

PREGNANCY RATES IN MARES INSEMINATED WITH 0.5 OR 1 MILLION  
SPERM USING HYSTEROSCOPIC OR TRANSRECTALLY GUIDED DEEP-HORN  
INSEMINATION TECHNIQUES

A Thesis

by

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## ABSTRACT

Placement of sperm deep in the equine uterine horn allows fewer sperm to be inseminated while maintaining acceptable fertility, and has been promoted for use in circumstances when fertility would be expected to be low if standard insemination were used (e.g. semen from a subfertile stallion, or frozen-thawed semen). Two main deep-horn insemination techniques, transrectally guided (TRG) and hysteroscopic (HYS) insemination, have been developed for this purpose; however, there is some controversy regarding their comparative efficacy. This study was conducted to compare pregnancy rates when mares were inseminated by TRG or HYS, utilizing sperm numbers approaching and under the threshold for maximal fertility, resulting in reduced fertility. Pregnancy rates were not different between HYS and TRG techniques when  $1 \times 10^6$  or  $0.5 \times 10^6$  sperm were inseminated. Combined pregnancy rates for the two techniques were also not different. Pregnancy rates using a subthreshold number of sperm were not significantly affected by a deep-horn insemination technique.

Dilution of semen to less than  $20 \times 10^6$  sperm/mL has been reported to decrease semen quality in multiple species, a phenomenon known as the semen “dilution effect.” The sperm concentrations utilized in Experiment 1 were 5 and  $2.5 \times 10^6$ /mL (1 and  $0.5 \times 10^6$  sperm doses, respectively). This experiment was performed to evaluate whether the lower pregnancy rates obtained with  $0.5 \times 10^6$  sperm was due to lower quality plasma membrane integrity (PMI) and sperm motion characteristics (TMOT, PMOT, VCL, VAP, VSL, STR). Treatments evaluated included  $2.5 \times 10^6$  sperm/mL with the addition

of 0, 7.5, and 25% seminal plasma,  $30 \times 10^6$  sperm/mL, and 3:1 extender:semen. The  $2.5 \times 10^6$  sperm/mL treatments have lower initial PMI, TMOT, and PMOT, but they maintain their initial quality following 24 and 48 h of cool-storage. The sperm velocity and straightness parameters suggest that sperm swim faster but have a more circular pattern as seminal plasma increases to 25% at a given concentration. Based on the findings from this experiment, the semen “dilution effect” may not significantly alter stallion sperm characteristics when a commercially-available semen extender is used for semen dilution.

## **DEDICATION**

I dedicate this work to my husband.

Phil, thanks for your support and patience during my residency and graduate studies at Texas A&M University. You are my inspiration and my best friend. I love you.

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To all my colleagues that provided technical support during the project, Dr. Jessica Cohen, Dr. Candace Jacobson, Dr. Shalyn Bliss, Dr. Anna Hichborn, Dr. Justin

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## NOMENCLATURE

2.5/0	2.5 x 10 <sup>6</sup> Sperm/mL with 0% Seminal Plasma Added
2.5/7.5	2.5 x 10 <sup>6</sup> Sperm/mL with 7.5% Seminal Plasma Added
2.5/25	2.5 x 10 <sup>6</sup> Sperm/mL with 25% Seminal Plasma Added
30	30 x 10 <sup>6</sup> Sperm/mL
3:1/30	Semen Diluted 3 Parts Semen to 1 Part Extender Than Diluted to 30 x 10 <sup>6</sup> Sperm/mL Prior to Analysis
3:1SD	Semen Diluted 3 Parts Semen to 1 Part Extender
AI	Artificial Insemination
DHI	Deep-Horn Insemination
Experiment 1	Chapter II Experiment
Experiment 2	Chapter III Experiment
HYS	Hysteroscopic Deep-Horn Insemination
INRA-T	INRA 96 (IMV, Maple Grove, MN, USA) containing 1 mg/mL (w:v) ticarcillin disodium and clavulanate potassium (Timentin®; GlaxoSmithKline, Research Triangle Park, NC, USA)
PMI	Plasma Membrane Intact
PMS	Progressively Motile Sperm
SP	Seminal Plasma
T0	At the Time of Semen Collection
T24	Following 24 Hours of Cooled Storage at 7-8°C

T48	Following 48 Hours of Cooled Storage at 7-8°C
T-End	Following Insemination of All Mares for the Day
TRG	Transrectally guided Deep-Horn Insemination
UBI	Uterine Body Insemination
UGIUI	Ultrasound-Guided Intrauterine Injection
V:V	Volume to Volume Ratio
WBFSH	World Breeding Federation of Sport Horses
W:V	Weight to Volume



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## CHAPTER I

### INTRODUCTION AND LITERATURE REVIEW\*

#### I.1. Deep-Horn Insemination

Artificial insemination (AI) has become a common technique in the equine industry with most breed registries permitting its use. Traditional AI in the mare typically involves depositing a minimum of  $250$  to  $500 \times 10^6$  progressively motile sperm (PMS) in the uterine body of the mare [1]. To accommodate decline in motility following cooling to  $4-6^{\circ}\text{C}$  for 24 h, an insemination dose of  $1 \times 10^9$  PMS is commonly used as a cool-stored insemination dose [1]. A lower cool-stored insemination dose, such as the recommendation of  $\geq 600 \times 10^6$  PMS by the World Breeding Federation of Sport Horses (WBFSH), has also been recommended [2]. Frozen semen doses are not as standardized as fresh and cool-stored doses; however, the WBFSH recommends that a frozen semen dose have a minimum post-thaw progressive motility of 35% and contain a minimum of  $250 \times 10^6$  PMS [2].

In the last 15 years, a form of AI called deep-horn insemination (DHI) has been reintroduced to the equine industry. Deep-horn insemination involves deposition of a low volume of extended semen at or near the oviductal papilla ipsilateral to the preovulatory follicle. In a study comparing the percentage of sperm in the oviducts of

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mares 18 h following uterine body (UBI) or DHI with  $500 \times 10^6$  total sperm extended to a 10-mL volume, Rigby et al. [3] reported no difference in the percentage of sperm in the two oviducts (UBI = 0.0007%, DHI = 0.0006%). When comparing the percentage of recovered sperm found within the oviduct ipsilateral or contralateral to the preovulatory follicle, a higher percentage was found in the oviduct ipsilateral to the preovulatory follicle in mares inseminated with DHI (77%) versus UBI (54%). Based on these results, sperm deposition closer to the site of fertilization, rather than in the uterine body, may enhance fertility, in circumstances when sperm numbers are limited or compromised. Deep-horn insemination has been successfully used in conjunction with frozen-thawed sperm from fertile stallions [4-8], with fresh [9-14] or cool-stored [10,11,14] sperm from subfertile stallions, with sex-sorted sperm [15-18], and with epididymal sperm [19-21].

Three types of DHI have been described in the mare: hysteroscopic (HYS), transrectally guided (TRG), and ultrasound-guided intrauterine injection (UGIUI). Deep-horn insemination was first reported by two teams of investigators in 1998. Vazquez et al. [22] achieved a 30% (3/10) pregnancy rate when they placed 20  $\mu$ L of semen containing 7 million total sperm from a known fertile stallion onto the oviductal papilla using a flexible endoscope with an insemination catheter passed through its biopsy channel (HYS). Manning et al. [23] also used a videoendoscopic-assisted insemination procedure, attempted to cannulate the oviductal papilla at the time of insemination. Zero of 11 (0%) and 2 of 9 mares (22%) became pregnant when inseminated with 10 million (250  $\mu$ L), or 1 million (<160  $\mu$ L), morphologically normal, progressively motile sperm, respectively. In comparison, 4 of 12 (33%) and 2 of 12 mares (17%) of mares were

pregnant following insemination into the uterine body with  $100 \times 10^6$  or  $10 \times 10^6$  morphologically normal, progressively motile sperm, respectively.

Subsequent to the first two HYS studies, Buchanan et al. [15] introduced the transrectally guided deep-horn insemination (TRG) technique. These workers passed either a flexible plastic AI pipette (1-mL dose volumes) or a disposable implant gun (0.2-mL dose volumes) to the tip of the uterine horn ipsilateral to the preovulatory follicle utilizing transrectal manipulation, confirmed the pipette or gun location with transrectal ultrasonography, and then deposited semen at the tip of the uterine horn. They reported no difference in pregnancy rates when they inseminated  $25 \times 10^6$  progressively motile sperm (PMS) in a 1-mL volume (57%, 12/21),  $5 \times 10^6$  PMS in a 1-mL volume (30%, 3/10), and  $5 \times 10^6$  in a 0.2-mL volume (4/10, 40%) utilizing the TRG technique. A significant difference, however, did exist between the TRG pregnancy rates and the pregnancy rate obtained when  $500 \times 10^6$  PMS was inseminated utilizing a conventional UBI (18/20, 90%).

The third and less commonly used technique in a commercial setting is the ultrasound-guided intrauterine injection (UGIUI). It is a technique that was first developed to inject a treatment solution into the uterine lumen of non-pregnant mare 12 days post-ovulation without traversing the mare's cervix [24]. It was then modified into a method for embryo transfer [25,26] and DHI [25]. Utilizing a transvaginal probe handle fitted with a 5 MHz convex-array transducer and needle guide, Silva et al. [25] inserted a 17-gauge needle, containing an epidural catheter (0.85 x 0.45 x 1000 mm) preloaded with the inseminate and attached to a 1-mL syringe, through the vaginal fornix

into the tip of the uterine horn (positioned against the vaginal fornix against the transducer by transrectal manipulation). The epidural catheter was then passed to the tip of the uterine horn, semen was deposited, and the tip of the uterine horn was manually held in a ventral position for 3 minutes to facilitate pooling of semen against the oviductal papilla. These workers achieved a 50% (5/10) pregnancy rate with an insemination dose containing  $20 \times 10^6$  progressively motile sperm.

Each of these DHI techniques has advantages and disadvantages. The hysteroscopic technique allows direct visualization of the oviductal papilla prior to semen deposition. However, the equipment is expensive and requires proper cleaning and sterilization to prevent iatrogenic infection (e.g. *Escherichia coli* and *Pseudomonas aeruginosa*), especially from contamination of the internal channels of the endoscope [27-31]. In addition, care must be taken to ensure that disinfectant solutions do not leave spermicidal residues within or on the endoscope. The sterilization and washing procedure is lengthy, and with the potential of spermicidal residues, the endoscope cannot be sterilized between mares that require insemination within a short period of time. If cleaning is done improperly post-insemination, residual semen may remain within the biopsy channel of the endoscope, creating the potential for inseminating with the wrong stallion's semen when the endoscope is used to breed with semen from more than one stallion. Another consideration is the possibility of inseminating the incorrect uterine horn if insemination is based only on visualization; this is the primary reason Lindsey et al. [17] advocated confirming endoscope location transrectally prior to deposition of semen.



The TRG technique avoids the need for expensive equipment but does not allow visualization to confirm that direct semen deposition onto the oviductal papilla has occurred. To compensate for this weakness, some proponents of TRG advocate holding the tip of the uterine horn in a ventral location during and for a short time after insemination to allow pooling of the inseminate on or near the oviductal papilla. Transrectally guided deep-horn insemination has the benefit of using a sterile disposable insemination pipette, eliminating the possibility of contamination of the uterus from the equipment and the need to clean or sterilize equipment between uses. It can be performed in a relatively short period of time but does require greater expertise than HYS to obtain optimal results.

The UGIUI technique is probably the most invasive of the three DHI techniques. It does not allow direct visualization of the oviductal papilla, but semen deposition can be visualized ultrasonographically. As with TRG, the tip of the uterine horn can be held in a ventral location during and after insemination, thereby theoretically allowing pooling of the inseminate on or near the oviductal papilla. It probably requires the greatest expertise of all three DHI techniques but once the expertise is possessed it is reported to only take approximately 48 seconds from insertion of the UGIUI equipment into the vagina to completion of the procedure [25]. Like the HYS technique, UGIUI requires relatively expensive equipment that requires proper cleaning and sterilization with the associated risks and labor costs (as described above with HYS). Even if rare, the risk of introducing bacteria into the peritoneal cavity seems possible since the UGIUI equipment is likely to become contaminated with bacteria from the caudal reproductive

tract [32] before the needle is introduced into the peritoneal cavity. Due to its procedure similarities to transvaginal aspiration of ovarian follicles, the risk of severe internal hemorrhage is also a rare but potential possibility with UGIUI [33].

Whether DHI results in the development or prevention of endometritis has been controversial. Lindsey et al. [17] suggested that the manipulation required to pass the pipette to the tip of the uterine horn with TRG could traumatize the endometrial wall, creating an inflammatory environment unsuitable for sperm survival and embryonic development. Schiemann et al. [34] reported that endoscopic examination of the diestrus uterus and the associated necessity of distending the uterus with air resulted in a 100% incidence (8/8) of histopathologic endometritis with isolation of pathogenic bacteria in six of these mares five days after the procedure, suggesting that the hysteroscopic procedure is inflammatory to the uterus and may be associated with induction of bacterial endometritis. When Sieme et al. [4] compared pregnancy rates following insemination of fresh semen in a clinical setting, he reported that HYS (5/15, 33%) resulted in lower pregnancy rates than UBI (16/19, 84%) in problem mares, but no effect of insemination technique was noted in normal mares (27/38, 71% and 18/38, 47%, respectively). These findings suggest that detrimental effects of the hysteroscopic procedure might be especially pronounced in mares with suboptimal uterine environments.

