,  
Texas Tech University  
Lubbock, 2005