Controlled studies evaluating the relationship of DHI to endometritis have shown that DHI does not induce more inflammation within the uterus than does conventional UBI in reproductively normal mares [35,36]. Güvenc et al. [35] evaluated the amount of

inflammation present in the uterine lumen of normal mares 24 h post-insemination with  $20 \times 10^6$  and  $200 \times 10^6$  sperm (0.5 mL) following UBI or TRG (i.e. 4 treatment groups). Specifically, they evaluated the amount of intrauterine fluid present ultrasonographically (compared to amount present at time of insemination), then obtained uterine fluid samples by first absorbing fluid into a tampon and then performing uterine lavage. The uterine fluid was analyzed for aerobic microbial growth, number of polymorphonuclear leukocytes per mL (PMN/mL), and lysozyme and trypsin-inhibitor capacity (TIC) levels. These investigators concluded that TRG and insemination dose (concentration) did not affect the level of inflammation 24 h post-insemination in normal mares. Ferrer et al. [36] evaluated the amount of inflammation present in normal mares and in mares with delayed uterine clearance 48 h post-insemination with either  $1 \times 10^9$  sperm (20 mL) with UBI,  $5 \times 10^6$  sperm (0.5 mL) with HYS, semen extender (0.5 mL) with HYS, or sperm-free seminal plasma (0.5 mL) with HYS. Severity of the inflammatory response was determined by ultrasonographically evaluating the amount of intrauterine fluid at 24 and 48 h post-insemination, and by evaluating the total number, concentration, and percentage of neutrophils in intrauterine fluid samples collected 48 h post-insemination with a tampon. The severity of post-breeding inflammation 48 h post-insemination did not differ between UBI and HYS in normal and delayed clearance mares. Delayed clearance mares, however, had more intrauterine fluid following HYS than following UBI at 24 h post-insemination. These investigators concluded that HYS was not contraindicated in mares with delayed uterine clearance, but that it should not be used as method to reduce post-breeding endometritis in these mares.

Results of HYS and TRG studies in 1998 and 2000 [15,23] did not support the use of DHI over a conventional uterine body insemination. Two studies presented at the 2002 International Symposium on Equine Reproduction [37,38], however, indicated that use of DHI could allow fewer sperm to be used than was needed for conventional UBI, with comparable pregnancy rates. In 2003, Morris et al. [39] substantiated these findings. Utilizing 86 mares inseminated with frozen-thawed semen from identical twin stallions, they found that pregnancy rates with  $14 \times 10^6$  PMS were not significantly different between mares inseminated utilizing conventional UBI (8/12, 67%) or HYS (9/14, 64%) techniques. When the insemination dose was decreased to  $3 \times 10^6$  PMS sperm, deposition of semen into the tip of the uterine horn ipsilateral to the preovulatory follicle resulted in higher pregnancy rates (16/34, 47%) than did deposition of semen immediately cranial to the internal cervical os (2/14, 14%), or at the tip of the uterine horn contralateral to the preovulatory follicle (1/12, 8%). An endoscopic technique was used for insemination at all 3 of these sites to eliminate confounding factors created by its use. These findings indicated that the benefits of HYS are not seen until the insemination dose contains less sperm than needed to achieve acceptable pregnancy rates with UBI.

Morris et al [40] further showed that a threshold level of sperm numbers exists that even HYS cannot overcome to sustain optimal pregnancy rates. In this study, optimal pregnancy rates were achieved when mares were inseminated with  $10 \times 10^6$  (60%),  $5 \times 10^6$  (75%), or  $1 \times 10^6$  (64%) fresh motile sperm following Percoll® (Sigma Chemical Co., St. Louis, MO, USA) gradient separation (to improve sperm quality

within the inseminate) from two stallions. Pregnancy rates were less when the insemination dose was decreased to  $0.5 \times 10^6$  (29%),  $0.1 \times 10^6$  (22%), and  $0.001 \times 10^6$  (10%).

Studies examining the motility and morphology of the sperm found in the oviducts of mares post-insemination have shown the presence of a higher percentage of sperm with normal morphology [41] and motility [42,43] than was present within the inseminate. Scott et al. found that morphologically normal sperm accumulate in the folds of the oviductal papilla, suggesting that the uterotubal junction (UTJ) may act as a sperm reservoir and “gatekeeper” to the oviduct [44]. Mares bred to subfertile stallions had fewer sperm in their oviducts 4 h after breeding than did mares bred to fertile stallions [41]. These studies suggest that increasing the reservoir of morphologically normal, motile sperm on the UTJ and within the oviduct could potentially enhance fertility especially in circumstances where subfertile semen is involved. The above described studies, however, did not evaluate other measures of sperm quality in the population of sperm found within the oviducts.

Woods et al. [45] were not able to increase the pregnancy rates of a stallion with teratospermia (18-26% morphologically normal sperm) by changing the site of insemination of  $25 \times 10^6$  total sperm from the uterine body (4/14, 29%) to the tip of the uterine horn (4/14, 29%). Mari et al. [12] improved per cycle pregnancy rates for a commercial breeding stallion with teratospermia and oligospermia via implementation of TRG with  $50 \times 10^6$  morphologically normal, progressively motile sperm (1 mL). Semen was processed with cushion (iodixanol solution) centrifugation alone or in combination

with discontinuous density gradient centrifugation (90:45% silicon coated silica colloids, RediGrad™; Amersham Biosciences, NJ, USA). To obtain an insemination dose of  $50 \times 10^6$  morphologically normal, progressively motile sperm,  $\sim 500 \times 10^6$  post-centrifugation sperm or  $\sim 130 \times 10^6$  RediGrad™ purified sperm were needed. The semen processed through the RediGrad™ (44/71, 62%) resulted in higher pregnancy rates than did the semen processed with cushion centrifugation only (30/71, 42%). Both pregnancy rates were, however, better than that for the previous season (27/105, 26%). The study suggests that the presence of abnormal sperm interferes with normal fertilization or with development of a viable pregnancy. It also suggests that selecting for sperm quality prior to insemination could enhance fertility of some stallions. Similar improvements in fertility have been reported with semen processed with cushion (iodixanol solution) centrifugation only and with cushion centrifugation followed by discontinuous (80:40% density gradient) or single-layer (80% density gradient) density gradient centrifugation with the colloidal silica-particle solution EquiPure™ (80% density gradient, EquiPure Bottom Layer™; 40% density gradient, EquiPure Top Layer™; Nidacron International AB, Mölndal, Sweden) [10,11,14].

Studies comparing the relative effectiveness of HYS and TRG (but not UGIUI) have been performed. Lindsey et al. [17] reported higher pregnancy rates with HYS (5/10, 50%) than with a modified TRG technique (0/10, 0%) following insemination of  $5 \times 10^6$  motile sperm following Percoll® gradient separation. Brinsko et al. [46] reported no difference between HYS and TRG (12/18, 66% and 10/18, 56%, respectively) following insemination of  $5 \times 10^6$  total sperm ( $3.1 - 3.9 \times 10^6$  progressively motile

sperm). In a follow-up study utilizing the TRG technique described by Brinsko et al. [46], Lindsey et al. [18] reported no difference in pregnancy rates (12/22, 56% and 9/24, 38%, for HYS and TRG, respectively) following insemination with  $20 \times 10^6$  sex-sorted sperm. Nevertheless, based on the earlier paper by Lindsey et al., it was suggested that HYS may be the preferred method of DHI when less than 25 to  $50 \times 10^6$  sperm are used, especially if the semen is frozen-thawed or sex-sorted [47].

The findings of the above-referenced studies indicated that differences in pregnancy rates related to insemination technique may not be detected if “excess” sperm are available to compensate for limitations in the technique [48,49]. Thus, to best demonstrate differences between techniques, assessment should be made using a subthreshold number (i.e. subfertile level) of sperm. The importance of utilizing subthreshold insemination doses was demonstrated by Morris et al. [39] in their comparison of uterine insemination sites (described above). Previous studies comparing HYS to TRG have not been conducted using subthreshold insemination doses.

The objective of the studies reported here was to determine if HYS or TRG techniques provided equivalent pregnancy rates when a subfertile dose of sperm was inseminated. A preliminary study was performed to identify an insemination dose below the threshold level for normal pregnancy rates for one particular stallion. The objective of the main study was to compare the pregnancy rates achieved after HYS or TRG techniques utilizing a subthreshold insemination dose from this stallion.

## **I.2. Effects of High Dilution on Sperm Quality**

To perform the low-dose inseminations performed in the deep-horn insemination study, the semen was diluted to 2.5 to 5 million sperm/mL. Excessive dilution of semen (below 20 million sperm/mL) has been demonstrated to have detrimental effects on sperm quality especially as duration of exposure to the diluent increases. This phenomenon known as the “dilution effect” was first described by Milovanov in 1934 [50]. He evaluated the ability of sperm within an ejaculate to resist immobilization following dilution with increasing proportions (volume of diluent to volume semen) of 1% sodium chloride.

Multiple hypotheses have been made regarding etiology of the “dilution effect.” Milovanov [50] attributed the loss of sperm motility to sodium chloride toxicity. Subsequent researchers proposed that it is due to the loss of adequate proportions of protective seminal plasma components [51-54], and/or intracellular and/or cell-surface components from the sperm [51-53,55-58] triggering a capacitation-like state and ultimately premature acrosome reaction [59]. Researchers studying the hydrodynamics of sperm motility have postulated an alternative hypothesis: they determined that the flagellar undulations of sperm induce motions in the fluid medium that move enzymes, ions, and other dissolved chemicals and gases to and from the plasma membranes of other sperm in close proximity, especially when sperm are within one body length from each other. As sperm concentration decreases, the distance between sperm is increased, which



results in impaired exchange of the components within the liquid medium that are important to maintain sperm viability [60]

The “dilution effect” has been described in multiple species including the rabbit [51,52,55,61], ram [52,54,57,61], bull [52,56-58,62-66], boar [59], dog [53], stallion [67,68], and human [52,69]. In all these species, this phenomenon occurs when the neat semen is diluted to less than 20 million sperm/mL [51-59,61,65,66,69,70]; even though neat semen concentration [71], diluent type and seminal plasma concentration varies within species and individuals. Ejaculates with low neat semen concentrations, however, tend to have more severe effects than do ejaculates with high neat semen concentrations at equivalent dilution rates (v:v), suggesting that a lower final sperm concentration is more detrimental to sperm quality than is simply dilution of seminal plasma [62,65,66,69,70]. Regardless of initial sperm concentration, the severity of the “dilution effect” appears to increase as the diluent to neat semen ratio (v:v) increases [51-59,61-66,69].

The type of diluent used does not appear to alter the concentration at which the “dilution effect” is seen; however, it does influence the severity of the effect [52-59,61,63,64,66,69,72]. Seminal plasma is not a suitable diluent for maintenance of sperm quality [58,69,70,73]. Supplementation of seminal plasma to other diluents, however, has reduced the detrimental effects of high dilution on sperm motility (rabbit [55], bull [57,58], ram [57], and dog [53]), plasma membrane integrity (ram [54], bull [66], and boar [59]), and mitochondrial

membrane potential (bull [66] and boars [59]). Isotonic semen diluents with suitable levels of buffers, electrolytes, energy sources and antibiotics have been found to improve the overall sperm quality of extended semen samples and to diminish the severity of the “dilution effect” [54]. High molecular weight compounds such as egg yolk [56], milk [70], and serum proteins [61,74] exhibit protective effects on highly diluted semen and are common components of the above semen diluents.

When comparing motility of rabbit sperm following dilution with Baker’s solution to  $20$ ,  $2.8$ ,  $0.4$  and  $0.06 \times 10^6$  sperm/mL, Emmens and Swyer [55] reported that sperm motility decreased as the level of dilution increased. Adding sperm-free seminal plasma to the Baker’s solution at a concentration (v:v) equivalent to the  $20 \times 10^6$  sperm/mL suspension improved sperm motility at the  $0.4 \times 10^6$  sperm/mL concentration. Further improvements in sperm motility were seen when the source of the Baker’s solution and seminal plasma diluent was the supernatant of a semen suspension allowed to incubate overnight at a concentration of  $20 \times 10^6$  sperm/mL, but sperm motility did not necessarily return to the level of the  $20 \times 10^6$  sperm/mL suspensions. These findings suggest a partial protective effect of seminal plasma in Baker’s solution especially when the seminal plasma may contain factors released either from the sperm plasma membrane or from within the sperm.

To evaluate the effect of diluent to seminal plasma ratio in the highly diluted samples, Emmens and Swyer [55] subjected  $20 \times 10^6$  sperm/mL

suspensions (diluted in Baker's solution) to two centrifugation treatments followed by resuspension to  $20 \times 10^6$  sperm/mL with either fresh Baker's solution or its own supernatant. The semen resuspended in the Baker's solution was estimated to have equivalent diluent to seminal plasma ratios as the  $0.6 \times 10^6$  sperm/mL simple dilution samples (described in the previous paragraph). This semen resulted in higher sperm motilities than did the semen resuspended in its own supernatant. However, if all of the seminal plasma was removed utilizing six centrifugation treatments followed by resuspension with fresh Baker's solution to  $20 \times 10^6$  sperm/mL, sperm motility was almost completely eliminated.

Resuspension of the sperm following the final centrifugation with Baker's solution containing 10% seminal plasma revitalized the motility to the level of the control semen sample (centrifuged semen resuspended into its own supernatant). The better motility of  $20 \times 10^6$  sperm/mL than  $0.4 \times 10^6$  sperm/mL preparations at the same seminal plasma concentration suggests that the lower sperm motility in the  $0.4 \times 10^6$  sperm/mL suspension is likely due to a lower sperm concentration.

Secondly, sperm motility was improved in Baker's solution when seminal plasma was present, but the amount of seminal plasma needed was very small (present after 2 centrifugation but not after 6 centrifugations). Finally, the findings of this study demonstrate that immotile does not mean "dead" and that Baker's solution containing 10% seminal plasma can rejuvenate motility of sperm immobilized by the lack of seminal plasma.

Garner et al. [65] evaluated plasma membrane integrity (SYBR-14 and propidium iodide), mitochondrial membrane potential (JC-1), and motility of bull sperm following 24 h of cool-storage (5°C) or after cryopreservation at 2, 10, 20, 40, and 60 x 10<sup>6</sup>/mL. They found that all three sperm parameters decreased as the level of dilution increased. The plasma membrane integrity was more severely affected than the mitochondrial membrane potential and the motility. Supplementing the lower four semen concentrations with seminal plasma at the concentration (v:v) found in the 60 x 10<sup>6</sup> sperm/mL reduced the severity of damage to plasma membrane integrity, mitochondrial membrane potential and motility but did not fully eliminate the “dilution effect.”

No studies evaluating the “dilution effect” in stallions at less than 20 x 10<sup>6</sup> sperm/mL on *in vitro* semen parameters were found on literature search. Varner et al. [70] evaluated the effects of simple dilution of stallion semen with a skim milk-glucose extender (Kenney extender) to concentrations greater than 20 x 10<sup>6</sup> sperm/mL (25, 50, and 100 x 10<sup>6</sup> sperm/mL; control = undiluted semen) on sperm motility following room-temperature (25°C) storage for 0.5, 3, 6, 12, and 24 h. They further evaluated the effects of neat semen concentration and seminal plasma content by comparing the motility of the sperm-rich fraction to that representative of the total ejaculate (sperm-rich fraction diluted 1:1 with the sperm-poor). Over all of the dilutions, mean total sperm motility at 12 and 24 h of storage and mean progressively motility at 24 h was greater in the sperm-rich fractions than in the total ejaculate. In addition, mean total sperm motility was

higher at  $25 \times 10^6/\text{mL}$  than at  $50 \times 10^6/\text{mL}$  starting at 12 h of storage, higher than at  $100 \times 10^6/\text{mL}$  starting at 3 h of storage, and higher than that for undiluted samples starting at 0.5 h of storage. The  $25 \times 10^6/\text{mL}$  treatment also had higher mean progressive motility than did the other three semen concentrations following 3 to 24 h of storage. The changes in motility in this study were not due to the “dilution effect” as the  $25 \times 10^6/\text{mL}$  samples performed better than the less dilute samples. They were due either to higher sperm concentration causing reduction of motility, possibly due to accumulation of metabolic byproducts, or to the higher percentage of seminal plasma (v:v) in the more concentrated samples, with higher levels of seminal plasma resulting in lower sperm motility parameters especially as storage time increased.

The percentage of seminal plasma in a semen suspension that optimizes semen quality following storage is dependent on the composition of the diluent. In Kenney (skim-milk/glucose) -based extenders, sperm motility is optimized in 5 – 20% seminal plasma (v:v) [75]. However, when Kenney-based extender is supplemented (65:35) with high-potassium Tyrode’s medium (KMT), sperm motility is optimized when all seminal plasma is removed from the semen suspension [76-78]. DNA integrity following 24 and 48 h of cool-storage ( $5^\circ\text{C}$ ), however, is optimized in both the Kenney-based and KMT extenders when no seminal plasma is present [78].

Insemination of stallion semen at less than  $20 \times 10^6$  sperm/mL, the sperm concentration where the “dilution effect” is seen *in vitro* in other species, has had

mixed effects on fertility. In one study, conventional UBI of  $250 \times 10^6$  PMS resulted in both lower and similar pregnancy rates when the sperm concentration was  $5 \times 10^6$  PMS/mL (50 mL) compared to a  $25 \times 10^6$  PMS/mL (10 mL); however, different stallions were used in this study which may account for differences in pregnancy rates [67]. In another study, pregnancy rates were lower with UBI of  $250 \times 10^6$  PMS at  $2.5 \times 10^6$  PMS/mL (100 mL) compared to  $25 \times 10^6$  PMS/mL (10 mL) [68]. It is not clear what may have caused this lower fertility since total sperm numbers in the inseminate were not reported.. The “dilution effect” may have reduced the sperm quality, but other possibilities suggested by the authors include retrograde sperm loss due to expulsion of the higher volume treatment resulting in an effectively lower sperm dose, or detrimental effects directly related to instilling large volumes of semen. This latter possibility was later disproven [79].

Because of the limited information available on the effect of dilution on equine sperm viability and motility, and its direct relationship to use in low-dose insemination programs, a study was conducted to evaluate the *in vitro* quality of stallion sperm subjected to the high levels of dilution. Sperm parameters evaluated included sperm motion characteristics and plasma membrane integrity.

**CHAPTER II**

**PREGNANCY RATES IN MARES INSEMINATED WITH**

**0.5 OR 1 MILLION SPERM USING HYS OR TRG\***

**II.1. Materials and Methods**

A preliminary study and a main study were conducted to compare the pregnancy rates associated with two different deep-horn insemination techniques, TRG and HYS, when a subthreshold insemination dose was used. The experiments were conducted during the summer and fall months of 2007 and 2008. All experimental procedures were performed according to the United States Government Principles for Utilization and Care of Vertebrate Animals Used in Testing, Research and Training and were approved by the Laboratory Animal Care Committee at Texas A&M University.

The preliminary study was conducted to identify the number of total sperm in an insemination dose that would result in suboptimal pregnancy rates for a specific stallion. The stallion, a 1989 American Quarter Horse, was a stallion of known high fertility. This stallion was selected to eliminate possible confounders being introduced by male-factor subfertility.

The subsequent study compared pregnancy rates at Days 14 to 16 after ovulation following TRG and HYS utilizing the subthreshold insemination dose identified in the

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preliminary study. Mares with complicating reproductive factors were excluded from this study to eliminate possible confounders being introduced by female-factor subfertility. In this study, each mare was inseminated with each deep-horn insemination technique in a cross-over design to control for the effect of the mare.

### ***II.1.1. Preliminary study***

#### ***II.1.1.1. Mare management***

Twenty-eight mares ranging in age from 3 – 18 yrs (mean 6.5; median 6) were inseminated utilizing either TRG or conventional uterine body insemination for a total of 44 estrous cycles. Mares were monitored from August through October of 2007 utilizing palpation and ultrasonographic evaluation (B-mode, real time ultrasonography, 5MHz linear transducer, Sonovet 600; Universal Solutions, Inc., Bedford Hills, NY, USA) of the reproductive tract per rectum to determine estrous cycle stage. Sexual receptivity to a stallion was also intermittently assessed to facilitate determination of estrous cycle stage. Evaluations were performed only on weekdays as the mares were inaccessible on the weekends.

If the mares had a preovulatory follicle  $\geq 32$  mm in diameter and prominent signs of uterine edema on ultrasonography, the mare was treated with human chorionic gonadotropin (hCG; Chorulon®; Intervet, Millsboro, DE, USA; 2500 IU, iv), or BioRelease Deslorelin (BET Pharm, Lexington, KY, USA; 1.5 mg, im). The same day or the following day, if a preovulatory follicle was still present, mares were inseminated with  $1.0 \times 10^6$  or  $0.5 \times 10^6$  total sperm utilizing TRG, or with a minimum of  $1 \times 10^9$  progressively motile sperm utilizing conventional uterine body insemination. With the



TRG method, sperm were inseminated in the horn ipsilateral to the dominant follicle. In mares with more than one follicle >30 mm, the sperm were placed in the horn ipsilateral to the follicle showing the most pronounced preovulatory characteristics (softness on palpation, thickening of the follicle lining and loss of circularity on ultrasonographic evaluation). Mares were inseminated only once per cycle. Ovulation was confirmed by daily transrectal ultrasonographic examination except when these examinations would have occurred over the weekend; then ovulation was confirmed the following Monday. Pregnancy determination was made utilizing transrectal palpation and ultrasonography 13 to 16 days from the first day that the ovulation was detected (n = 42) or following uterine flush for embryo recovery 7 days post-ovulation (n = 2). Mares determined to be pregnant 13 to 16 days post-ovulation and mares subjected to uterine flush 7 days post-ovulation were treated with cloprostenol sodium (Estrumate®; Schering-Plough Animal Health Corp., Union, NJ, USA; 250 µg, im), or dinoprost tromethamine (Lutalyse®; Upjohn Pharmacia, Kalamazoo, MI, USA; 5 mg, im) to terminate pregnancy unless continuation of pregnancy was desired for other purposes (e.g., fetal sexing laboratory). Non-pregnant mares 13 to 16 days post-ovulation were either administered the same luteolytic regimen as for the pregnant mares, to return the mare to estrus, or they were allowed to return to estrus naturally.

If the mares were not found in estrus after this time, they were periodically monitored as described above until they returned to estrus naturally, or they were treated with a luteolytic agent to terminate the luteal phase and return them to estrus. If it was known that the most recent ovulation occurred  $\geq 7$  days previously, the mare was

scheduled for recheck evaluation 1 to 4 days later based on the diameter of the largest follicle (40 to <15 mm, respectively). If it was not known, when the mare last ovulated, the mare was re-evaluated 1 to 3 days after treatment with a luteolytic agent to determine whether luteolysis had occurred. In some mares, during the estrus following luteolysis, ovulation was induced by treatment with hCG, then luteolytic treatment was administered 7 days after this ovulation, and mares were used for the study on the subsequent estrus.

In this preliminary study, the insemination technique used was dictated based on the needs of the TAMU Section of Theriogenology. If it was imperative that the mares became pregnant (i.e. for the use in laboratories and other research projects), conventional uterine body insemination utilizing  $\geq 1.0 \times 10^9$  progressively motile sperm was performed to optimize fertility rates. Otherwise, mares were bred with TRG. The dose used in the TRG breeding was not randomized; rather, it was determined based on equality of number of mares inseminated with each dose (e.g., if there were 2 more mares inseminated with one dose, the next 2 mares would be inseminated with the other dose).

#### ***II.1.1.2. Semen collection***

Semen was collected from stallions using a Missouri-model artificial vagina (Nasco, Ft. Atkinson, WI, USA) lightly lubricated with 3 – 5 mL of sterile, non-spermicidal lubricating jelly (Priority Care; First Priority, Inc., Elgin, IL, USA) and fitted with an in-line nylon gel filter (Animal Reproduction Systems, Chino, CA, USA). A mare exhibiting behavioral estrus was used for sexual stimulation. The mount source

was a breeding phantom. Immediately prior to collection, the stallion's erect penis was cleansed with warm water and thoroughly dried.

### ***II.1.1.3. Semen processing***

#### ***II.1.1.3.1. General semen processing***

Immediately following semen collection, the gel filter was removed from the collection bottle to prevent leakage of the gel into the gel-free semen. The gel-free semen was transported to an adjacent laboratory and placed in a 37°C incubator. The total number of sperm in the ejaculate was determined by measuring the volume of the neat semen using a 100-mL graduated cylinder and measuring the concentration using the NucleoCounter® SP-100™ (ChemoMetec A/S, Allerød, Denmark).

An aliquot of neat semen was diluted with pre-warmed (37°C) semen extender (INRA 96; IMV, Maple Grove, MN, USA) to a final concentration of  $30 \times 10^6$  sperm/mL and a volume of 1.0 mL in a 1.2-mL Cryogenic vial (Corning Life Sciences, Lowell, MA, USA) using the following formulas:

- Volume of raw semen required =  $(30 \times 10^6 \text{ sperm/mL}) \div (\text{raw semen concentration}) * (1.0 \text{ mL})$
- Volume of extender =  $(1.0 \text{ mL}) - (\text{volume of raw semen required})$

The Cryogenic vial was placed directly on a 37°C covered slide warmer for 10 minutes prior to evaluation of initial sperm motion characteristics using a computer-assisted sperm motion analyzer (CASMA; IVOS Version 12.2L; Hamilton Thorne Biosciences, Beverly, MA, USA).

### ***II.1.1.3.2. Semen processing for deep-horn insemination dose***

A 3:1 extended semen sample was made by adding 0.5 mL of neat semen to a 2.0-mL Cryogenic vial (Corning Life Sciences, Lowell, MA, USA) containing 1.5 mL of pre-warmed (37°C) INRA 96. The concentration of this 3:1 extended semen sample was measured using the NucleoCounter® SP-100™.

To obtain TRG insemination doses of  $1 \times 10^6$  and  $0.5 \times 10^6$  sperm in 200  $\mu$ L, final dilution of semen to  $5 \times 10^6$  or  $2.5 \times 10^6$  sperm/mL, respectively, was made. Depending on the number of mares to be bred that day, a 1- or 2-mL aliquot of semen at the above concentrations was made utilizing the 3:1 extended semen sample, pre-warmed (37°C) INRA 96, and frozen-thawed, sperm-free seminal plasma to ensure that each inseminate contained 7.5% seminal plasma [75,77,80]. The amount of each of these components was determined using the following formulas:

- volume of sample desired = X + Y + Z
  - X = volume of 3:1 extended semen
  - Y = volume of INRA 96
  - Z = volume of frozen-thawed sperm-free seminal plasma
- $X = (\text{concentration of sample desired}) \div (\text{concentration of 3:1 extended semen}) * (\text{volume of sample desired})$
- combined amount of INRA 96 and frozen-thawed, sperm-free seminal plasma =  $(\text{volume of sample desired}) - X$
- % of seminal plasma in final inseminate if no seminal plasma was added =  $[(25\%) * (X) \div (\text{volume of sample desired})] * 100$

- % of seminal plasma in INRA 96 to make a final inseminate containing 7.5% seminal plasma = 7.5% - (% of seminal plasma in final inseminate if no seminal plasma was added)
- Z = (combined amount of INRA 96 and frozen-thawed, sperm-free seminal plasma) \* (% of seminal plasma in INRA 96 to make a final inseminate containing 7.5% seminal plasma)
- Y = (combined amount of INRA 96 and frozen-thawed, sperm-free seminal plasma) - Z

For example, to make 1.0 mL of an inseminate that was  $5 \times 10^6$  sperm/mL from a 3:1 semen sample that was  $75 \times 10^6$  sperm/mL:

- $X = (5.0 \times 10^6 \text{ sperm/mL}) \div (75 \times 10^6 \text{ sperm/mL}) * (1.0 \text{ mL})$ 
  - 0.067 mL = volume of 3:1 extended semen
- combined amount of INRA 96 and frozen-thawed, sperm-free seminal plasma = (1.0 mL) – (0.067 mL)
  - 0.933 mL
- % of seminal plasma in final inseminate if no seminal plasma was added =  $[(25\%) * (0.067\text{mL}) \div (1.0 \text{ mL})] * 100$ 
  - 1.67%
- % of seminal plasma in INRA 96 to make a final inseminate containing 7.5% seminal plasma = (7.5%) – (1.67%)
  - 5.83%

- $Z = (0.933 \text{ mL}) * (5.83\%)$ 
  - $0.054 \text{ mL} = \text{volume of frozen-thawed, sperm-free seminal plasma}$
- $Y = (0.933 \text{ mL}) - (0.054 \text{ mL})$ 
  - $0.879 \text{ mL} = \text{volume of INRA 96}$

The concentrations of the insemination doses were evaluated using the NucleoCounter® SP-100™. Samples not within  $0.3 \times 10^6/\text{mL}$  of the desired final concentration were discarded. Calculations were checked twice. If samples were discarded new insemination doses were made, and evaluated using the procedures as previously described.

#### ***II.1.1.3.3. Semen processing for conventional uterine body insemination dose***

A minimum of  $1 \times 10^9$  progressively motile sperm was diluted with INRA 96 to make an insemination dose that contained  $\geq 1$  part INRA 96 to 1 part neat semen (v:v) and had a final volume of 10 – 60 mL. Fulfilling this criteria meant that the maximum volume of neat semen per insemination dose was  $\leq 30$  mL.

The following formula was used to determine the minimum volume of neat semen needed to make an insemination dose that contained  $\geq 1 \times 10^9$  progressively motile sperm:

- Volume of neat semen containing  $1 \times 10^9$  progressively motile sperm =  $(1 \times 10^9) \div [(\text{neat semen concentration}) * (\% \text{ of progressively motile sperm})]$

If the entire ejaculate was used for insemination, the volume of neat semen per insemination dose was determined using the following formula:

- Maximal volume of neat semen available per insemination = [(total volume of neat semen in ejaculate) \* (volume of neat semen in ejaculate) \* (% of progressively motile sperm)] ÷ (number of mares requiring insemination)

Based on the results of these two formulas and the above defined criteria, the appropriate volume of neat semen was diluted with the appropriate amount of INRA 96. The product was aspirated into either a 25 mL or 60 mL non-spermicidal, all-plastic, sterile syringe (NORM-JECT®; Henke Sass Tolf, Tuttlingen, Germany). The extended semen was inseminated utilizing a conventional uterine body insemination within one hour of semen collection.

#### ***II.1.1.4. Inseminations***

##### ***II.1.1.4.1. General insemination protocol***

The mares were placed in stocks for restraint. No tranquilization was used. The mares' tails were bagged in non-sterile rectal sleeves and tied up, and their perinea were prepared by scrubbing with 7.5% povidone-iodine surgical scrub, rinsing with water, and drying with paper towels.

##### ***II.1.1.4.2. Transrectally guided deep-horn insemination (TRG)***

Transrectally guided deep-horn insemination was performed as described by Brinsko et al. [46], except that a commercially available 75 cm flexible, intrauterine insemination catheter with an 80 cm flexible inner catheter (IUI Pipette with Inner Catheter; Minitube of America, Inc.<sup>TM</sup>, Verona, WI, USA) (Figure 1) attached to a 5 mL non-spermicidal, all-plastic, sterile syringe (NORM-JECT®; Henke Sass Wolf, Tuttlingen, Germany) was used. The syringe was preloaded with 3 mL of air. A sterile

sleeve was donned. Two hundred microliters of semen, as described in section II.1.1.3.2, was aspirated into an air-displacement pipette (PIPETMAN Classic™ P200; Gilson®, Inc., Middleton, WI, USA). The distal tip of the air-displacement pipette was inserted into the lumen of the tip of the 80 cm flexible inner catheter. The pipette plunger was slowly depressed as gentle negative pressure was gently applied by the syringe plunger. Care was taken to keep the semen at the distal end of the inner catheter (i.e. the end furthest from the syringe). The inner catheter was re-inserted into the outer catheter. The entire double catheter was bent 5 cm from the distal end to an approximate 45° angle. The catheter was carried transvaginally (Figure 2) and passed through the cervix. The arm was withdrawn from the vagina, inserted into the rectum (Figure 3), and the catheter was guided transrectally to the tip of the uterine horn ipsilateral to the dominant follicle. When the end of the catheter was confirmed by palpation to be at the end of the uterine horn, the plunger of the 5-mL syringe was depressed slowly to expel the semen into the uterine lumen (Figure 4). The syringe was detached, loaded with 3 mL of air, reattached, and then the air was expressed slowly through the catheter to aid in full evacuation of the contents. The inner catheter with the syringe attached was removed through the outer catheter, and evaluated for presence of semen (Figure 5). If semen was visible in the inner catheter, it was inserted again, and the process was repeated. After withdrawal of the inner catheter, the contents of the outer catheter were also evacuated (in case any semen pooled in the end of the outer catheter) by injecting 5 mL of air through the outer catheter (Figure 6). The tip of the uterine horn was directed ventrally via manipulation



per rectum throughout these procedures, and was held in this position, if possible, for up to 3 min after removal of the insemination device (Figure 7).

Figure 1. The 75 cm flexible, intrauterine insemination catheter with an 80 cm flexible inner catheter (IUI Pipette with Inner Catheter; Minitube of America, Inc.<sup>TM</sup>, Verona, WI, USA).



Figure 2. The insemination catheter was passed transvaginally through the cervix into the uterus. Note the attached 5-mL syringe containing 3 mL of air to the catheter.

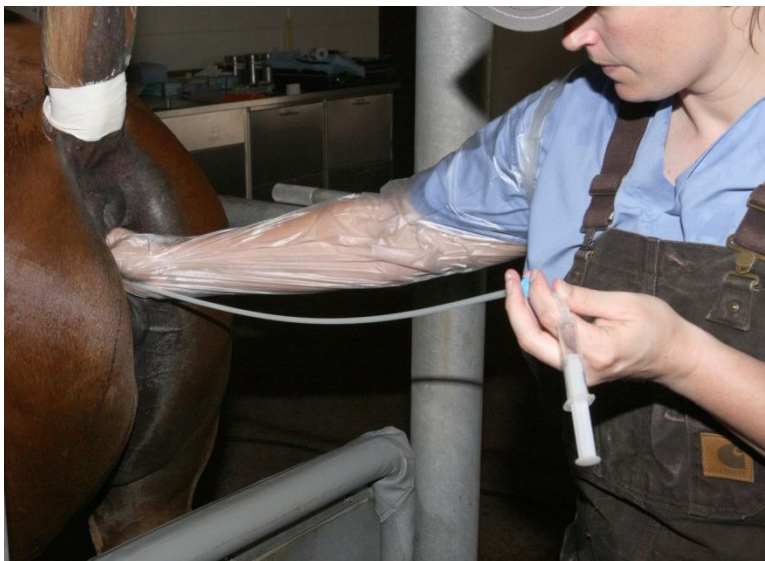


Figure 3. Insertion of the operator's arm in the rectum (after the catheter has been passed transvaginally in the uterus).



Figure 4. Slow depression of the 5-mL syringe plunger following confirmation of placement of the tip of the catheter at the end of the uterine horn ipsilateral to the preovulatory follicle.



Figure 5. Removal of the flexible inner catheter with examination of it for presence of semen. The inner catheter was reinserted into the outer catheter if semen was grossly visualized.



Figure 6. Passage of 5 mL of air through the outer catheter (following removal of the inner catheter) to remove any semen that may have pooled within the tip of it.



Figure 7. Removal of the outer catheter as the operator held the tip of uterine horn ipsilateral to the preovulatory follicle in a ventral position per rectum. Ventral orientation of the tip of the uterine horn was done throughout the insemination and if possible, for up to 3 minutes after removal of the outer catheter.



#### ***II.1.1.4.3. Conventional uterine body insemination (UBI)***

The 60-mL syringe containing the extended semen as described above was attached to a standard, sterile, equine insemination pipette. A sterile sleeve was donned. Five to six milliliters of air was aspirated into the semen syringe to aid in full evacuation of the semen from the syringe and insemination pipette. The insemination pipette was carried transvaginally and passed through the cervix. The distal end of the pipette was advanced into the cranial uterine body. To move the air bubble into a position that would allow it to “chase” all remaining semen from the syringe and pipette, the syringe tip was rotated to the 6 o’clock position and the plunger-end of the syringe was elevated to a 45°



angle. The cervix was held closed by the inseminator's hand and the plunger was slowly depressed until all semen was expelled into the uterus. The inseminator's arm was slowly withdrawn from the vagina to prevent aspiration of air into the vagina.

#### ***II.1.1.5. Embryo recovery***

Two mares underwent uterine lavage for embryo recovery 7 days after ovulation. The mares were restrained and the perineum prepared in the same manner as described above in section II.1.1.4.1, with the addition that mares were sedated with detomidine (Dormosedan®; Pfizer, Lees Summit, MO, USA; 3 to 4 mg, iv).

A sterile sleeve was donned. A sterile, equine lavage catheter (37 Fr, 75 mL, 36 in; Bioniche Life Sciences, Inc., Belleville, Ontario, Canada) was connected to an equine Y-tube set with spike (Bioniche Life Sciences Inc., Belleville, Ontario, Canada). The spike was inserted in a bag containing ViGRO™ Complete Flush Solution (Bioniche Life Sciences Inc., Belleville, Ontario, Canada) and the tubing was primed with flush solution. The equine lavage catheter was carried transvaginally and passed through the cervix. The catheter balloon was inflated with 30 – 40 mL of air. A sufficient volume of flush solution was instilled into the uterine lumen to entirely fill it (1 – 2 L). After approximately 200 – 500 mL of flush solution had been instilled, the operator's arm was withdrawn from the vagina and inserted into the rectum. After the uterine lumen was distended fully, the fluid was held in place for 2 minutes. The flush solution was then removed from the uterus by gravity flow, through a 150-mL embryo collection cup fitted with a 75-µm nylon mesh filter (Millipore Non-Vented Embryo Collection Filter; Bioniche Life Sciences, Inc., Belleville, Ontario, Canada). The uterine flush was

repeated two more times but without delay in removing the solution from the uterus. The mare was treated with 20 IU oxytocin iv at the completion of the third influx of solution to improve myometrial contraction and facilitate complete removal of the solution from the uterus. The uterus was intermittently, ballotted manually per rectum during the flushing process in an attempt to suspend the uterine contents within the fluid.

After the final flush, the catheter balloon was deflated and the lavage catheter was removed from the mare. With the flush catheter held in a vertical position with the tip uppermost, it was allowed to fill with flush solution to the level of the catheter balloon. The flush solution was then released through the efflux portion of the tubing into the embryo collection cup. This process was performed 2 – 3 times in an attempt to ensure that any embryo present no longer remained within the catheter and tubing.

One centimeter depth of flush solution was maintained above the filter in the embryo collection cup. The cup was then transported into an adjacent laboratory. The cup was inverted and its contents were thoroughly rinsed into an embryo search dish (100 x 15 mm round searching grid dish; Bioniche Life Sciences, Inc., Belleville, Ontario, Canada) using a 20-mL all-plastic syringe fitted with a 20-gauge 1.5-in needle. The entire dish was systematically searched for the presence of an embryo  $\geq 2$  times using a stereo microscope (13.4-80x).

## ***II.1.2. Main study***

### ***II.1.2.1. Mare management***

For this experiment, 37 mares ranging in age from 4 to 19 y (mean 9.2; median 8) were used. The time of ovulation was synchronized by daily intramuscular

administration of progesterone (150 mg) and estradiol-17 $\beta$  (10 mg) in oil (Hagyard Pharmacy, Lexington, KY, USA) for 10 d [81], with a single intramuscular injection of 250  $\mu$ g cloprostenol sodium administered on the 10th day of treatment. BioRelease Deslorelin (BET Pharm, Lexington, KY, USA, 1.5 mg) was administered im once a preovulatory follicle  $\geq$ 32 mm diameter and prominent uterine edema were detected. At the time of deslorelin administration, mares were assigned in random pairs to be inseminated the following day with  $1 \times 10^6$  sperm by either HYS or TRG. Sperm were inseminated in the horn ipsilateral to the dominant follicle. In mares with more than one follicle  $>$ 30 mm, the sperm were placed in the horn ipsilateral to the follicle showing the most pronounced preovulatory characteristics (softness on palpation, thickening of the follicle lining and loss of circularity on transrectal ultrasonographic examination). Mares were inseminated only once, and were examined by transrectal ultrasonography daily thereafter to confirm ovulation and at 14 to 16 d post-ovulation for pregnancy determination.

At 14 to 16 days after ovulation, mares were treated with 250  $\mu$ g, cloprostenol im to terminate pregnancy and/or return the mare to estrus. All mares were then allowed a minimum of one estrous cycle without treatment. Thirty-three mares were then treated again for estrus synchronization as described above. Mare management, semen collection and insemination were the same as for the previous replicate except that mares were assigned to the opposite treatment group (HYS or TRG), and were inseminated with  $0.5 \times 10^6$  sperm.

### ***II.1.2.2. Semen collection***

Semen was collected as described above in section II.1.1.2.

### ***II.1.2.3. Semen processing***

Immediately following semen collection, the gel filter was removed from the collection bottle to prevent leakage of the gel into the gel-free semen. The gel-free semen was transported to an adjacent laboratory and placed in a 37°C incubator. The total number of sperm in the ejaculate was determined by measuring the volume of the neat semen using a 100-mL graduated cylinder and measuring the concentration using the NucleoCounter® SP-100™.

An aliquot of neat semen was diluted with pre-warmed (37°C) INRA 96 containing 1 mg/mL (w:v) ticarcillin disodium and clavulanate potassium (Timentin®; GlaxoSmithKline, Research Triangle Park, NC, USA), hereafter referred to as INRA-T, to a final concentration of  $30 \times 10^6$  sperm/mL and a volume of 4.0 mL in a 4-mL Cryogenic vial (Corning Life Sciences, Lowell, MA, USA) using the following formulas:

- Volume of raw semen required =  $(30 \times 10^6 \text{ sperm/mL}) \div (\text{raw semen concentration}) * (4.0 \text{ mL})$
- Volume of extender =  $(4.0 \text{ mL}) - (\text{volume of raw semen required})$

One milliliter aliquots of the  $30 \times 10^6$  sperm/mL extended semen were pipetted into three 1.2-mL Cryogenic vials. One of these vials was placed directly on a 37°C covered slide warmer for 10 minutes prior to evaluation of initial sperm motion characteristics using a CASMA. The second and third vials were packaged in a commercial semen-transport



container (Equitainer®I; Hamilton Research, Inc., South Hamilton, MA, USA), as recommended by the manufacturer with sufficient ballast to control the cooling rate, for evaluation of sperm motion characteristics immediately following the deep-horn insemination of the final mare of the day and following 24 h of cooled storage (7-8°C).

A 3:1 extended semen sample was made by adding 1.0 mL of neat semen to a 4-mL Cryogenic vial containing 3.0 mL of pre-warmed (37°C) INRA-T. The concentrations of this 3:1 extended semen sample were measured using the NucleoCounter® SP-100™.

A 5.0-mL aliquot of either  $5 \times 10^6$  sperm/mL or  $2.5 \times 10^6$  sperm/mL was made by diluting the appropriate amount of 3:1 extended semen with INRA-T containing 5% (v:v) frozen-thawed, sperm-free seminal plasma into 5-mL Cryogenic vials (Corning Life Sciences, Lowell, MA, USA). Seminal plasma (SP) was added to the INRA-T to ensure that each inseminate contained a minimum of 5% seminal plasma to preserve semen quality [75,77,80]. The following formulas were used to produce this semen aliquot:

- volume of sample desired = X + Y
  - X = volume of 3:1 extended semen
  - Y = volume of INRA-T with 5% frozen-thawed, sperm-free seminal plasma (v:v)
- X = (concentration of sample desired) ÷ (concentration of 3:1 extended semen) \* (volume of sample desired)
- Y = (volume of sample desired) - X

For example, to make 5.0 mL of an inseminate that was  $2.5 \times 10^6$  sperm/mL from a 3:1 semen sample that was  $75 \times 10^6$  sperm/mL:

- $X = (2.5 \times 10^6 \text{ sperm/mL}) \div (75 \times 10^6 \text{ sperm/mL}) * (5.0 \text{ mL})$ 
  - 0.167 mL = volume of 3:1 extended semen
- $Y = (5.0 \text{ mL}) - (0.167 \text{ mL})$ 
  - 4.833 mL = volume of INRA-T with 5% frozen-thawed, sperm-free seminal plasma (v:v)

Final concentration was evaluated with the NucleoCounter® SP-100™. The sample was adjusted as needed until the concentration reported by the NucleoCounter® SP-100™ was within  $0.3 \times 10^6$  sperm/mL. The vial containing the remaining 3:1 extended semen was placed in the Equitainer®I as for the  $30 \times 10^6$  sperm/mL samples for evaluation following 24 h of cooled storage.

One milliliter aliquots of the  $5.0 \times 10^6$  sperm/mL or  $2.5 \times 10^6$  sperm/mL extended semen were pipetted into three 1.2-mL Cryogenic vials. The remaining 2 mL of extended semen was transferred from the original 5-mL Cryogenic vial to a 2-mL Cryogenic vial. One of the 1.2-mL Cryogenic vials was placed immediately on a 37°C covered slide warmer for 10 minutes prior to evaluation of initial sperm motion characteristics using a CASMA. The 2-mL Cryogenic vial and the remaining two 1.2-mL Cryogenic vials were packaged in the same Equitainer®I as the  $30 \times 10^6$  sperm/mL samples as described above. The 2-mL Cryogenic vial was stored for deep-horn insemination. The 1-mL extended semen samples were stored for evaluation of sperm

motion characteristics immediately following the deep-horn insemination of the final mare of the day and following 24 h of cooled storage.

Immediately prior to breeding each mare, a total of 210  $\mu\text{L}$  of semen extended to either  $5 \times 10^6$  sperm/mL or  $2.5 \times 10^6$  sperm/mL was placed in a 1.5-mL microcentrifuge tube for later loading into the appropriate insemination catheter. Losses within the 1.5-mL microcentrifuge tube were evaluated (data not included) and estimated to be approximately 10  $\mu\text{L}$ , thus delivering 200  $\mu\text{L}$  from the catheter during insemination.

#### ***II.1.2.4. Inseminations***

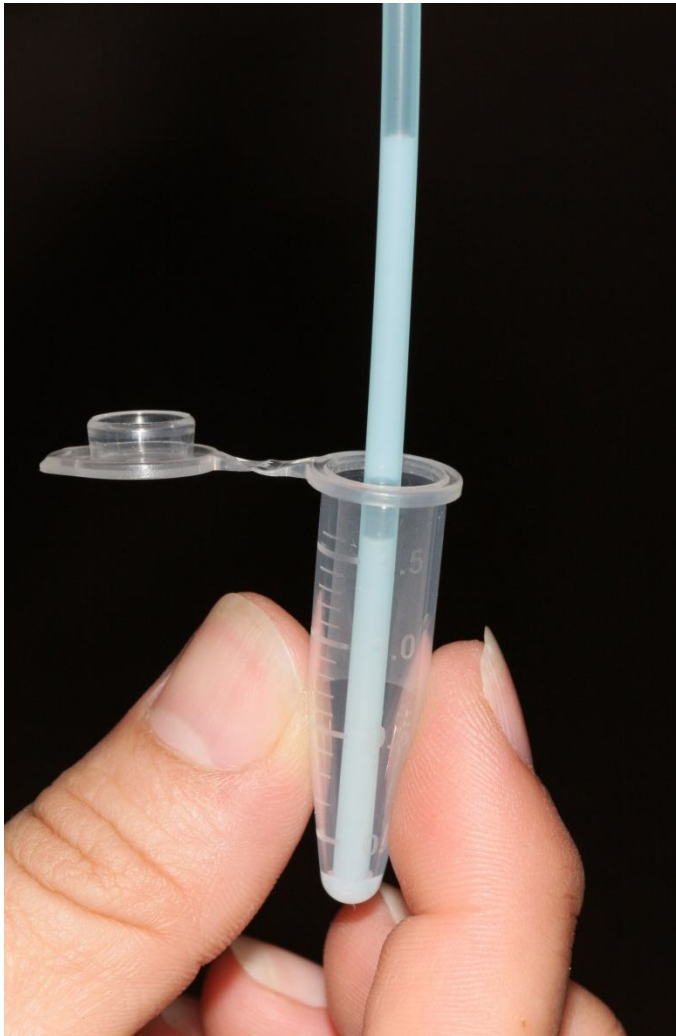
##### ***II.1.2.4.1. General insemination protocol***

The mares were restrained and prepared for insemination as described in section II.1.1.4.1, except they were sedated with detomidine (4 to 8 mg, iv).

##### ***II.1.2.4.2. Transrectally guided deep-horn insemination (TRG)***

Transrectally guided deep-horn insemination was performed as described in the preliminary study (section II.1.1.4.2) except that loading of the semen into the TRG catheter was modified so that the TRG and HYS catheters could be loaded in a similar manner. Specifically, the operator donned a sterile sleeve, inserted the distal end of the inner catheter into the 1.5-mL microcentrifuge tube, and drew the semen dose into the catheter by negative pressure with the syringe which was preloaded with 3 mL of air (Figure 8).

Figure 8. Aspiration of semen into the distal end of the inner catheter from a 1.5-mL microcentrifuge tube.



#### ***II.1.2.4.3. Hysteroscopic insemination (HYS)***

For HYS, semen was held in a 1.5-mL microcentrifuge tube, as described above with the TRG technique. The distal end of the inner cannula of an intra-fallopian insemination catheter (Cook Veterinary Products®, Spencer, IN, USA) was placed into the vial (Figure 9), and the semen was aspirated using a 3-mL syringe preloaded with 2

mL of air. The operator donned a sterile sleeve and carried a 1 m flexible video-endoscope (Olympus GIF Type Q180, Olympus America Inc., Irving, TX, USA) transvaginally and passed it through the cervix. The arm was withdrawn from the vagina, inserted into the rectum, and the endoscope was guided transrectally to the middle of the uterine horn ipsilateral to the dominant follicle (Figure 10), as described by Sieme et al. [4]. The proximal end of the uterine horn was compressed around the endoscope, the distal uterine horn was insufflated, and the endoscope was advanced until the oviductal papilla was visualized. The intra-fallopian insemination catheter was advanced through the biopsy channel of the endoscope, then the inner catheter was advanced until it was seen to be adjacent to the oviductal papilla, at which time the semen was expelled onto the papilla. The syringe was refilled with air and this air was injected through the catheter until residual semen could no longer be expelled (Figure 11). The entire catheter was drawn back into the endoscope channel, and the endoscope withdrawn to the base of the uterine horn. The air was aspirated from the uterine horn only if it was not seen to promote semen migration towards the uterine bifurcation. The endoscope and catheter were then withdrawn from the mare's reproductive tract.

Figure 9. Aspiration of semen into the distal end of the inner cannula of an intra-fallopian insemination catheter (Cook Veterinary Products®, Spencer, IN, USA) from a 1.5-mL microcentrifuge tube.

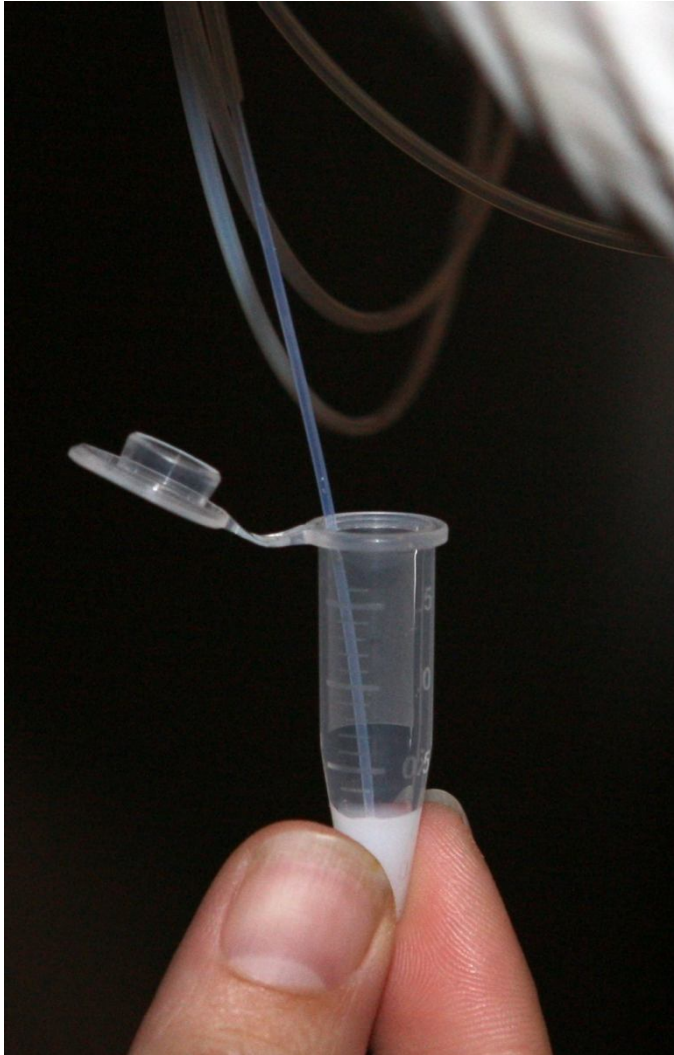


Figure 10. The arm was withdrawn from the vagina, inserted into the rectum, and the endoscope was guided transrectally to the middle of the uterine horn ipsilateral to the dominant follicle.



Figure 11. Semen was expelled onto the oviductal papilla. Then, the 3-mL syringe was refilled with air and injected through the catheter until residual semen could no longer be expelled.



### ***II.1.2.5. Cleaning of hysteroscopic equipment***

#### ***II.1.2.5.1. Cleaning of the endoscope***

The 1-m flexible video-endoscope used in this study was purchased specifically for this study and HYS. It was not exposed to disinfecting agents such as glutaraldehyde prior to or during the study, to ensure that no spermicidal residue was present in or on the endoscope. The endoscope was, however, cleaned with a combination of high pressure air, sterile saline, deionized water, and isopropyl alcohol (70%) before the study and after the final deep-horn insemination of each day. Specifically, high pressure air was blown through the biopsy channel until fluid was no longer expelled. A clean 4x4 gauze sponge soaked in sterile saline followed by a dry sponge to wipe off the insertion tube and distal head. The suction, air/water, and biopsy valves were removed from their respective valve housing within the control body of the endoscope. The valves were rinsed with deionized water, then placed in a sterile cup containing isopropyl alcohol (70%) until completion of the sterilization process for the rest of the endoscope.

Aseptic technique was used for the remainder of the cleaning of the endoscope. One person held the control body of the endoscope so that the insertion and universal/light guide tubes hung vertically. The water-resistant cap was placed on the electrical connector, completely sealing the electrical components from water contamination. The injection tube was attached to the air pipe, suction connector, and the water/air connector. The injection tube intake filter was submerged in a sterile cup containing deionized water and a 60-mL syringe was attached to the air/water channel port of the injection tube. Deionized water was then injected into the air/water channel,



60 mL at time, for a total of 300 – 500 mL. To clean the full length of the air/water channel, the endoscope holder plugged the opening to the air/water channel in the control body with an index or middle finger, and intermittently allowed some water to escape through the opening. This process was repeated with the suction channel except that the biopsy channel opening and the suction channel opening in the control body both were held closed by the endoscope holder with the right and left index or middle fingers. Then, 60 mL of deionized water was injected into the openings of the biopsy, suction, and water/air channels in the control body. This entire process of both channels and all 3 channel openings was repeated using isopropyl alcohol (70%) to promote drying and inhibit bacterial growth. The channels and channel openings were then fully dried with high pressure air filtered through a 0.2  $\mu$ m hydrophobic PTFE filter (Millex®-FG Syringe Filter Unit, Hydrophobic PTFE, 50 mm; © EMD Millipore Corporation, Billerica, MA, USA). The injection tubes were disconnected and the entire outer surface of the endoscope and the injection tubes were thoroughly cleaned with sterile 4x4 gauze sponges soaked in deionized water, followed by sponges soaked in isopropyl alcohol (70%). The insertion tube of the endoscope was inserted into a sterile obstetrical sleeve and then hung from the endoscope rack. The suction, water/air, and biopsy valves were placed in a sterile cup to dry overnight.

The endoscope was not subjected to the above described cleaning and sterilization procedures between inseminations within the same day due to practicality and the potential spermicidal effects of the deionized water and isopropyl alcohol if not given sufficient time to evaporate. Instead, after each insemination, high pressure air was

blown through the biopsy channel until fluid was no longer expelled. Then, the insertion tube and distal head of the endoscope was aseptically cleaned using a sterile 4x4 gauze sponge soaked in 0.9% saline, then dried with a dry, sterile 4x4 gauze sponge.

#### ***II.1.2.5.2. Cleaning of the intra-fallopian insemination catheters***

The intra-fallopian catheter consists of an inner and outer cannula (Figure 12). It has a plastic case (Figure 13) that prevents damage to the catheter when not in use. All three of these components were individually cleaned/sterilized, re-assembled and packaged into a new, clean, gallon storage bag (Ziploc® brand Storage Bags with the Smart Zip® Seal; S. C. Johnson & Sons, Inc., Racine, WI, USA).

Figure 12. The inner and outer cannulas of the intra-fallopian insemination catheter (Cook Veterinary Products®, Spencer, IN, USA). The inner cannula can be seen coming out of the distal end of the outer cannula.

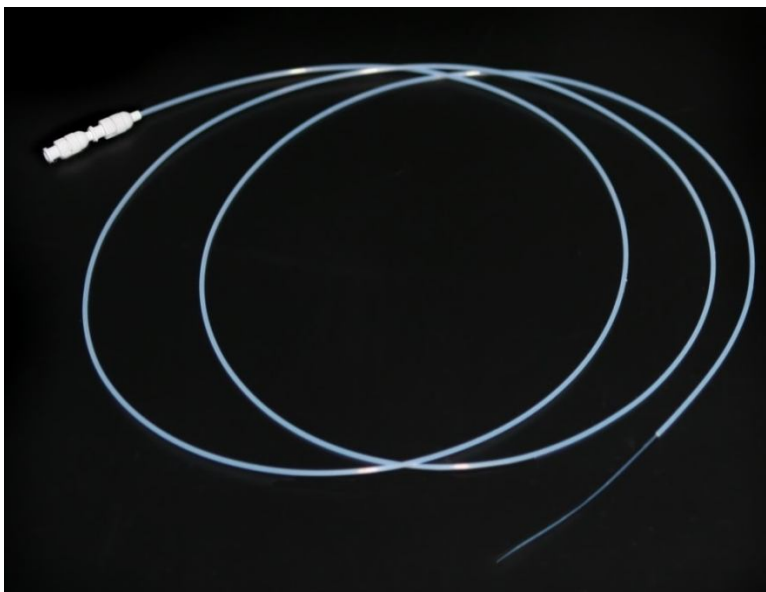


Figure 13. The intra-fallopian insemination catheter (Cook Veterinary Products®, Spencer, IN, USA) contained within the plastic tubing packaging.



The outer plastic case and the inner and outer cannulas were disassembled and wiped off with a dry, clean, 4x4 gauze sponge. Using aseptic technique, each component was flushed with deionized water, followed by an equal volume of isopropyl alcohol. The volume of flush and syringe size used for the outer plastic case, outer cannula, and inner cannula were 180-mL/60-mL syringe, 120-mL/20-mL syringe, and 60-mL/12-mL syringe, respectively. After each component was flushed, it was placed in a container with isopropyl alcohol and allowed to soak until all the catheters and cases used that day had been flushed and were in alcohol. Then, in the same order that the catheters had been cleaned, they were flushed with high pressure air filtered through a 0.2  $\mu\text{m}$  hydrophobic PTFE filter to remove all remaining alcohol from the lumen, and their outer

surfaces were dried with sterile 4x4 gauze sponges. The catheters were then reassembled and inserted into their plastic case as described above.

### ***II.1.3. Seminal plasma (SP) processing***

Two ejaculates were collected from the same stallion used in the experiments to obtain sperm-free seminal plasma. Semen was collected as described in section II.1.1.2. Following semen collection, the semen was placed into two 15-mL polypropylene conical-bottom tubes (Corning Life Sciences, Lowell, MA, USA), and centrifuged at 2000 x g for 10 min (IEC Centra CL2; Thermo Scientific, Waltham, MA, USA). The seminal plasma was decanted into 20-mL all-plastic syringes (2-part disposable syringes HSW Norm-Ject®; Henke Sass Wolf, Tuttlingen, Germany) fitted with tandem 5.0 µm and 1.2 µm nylon filters (Cameo 30N Syringe Filter, Nylon, 30 mm; Sigma-Aldrich, St. Louis, MO, USA). The seminal plasma was passed through the filters into a 50-mL conical vial (Corning Life Sciences, Lowell, MA, USA) to remove any remaining sperm. Aliquots (1 mL) of seminal plasma were stored in 1.5-mL snap-cap microcentrifuge tubes (disposable/conical economy micro tubes with snap caps; VWR International, USA) which were frozen and stored in a freezer at -80°C until used.

### ***II.1.4. Measurement of sperm concentration***

A NucleoCounter® SP-100™ (ChemoMetec A/S, Allerød, Denmark) was used for all semen concentration measurements. It utilizes a proprietary reagent, Reagent S-100 (ChemoMetec A/S, Allerød, Denmark) to permeabilize the sperm plasma membranes and propidium-iodide to label the DNA in the permeabilized spermatozoa. The dilution factors used in these experiments were 1:100 for raw semen and for 3:1

extended semen (10  $\mu$ L of semen to 1.0 mL of Reagent S-100), and 1:10 for  $2.5 \times 10^6$  and  $5.0 \times 10^6$  sperm/mL (100  $\mu$ L of semen to 1.0 mL of Reagent S-100).

To prepare the dilutions, each semen sample was thoroughly mixed and the appropriate volume of semen was drawn up into a positive displacement pipette (10  $\mu$ L with a 1-10- $\mu$ L pipette; 100  $\mu$ L with a 10-100- $\mu$ L pipette). The sides of the pipette tip were wiped with a delicate task wiper (Kimtech Science<sup>TM</sup> Kimwipes®, Kimberly-Clark Professional<sup>TM</sup>, Roswell, GA, USA) to remove excess semen. The semen was dispensed into the 1.2-mL Cryogenic vial preloaded with 1.0 mL of Reagent S-100. The vial was inverted a minimum of 10-15 times to thoroughly mix its contents. The tip of the SP-1 Cassette (ChemoMetec A/S, Allerød, Denmark) was immersed into the reagent-semen solution, the cassette piston depressed, pulling the appropriate amount of solution into the cassette. The cassette was inserted into the NucleoCounter® and the total sperm/mL was measured.

#### ***II.1.5. Evaluation of sperm motion characteristics (sperm motility)***

Because the inseminations for each insemination dose did not occur on the same day, sperm motility characteristics were evaluated to ensure that mares included in the analysis of pregnancy rates were being inseminated with similar quality semen. All semen samples were evaluated using computer-assisted motion analyzer (CASMA; IVOS version 12.2L; Hamilton Thorne Biosciences, Beverly, MA, USA) following 10 minutes of warming at 37°C on a covered slide warmer. The extended semen was thoroughly mixed then 6  $\mu$ L of the semen was loaded into a warmed (37°C) disposable counting chamber (Leja 20  $\mu$ m, 2 chamber slide; Leja Products B.V., Nieuw-Vennep,

The Netherlands). The slide was placed onto a warmed stage and inserted into the CASMA. For the  $30 \times 10^6$  sperm/mL samples, a minimum of 10 microscopic fields and 500 sperm cells were analyzed per sample. For the  $5.0 \times 10^6$  and  $2.5 \times 10^6$  sperm/mL, all microscopic fields on a slide were analyzed to compensate for the low sperm concentration in the samples. The preset values for the IVOS system were as follows: 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 for these studies were total sperm motility (TMOT), progressive sperm motility (PMOT, %), curvilinear velocity (VCL,  $\mu$ /s), average-path velocity (VAP,  $\mu$ /s), straight-line velocity (VSL,  $\mu$ /s), and straightness (STR, %).

### ***II.1.6. Statistical analysis***

#### ***II.1.6.1. Pregnancy rates***

Pregnancy rates between groups were compared using Chi-square analysis. Fisher's exact test was used when a value of less than 5 was expected for any square. For all analyses,  $P < 0.05$  was considered significantly different.

#### ***II.1.6.2. Sperm motion characteristics (sperm motility)***

A mixed model analysis of variance (SAS Institute, Cary, NC) was used to determine differences in treatment (sperm concentration). Parameters expressed as a percentage (TMOT, PMOT, and STR) were arcsin square root transformed to normalize

the data prior to analysis. The effects of stallion, ejaculate and ejaculate within stallion were considered random.

## **II.2. Results**

### ***II.2.1. Preliminary study***

Twenty-eight mares were inseminated utilizing either TRG or conventional uterine body insemination for a total of 44 estrous cycles. Conventional uterine body insemination ( $\geq 1 \times 10^9$  progressively motile sperm) was used on 18 cycles; whereas, TRG was used on 14 and 12 cycles for  $1 \times 10^6$  and  $0.5 \times 10^6$  total sperm, respectively. Exact day of ovulation was determined for 40 cycles. The four cycles with unconfirmed days of ovulation (2 –  $0.5 \times 10^6$  TRG; 2 –  $1 \times 10^6$  TRG) were in mares bred on Friday that could not be examined over the weekend due to inaccessibility to the mares. All four had a prominent corpus luteum the following Monday. In these four mares, the two mares that received an ovulatory induction agent the day before breeding (1 –  $0.5 \times 10^6$ ; 1 –  $1 \times 10^6$ ) were pregnant while the two mares that received the ovulatory induction agent the day of insemination (1 –  $0.5 \times 10^6$ ; 1 –  $1 \times 10^6$ ) were not pregnant.

In the 40 cycles for which the exact day of ovulation was determined (based on daily transrectal ultrasonographic examinations starting the day the mares were treated with an ovulatory induction agent), ovulation occurred the same day as insemination (n = 1), and within 24 (n = 30), 48 (n = 8), and 96 (n = 1) hours of insemination. Mares bred with conventional uterine body insemination ovulated within 24 h (n = 13) and 48 h (n = 5) of insemination. All but 3 of these mares were pregnant; the three non-pregnant mares ovulated within 24 h of insemination. Mares inseminated with  $1.0 \times 10^6$  sperm

ovulated within 24 (n = 9), 48 (n = 2), and 96 (n = 1) of insemination. A total of 5 pregnancies resulted, all from breedings that occurred within 24 h of insemination.

Mares inseminated with  $0.5 \times 10^6$  sperm ovulated within 0 (n = 1), 24 (n = 8), and 48 (n = 1) hours of insemination. Two mares were pregnant; these mares ovulated within 24 or 48 h of ovulation.

Pregnancy rates were higher for conventional uterine body insemination (UBI) than for TRG with  $1 \times 10^6$  sperm (15/18, 83% vs. 6/14, 43%, respectively; P = 0.03) and with  $0.5 \times 10^6$  sperm (15/18, 83% vs. 3 /12, 25%, respectively; P = 0.002) (Table 1). Pregnancy rates were not different between TRG insemination doses (6/14, 43% vs. 3/12, 25%, respectively; P = 0.4) (Table 1).

Table 1. Pregnancy rates relative to time of ovulation (n = 44) for mares bred with conventional uterine body insemination (UBI) using  $\geq 1$  billion progressively motile sperm or with TRG using either 1 or  $0.5 \times 10^6$  sperm from one Quarter Horse stallion.

Day of Ovulation Relative to Insemination (hrs)	Insemination Dose & Method		
	$\geq 1 \times 10^9$ – UBI	$1 \times 10^6$ – TRG	$0.5 \times 10^6$ – TRG
0	--	--	0 / 1 (0%)
$\leq 24$	10 / 13 (85%) <sup>a</sup>	5 / 9 (56%) <sup>ac</sup>	1 / 8 (13%) <sup>bc</sup>
$\leq 48$	5 / 5 (100%) <sup>a</sup>	0 / 2 (0%) <sup>bc</sup>	1 / 1 (100%) <sup>ac</sup>
$\leq 96$	--	0 / 1 (0%)	--
Unknown <sup>*</sup>	--	1 / 2 (50%) <sup>a</sup>	1 / 2 (50%) <sup>a</sup>
Total	15/18 (83%) <sup>a</sup>	6 / 14 (43%) <sup>b</sup>	3 / 12 (25%) <sup>b</sup>

<sup>\*</sup> Unknown = mares bred on a Friday and next examination was Monday

<sup>abc</sup> Within rows, rates with different superscripts differed (P < 0.05)



## ***II.2.2. Main study***

### ***II.2.2.1. Pregnancy rates***

Of the 37 mares that started this experiment, nine were eliminated due to development of hemorrhagic anovulatory follicles ( $n = 3$ ), failure to ovulate within 72 h after insemination ( $n = 2$ ), presence of excessive endometrial cysts ( $n = 1$ ), excessive intrauterine fluid accumulation after breeding ( $n = 2$ ), or presence of hyperechoic intrauterine fluid at the end of the second estrus synchronization ( $n = 1$ ). This resulted in 28 mares that were inseminated using both techniques (Table 2). All 28 mares inseminated with  $1 \times 10^6$  sperm and 26/28 mares inseminated with  $0.5 \times 10^6$  sperm ovulated within 24 h of insemination. The remaining 2 mares inseminated with  $0.5 \times 10^6$  sperm ovulated within 48 h of insemination; both were bred by HYS and neither was pregnant. There was no significant difference in pregnancy rates between HYS and TRG methods when mares were inseminated with  $1 \times 10^6$  sperm (HYS 10/13, 77% vs TRG 11/15, 73%;  $P = 1$ ) or with  $0.5 \times 10^6$  sperm (HYS 3/15, 20% vs TRG 4/13, 31%;  $P = 0.7$ ). Pregnancy rates were not different between insemination techniques when the insemination doses of 0.5 or  $1 \times 10^6$  sperm were combined (HYS 13/28, 46% vs TRG 15/28, 54%;  $P = 0.8$ ). Pregnancy rates were higher in mares inseminated with  $1 \times 10^6$  sperm than in mares inseminated with  $0.5 \times 10^6$  sperm (21/28, 75% vs. 7/28, 25%, respectively;  $P < 0.01$ ). This difference in pregnancy rates between sperm doses held true even when mares that ovulated more than 24 h after insemination were excluded (21/28, 75% vs. 7/26, 27%, respectively;  $P < 0.001$ ).

Table 2. Pregnancy rates in mares (n = 28) inseminated hysteroscopically (HYS) or transrectally (TRG) with either 1.0 or 0.5 x 10<sup>6</sup> sperm from one Quarter Horse stallion.

Insemination Method	Number of Sperm Inseminated		
	1 x 10 <sup>6</sup>	0.5 x 10 <sup>6</sup>	Combined 1 and 0.5 x 10 <sup>6</sup>
HYS	10/13 (77%) <sup>a</sup>	3/15 (20%) <sup>b</sup>	13/28 (46%)
TRG	11/15 (73%) <sup>a</sup>	4/13 (31%) <sup>b</sup>	15/28 (54%)
Total	21/28 (75%) <sup>a</sup>	7/28 (25%) <sup>b</sup>	

<sup>ab</sup> Within adjacent columns, rates without a common subscript differed (P < 0.05).

#### ***II.2.2.2. Sperm motion characteristics (sperm motility)***

To ensure that mares included in the analysis of pregnancy rates were being inseminated with similar quality semen, sperm motion characteristics were evaluated on every ejaculate at the time of collection (T0), following insemination of all mares for the day (T-End), and following 24 h of cooled storage (T24) at 7-8°C. The TMOT and PMOT for T0 and T-End for each ejaculate included in the pregnancy rate data are included in Table 3-4. Because difference in sperm quality often become more evident following storage, the mean and standard error the mean ( $\pm$  SEM) were calculated for TMOT, PMOT, VCL, VAP, VSL, and STR for the ejaculates at T24 (Table 5). The 3:1 samples had a mean sperm concentration of 55 x 10<sup>6</sup>/mL (range = 23 to 138 x 10<sup>6</sup>/mL).

Table 3. Total and progressive motility at the time of semen collection (T0) and following insemination of all mares for the day (T-End) for the ejaculates used for the  $1.0 \times 10^6$  insemination dose.

Ejaculate	Semen Samples					
	T0 / $30 \times 10^6$ *		T-End / $30 \times 10^6$ *		T-End / $5 \times 10^6$ *	
	TMOT <sup>§</sup>	PMOT <sup>§</sup>	TMOT <sup>§</sup>	PMOT <sup>§</sup>	TMOT <sup>§</sup>	PMOT <sup>§</sup>
1	97	77	--	--	92	84
2	95	69	89	74	77	71
3	92	70	88	70	85	76
4	96	78	89	71	77	66
5	95	68	89	64	83	65
6	91	67	91	67	81	64
7	94	70	94	70	83	73
8	95	74	94	79	91	87
9	96	74	96	74	91	87
10	97	74	96	72	91	73
Mean (Range)	95 (91-97)	72 (67-78)	92 (88-96)	71(64-79)	85 (77-92)	75 (64-87)

<sup>§</sup> TMOT = total sperm motility (%)

PMOT = progressive sperm motility (%)

\* T0 /  $30 \times 10^6$  =  $30 \times 10^6$  sperm/mL at the time of semen collection

T-End /  $30 \times 10^6$  =  $30 \times 10^6$  sperm/mL following insemination of all mares for the day

T-End /  $5 \times 10^6$  =  $5 \times 10^6$  sperm/mL following insemination of all mares for the day

Table 4. Total and progressive motility at the time of semen collection (T0) and following insemination of all mares for the day (T-End) for the ejaculates used for the  $0.5 \times 10^6$  insemination dose.

Ejaculate	Semen Samples					
	T0 / $30 \times 10^6$ *		T-End / $30 \times 10^6$ *		T-End / $2.5 \times 10^6$ *	
	TMOT <sup>§</sup>	PMOT <sup>§</sup>	TMOT <sup>§</sup>	PMOT <sup>§</sup>	TMOT <sup>§</sup>	PMOT <sup>§</sup>
1	96	77	92	82	84	80
2	95	68	89	72	69	61
3	96	75	92	79	79	73
4	97	79	88	74	73	70
5	91	61	86	65	81	75
6	90	67	88	70	70	63
7	94	75	91	77	87	75
Mean (Range)	94 (90-97)	72 (61-79)	89 (86-92)	74 (65-79)	78 (69-87)	71 (61-80)

<sup>§</sup> TMOT = total sperm motility (%)

PMOT = progressive sperm motility (%)

\* T0 /  $30 \times 10^6$  =  $30 \times 10^6$  sperm/mL at the time of semen collection

T-End /  $30 \times 10^6$  =  $30 \times 10^6$  sperm/mL following insemination of all mares for the day

T-End /  $2.5 \times 10^6$  =  $2.5 \times 10^6$  sperm/mL following insemination of all mares for the day

The TMOT for the  $1.0 \times 10^6$  insemination dose at T0 ( $30 \times 10^6$ ), T-End at  $30 \times 10^6$  sperm/mL, and T-End at  $5 \times 10^6$  sperm/mL had means of 95% (range 91 to 97%), 92% (range 88 to 96%), and 85% (range 77 to 92%), respectively. The PMOT for the  $1.0 \times 10^6$  insemination dose at T0, T-End at  $30 \times 10^6$  sperm/mL, and T-End at  $5 \times 10^6$  sperm/mL had means of 72% (range 67 to 78%), 71% (range 64 to 79%), and 75% (range 64-87%), respectively. The TMOT for the  $0.5 \times 10^6$  insemination dose at T0, T-End at  $30 \times 10^6$  sperm/mL, and T-End at  $2.5 \times 10^6$  sperm/mL had means of 94% (range 90 to 97%), 89% (range 86 to 92%), and 78% (range 69-87%), respectively. The PMOT for the  $0.5 \times 10^6$  insemination dose at T0, T-End at  $30 \times 10^6$  sperm/mL, and T-End at  $2.5 \times 10^6$  sperm/mL means of 71% (range 61 to 79%), 74% (range 65 to 79%), and 71% (range 61-80%), respectively.

Table 5. Sperm motion characteristics following 24 h of cooled storage (T24) in an Equitainer®I (mean  $\pm$  SEM).

Motility Parameter <sup>§</sup>	Semen Samples			
	$2.5 \times 10^6$ *	$5 \times 10^6$ *	$30 \times 10^6$ *	3:1SD*
TMOT	83 (2) <sup>b</sup>	85 (2) <sup>b</sup>	90 (1) <sup>a</sup>	87 (2) <sup>ab</sup>
PMOT	68 (2) <sup>a</sup>	66 (3) <sup>a</sup>	57 (3) <sup>ab</sup>	52 (3) <sup>b</sup>
VCL	212 (7) <sup>ab</sup>	204 (9) <sup>b</sup>	225 (6) <sup>a</sup>	220 (8) <sup>ab</sup>
VAP	103 (4) <sup>b</sup>	102 (5) <sup>b</sup>	114 (3) <sup>a</sup>	114 (4) <sup>a</sup>
VSL	76 (2) <sup>a</sup>	72 (2) <sup>a</sup>	72 (2) <sup>a</sup>	73 (3) <sup>a</sup>
STR	75 (2) <sup>a</sup>	73 (3) <sup>a</sup>	62 (2) <sup>b</sup>	62 (1) <sup>b</sup>

<sup>§</sup> TMOT = total sperm 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 ( $[\text{VSL}/\text{VAP}]100$ ; %)

\*  $2.5 \times 10^6 = 2.5 \times 10^6$  sperm/mL (n = 7)  
 $5 \times 10^6 = 5 \times 10^6$  sperm/mL (n = 10)  
 $30 \times 10^6 = 30 \times 10^6$  sperm/mL (n = 17)  
 3:1SD = 3:1 Simple Dilution (n = 17)

<sup>ab</sup> Within rows, rates with different superscripts differed (P < 0.05)

At T24 (Table 5), the TMOT was higher than  $2.5 \times 10^6$  and  $5 \times 10^6$  ( $P < 0.05$ ) but, similar to 3:1SD; and 3:1SD was similar to  $2.5 \times 10^6$  and  $5 \times 10^6$ . The PMOT for  $2.5 \times 10^6$  and  $5 \times 10^6$  was higher than 3:1SD ( $P < 0.05$ ); and  $30 \times 10^6$  was similar to  $2.5 \times 10^6$  and  $5 \times 10^6$ . The VCL for  $30 \times 10^6$  was higher than  $5 \times 10^6$  ( $P < 0.05$ ); and similar to  $2.5 \times 10^6$  and 3:1SD ( $P < 0.05$ ). The VAP for  $30 \times 10^6$  and 3:1SD were higher than  $2.5 \times 10^6$  and  $5 \times 10^6$  ( $P < 0.05$ ). The VSL was similar among all treatments ( $P \geq 0.05$ ). STR at  $2.5 \times 10^6$  and  $5 \times 10^6$  were higher than  $30 \times 10^6$  and 3:1SD ( $P < 0.05$ ).

### **II.3. Discussion**

Low-dose deep-horn insemination is commonly used in equine practice because it is believed that delivering a small volume of relatively concentrated semen closer to the site of fertilization (tip of the uterine horn ipsilateral to the site of ovulation) may improve fertility [1]. Fertility trials involving horses commonly utilize an insemination dose of 250 to  $500 \times 10^6$  progressively motile sperm for uterine body insemination, which should provide optimum pregnancy rates, regardless of inter-stallion variability. However, using supra-threshold sperm numbers can mask the ability of the experimental design to detect a difference in technique (i.e. HYS vs. TRG) due to excess sperm numbers. In the present study, we performed a preliminary trial to establish a sperm insemination number that was below the threshold for optimal fertility for the stallion in this study. The results of the preliminary trial indicated that for this stallion deep-horn insemination of either 1 or  $0.5 \times 10^6$  sperm resulted in pregnancy rates (43 and 25%, respectively) that were lower than if more sperm ( $\geq 1 \times 10^9$ ) were inseminated into the uterine body (83%). Establishing a “subthreshold” sperm level allowed us to evaluate

whether one technique (HYS or TRG) would provide higher pregnancy rates than the other.

In our main experiment, the efficacy of HYS and TRG techniques were similar; this finding agrees with previous studies utilizing  $5 \times 10^6$  total, cool-stored sperm [46] and  $20 \times 10^6$  sex-sorted sperm [18]. However, in contrast to the preliminary experiment, in this experiment only the  $0.5 \times 10^6$  insemination dose appeared to be below the threshold number required to achieve normal pregnancy rates. Thus, mares were inseminated with a subthreshold insemination dose using only one of the deep-horn insemination techniques, preventing the complete removal of mare variability. But, because all mares in this experiment were proven, fertile mares, the similarity in pregnancy rates between HYS and TRG achieved with  $0.5 \times 10^6$  sperm confirmed that these two techniques can be expected to achieve similar efficacy when below-threshold levels of sperm are inseminated. These results, therefore, contradict the recommendation of Squires [47] to utilize HYS when sperm numbers in the inseminate are less than 25 to  $50 \times 10^6$ . This finding also suggests that there is a subthreshold level of sperm, even in fertile stallions, that cannot be overcome by using the deep-horn technique. Therefore, there are clinical circumstances (e.g. stallions with inherently poor semen quality or frozen semen from some stallions) under which it cannot be assumed that either technique will improve fertility.

It is unclear why pregnancy rates with TRG insemination of  $1 \times 10^6$  sperm were higher in the main experiment than they were in the preliminary trial. Improvements in pregnancy outcome did, however, occur as the operator gained more expertise in the

TRG technique. In addition, they may have been due to administration of detomidine prior to insemination, since mares bred in the preliminary trial were not tranquilized as opposed to the mares in the main study. Detomidine is an  $\alpha_2$ -adrenergic receptor agonist that increases uterine contractions in the normal non-pregnant mare [82]; there initially was concern that it might interfere with uterine contractions necessary to propel sperm toward the oviduct, or cause premature evacuation of sperm from the uterus. However, the normal pregnancy rates (73 to 77%) achieved with insemination of  $1 \times 10^6$  sperm using either technique in this study, as well as the good pregnancy rates achieved in previous studies [46], suggested that detomidine administration prior to deep-horn insemination was not detrimental. Likewise, tranquilization of maiden mares prior to natural service did not reduce pregnancy rates [83]. Due to its ability to reduce rectal peristalsis and tone, detomidine may allow a more accurate and consistent placement of the intrauterine catheter at the tip of the uterine horn and it may facilitate the operator's ability to orient the tip of the uterine horn ventrally. We postulate that the ventral orientation of the uterine horn tip may promote pooling of the inseminate on or around the oviductal papilla. The value of holding the tip of the uterine horn ventrally, however, has not been critically evaluated.

From these findings, we conclude that whereas high fertility can be achieved with very low sperm numbers, there is a functional threshold below which fertility cannot be improved through deep-horn insemination techniques alone, even in a fertile stallion. Previous workers were not able to increase fertility in a subfertile stallion using TRG insemination of  $25 \times 10^6$  sperm (29% pregnancy rate) compared to that achieved



with a fertile stallion with good semen quality (63% pregnancy rate) [45]. Sperm quality (DNA quality, sperm morphology and motility) can be selected for by centrifugation through density gradients [84-87], which aids in removal of sperm with abnormal heads and midpieces, bent midpieces, bent tails, coiled tails and premature germ cells [84]. Low-dose deep-horn insemination of these purified samples may increase fertility in some circumstances [10-12,14].

Use of the hysteroscope may [4,34] or may not [4,9] increase the chance of endometritis. Schiemann et al. [34] reported histopathologic evidence of endometritis in all eight mares subjected to diagnostic hysteroscopy, and bacterial pathogens were isolated in 6/8 mares. Hysteroscope-derived post-insemination endometritis was not reported in one study following repeated inseminations [9]. Sieme et al. [4] reported no clinical effect of hysteroscopy in reproductively normal mares, but pregnancy rates were lower for HYS of fresh semen compared to standard uterine body insemination in subfertile mares, suggesting that mares susceptible to infection may be at greater risk with the HYS technique. Nie et al. [88] found that post-breeding endometritis was lower following the TRG technique compared to conventional uterine body insemination, whereas Güvenc et al. [35] found no difference in post-breeding endometritis following the same two techniques.

Despite routine sterilization between procedures, endoscopes used for human applications have been identified as a source for the biofilm-producing bacteria *Escherichia coli* and *Pseudomonas aeruginosa*, resulting in nosocomial infections [27-31]. These bacteria are known pathogens of the equine endometrium [89,90]. They were

isolated from 6 of 8 mares following diagnostic hysteroscopy in one study [34] and have been associated with reduced pregnancy rates in mares [89]. In contrast, the catheter used for TRG is inexpensive and a new catheter may be used for each mare, eliminating the possibility of nosocomial infection.

In summary, similar pregnancy rates were achieved with low-dose insemination using a subthreshold number of sperm when either hysteroscopically guided or transrectally guided techniques were used. Due to simplicity, lower cost, reduction of time involved, and reduction of the possibility of nosocomial infections, the transrectally guided technique may be the method of choice for low dose insemination in the mare.

**CHAPTER III**  
**EFFECT OF DILUTION AND SEMINAL PLASMA ON MOTION**  
**CHARACTERISTICS AND PLASMA MEMBRANE INTEGRITY**  
**OF STALLION SPERM**

**III.1. Materials and Methods**

A study was conducted to evaluate the sperm motion characteristics and plasma membrane integrity of stallion semen when processed to  $2.5 \times 10^6$  sperm/mL as used for low-dose insemination. Three ejaculates were collected from each of three stallions, two 20-yr old American Quarter Horses and a 9-yr old American Paint Horse, for a total of 9 ejaculates. The study was conducted in September 2009 in southeast Texas. All experimental procedures were performed according to the United States Government Principles for Utilization and Care of Vertebrate Animals Used in Testing, Research and Training and were approved by the Laboratory Animal Care Committee at Texas A&M University.

***III.1.1. Semen collection***

An ejaculate was collected daily as described in section II.1.1.2 from each of 3 stallions for 3 consecutive days, for a total of 9 ejaculates (3 ejaculates/stallion).

***III.1.2. Semen processing***

Immediately following semen collection, the gel filter was removed from the collection bottle to prevent leakage of the gel into the gel-free semen. The gel-free semen was placed in a 37°C incubator. The total number of sperm in the ejaculate was

determined by measuring the volume of the neat semen using a 10- mL graduated cylinder and measuring the concentration using the NucleoCounter® SP-100™ as detailed in Chapter II.

Using pre-warmed (37°C) INRA-T and neat semen, a 3:1 extended semen sample (3:1SD) and a  $30 \times 10^6$  sperm/mL sample (30) were made. The 3:1SD sample was made by adding 1.5 mL of neat semen into a 15-mL conical vial containing 4.5 mL INRA-T. A 5.0-mL semen sample with a concentration of  $30 \times 10^6$  sperm/mL (30) was made using the following formula and placed into a 5.0-mL Cryogenic vial:

- Volume of raw semen required =  $(30 \times 10^6 \text{ sperm/mL}) \div (\text{raw semen concentration}) * (5.0 \text{ mL})$
- Volume of extender =  $(5.0 \text{ mL}) - (\text{volume of raw semen required})$

The total sperm and non-plasma membrane-intact sperm concentrations were determined for both semen samples using the NucleoCounter® SP-100™ as described below in Section III.1.4. Aliquots for evaluation of initial sperm motion characteristics were made by pipetting 0.5 mL of the 3:1SD and 30 preparations into 0.5-mL Eppendorf tubes. The aliquots were then placed on a 37°C covered slide warmer for 10 minutes prior to evaluation. Two 1.2-mL aliquots were placed in separate 1.2-mL Cryogenic vials for evaluation following 24- and 48-h of cooled storage (7-8°C) in an Equitainer®I.

Five-milliliter semen samples with a concentration of  $2.5 \times 10^6$  sperm/mL were made by diluting the appropriate amount of 3:1 extended semen with INRA-T, INRA-T with 7.5% frozen-thawed, sperm-free seminal plasma (v:v), or INRA-T with 25% frozen-thawed, sperm-free seminal plasma (v:v); thus, a total of 3 samples were made at

$2.5 \times 10^6$  sperm/mL. These  $2.5 \times 10^6$  sperm/mL treatments extended with 0, 7.5 and 25% seminal were designated as 2.5/0, 2.5/7.5, and 2.5/25. The following formulas were used to produce these semen samples:

- volume of sample desired = X + Y
  - X = volume of 3:1 extended semen
  - Y = volume of semen extender
- $X = (2.5 \times 10^6 \text{ sperm/mL}) \div (\text{concentration of 3:1 extended semen}) * (5.0 \text{ mL})$
- $Y = (5.0 \text{ mL}) - X$

The total sperm and non-plasma membrane-intact sperm concentrations were determined for all  $2.5 \times 10^6$  sperm/mL semen samples using the NucleoCounter® SP-100™. A 0.5-mL aliquot of each of the  $2.5 \times 10^6$  sperm/mL samples was pipetted into a 0.5-mL Eppendorf tube, then placed on a 37°C covered slide warmer for 10 minutes prior to evaluation of initial sperm motion characteristics using a CASMA. Two 1.2-mL aliquots of each of the 2.5/25 samples were pipetted into separate 1.2-mL Cryogenic vials for evaluation following 24 and 48 h of cool storage (7-8°C) in an Equitainer®I.

Two Equitainers per stallion were used for the 24- and 48-h cooled storage samples to ensure that cooling was not disturbed when the 24-h samples were evaluated. Semen samples were packaged as recommended by the manufacturer with sufficient ballast to control the cooling rate. In addition to the above-described semen samples, a 2-mL Cryogenic vial containing 2 mL of INRA-T with 25% frozen-thawed, sperm-free

seminal plasma (v:v) was placed into each of the Equitainers. This extender was used to make a  $30 \times 10^6$  sperm/mL aliquot of the 3:1SD for motility analysis.

Following 24 and 48 h of cool storage, 0.5-mL aliquots of all semen samples were pipetted into 0.5-mL Eppendorf tubes for analysis. When the concentration of the 3:1SD sample was greater than  $30 \times 10^6$  sperm/mL, a 0.5-mL aliquot was made by diluting the appropriate amount of the sample with INRA-T containing 25% frozen-thawed seminal plasma (v:v) to a concentration of  $30 \times 10^6$  sperm/mL (3:1/30) using the following formulas:

- Volume of 3:1 extended semen required =  $(30 \times 10^6 \text{ sperm/mL}) \div (3:1 \text{ extended semen sperm concentration}) * (0.5 \text{ mL})$
- Volume of extender =  $(0.5 \text{ mL}) - (\text{volume of 3:1 extended semen required})$

All of these 0.5-mL aliquots were placed on a 37°C covered slide warmer for 10 minutes prior to evaluation of sperm motion characteristics using a CASMA. The semen remaining in each of the original 1.2-mL Cryogenic vials were evaluated for total sperm and non-plasma membrane-intact sperm concentrations using the NucleoCounter® SP-100™.

### ***III.1.3. Seminal plasma (SP) processing***

Prior to the experiment, one ejaculate was collected from each of the 3 stallions used in the experiment to obtain sperm-free seminal plasma. Semen was collected as described in section II.1.1.2 and processed as described in section II.1.3 to collect seminal plasma, which was then frozen and stored in a -80°C freezer, as described in section II.1.3, until used.

### ***III.1.4. Evaluation of sperm concentration and plasma membrane integrity***

The NucleoCounter® SP-100™ was the system used for determination of total sperm and non-plasma membrane-intact sperm concentrations. As described in section II.1.4, Reagent S-100 was used for measurement of total sperm concentration because it permeabilizes the plasma membrane, allowing propidium-iodide to label the DNA of all spermatozoa in the sample. To evaluate the concentration of the non-plasma membrane-intact sperm, phosphate-buffered saline (PBS, GIBCO® Dulbecco's Phosphate Buffered Saline 1X; Invitrogen; Grand Island, NY, USA) was substituted for the Reagent S-100. The dilution factors (DF) used in this experiment were prepared according to the following table (Table 6).

Table 6. NucleoCounter® SP-100™ dilution factors used in the evaluation of plasma membrane integrity.

Sperm Concentration (x 10 <sup>6</sup> /mL)	Dilution Factor	Diluent Volume (μL)	Semen Volume (μL)
<2.5	6	500	100
2.5-30	11	1000	100
30-180	101	1000	10
180-300	201	2000	10

Each semen sample was thoroughly mixed and the appropriate volume of semen was drawn up into a positive-displacement pipette (10 μL with a 1 – 10 μL pipette; 100 μL with a 10 – 100 μL pipette). The sides of the pipette tip were wiped with a Kimwipe to remove excess semen. The semen was dispensed into the 1.2- or 2.0-mL Cryogenic vial preloaded with the appropriate amount of Reagent S-100 or PBS. The vial was

inverted a minimum of 10-15 times to thoroughly mix its contents. The tip of the SP-1 Cassette (ChemoMetec A/S, Allerød, Denmark) was immersed into the reagent-semen solution, the cassette piston depressed, pulling the appropriate amount of solution into the cassette. The cassette was inserted into the instrument and the total sperm/mL was measured. The total sperm concentration for each semen sample was evaluated prior to evaluation of the non-plasma membrane-intact sperm to allow the SemenView™ program (Version 1.21; ChemoMetec A/S, Allerød, Denmark) to calculate the percentage of plasma membrane-intact sperm. If an error occurred in the order that the tests were run, the percentage of plasma membrane-intact sperm was calculated by hand using the following equation:

- % of plasma membrane-intact spermatozoa = [(total sperm concentration) – (non-plasma membrane-intact sperm concentration)] ÷ (total sperm concentration) \* 100%

### ***III.1.5. Evaluation of sperm motion characteristics (sperm motility)***

Sperm motion characteristics were evaluated as described in section II.1.5 using a CASMA. A minimum of 10 microscopic fields and 500 sperm cells were analyzed in the 30, 3:1SD and 3:1/30 samples. The range of the concentration of the 3:1 non-adjusted samples was 23 to 75 x 10<sup>6</sup> sperm/mL. For the 2.5/25 samples, all microscopic fields on a slide were analyzed to compensate for the low sperm concentration. Experimental endpoints for this study were total sperm motility (TMOT), progressive sperm motility (PMOT), curvilinear velocity (VCL), average-path velocity (VAP), straight-line velocity (VSL), and straightness (STR).



### ***III.1.6. Statistical analysis***

A mixed model analysis of variance (SAS Institute, Cary, NC) was used to determine differences due to treatment (sperm concentration). Parameters expressed as a percentage (TMOT, PMOT, PMI, and STR) were arcsin square root transformed to normalize the data prior to analysis. The effects of stallion, ejaculate and ejaculate within stallion were considered random.

### **III.2. Results**

A total of nine ejaculates was analyzed for each treatment except for the 3:1/30 which only included 7 samples because the 3:1SD were  $\leq 30 \times 10^6$  sperm/mL in two samples; the concentration of the 3:1SD samples ranged from 23 to  $75 \times 10^6$  sperm/mL ( $50 \times 10^6 \pm 3$ , mean  $\pm$  SEM). The seminal plasma concentration in the 30 treatments ranged from 11 to 32% (mean, 18%). The final seminal plasma concentration in the 2.5/0, 2.5/7.5 and 2.5/25 treatments were 0.9-2.6%, 8.4-10.1%, 25.9-27.6%, respectively.

At T0 (Table 7), the PMI and TMOT were higher in 30 and 3:1SD than all  $2.5 \times 10^6$  sperm/mL treatments ( $P < 0.05$ ). The PMOT for 30 and 3:1SD were higher than 2.5/7.5 and 2.5/25 ( $P < 0.05$ ), but similar to 2.5/0 ( $P \geq 0.05$ ). In general for VCL, VAP, and VSL, velocities were higher in those treatments with higher seminal plasma concentration (i.e.  $>11\%$ ; 2.5/25, 30, 3:1SD) compared to 2.5/0 and 2.5/7.5 ( $P < 0.05$ ). The STR was higher in the 2.5/0 than all the other treatments ( $P < 0.05$ ).

Table 7. Initial sperm motion characteristics and sperm plasma membrane integrity for 9 ejaculates (3 ejaculates per stallion) (mean  $\pm$  SEM).

Laboratory Parameter <sup>§</sup>	Semen Samples				
	2.5/0*	2.5/7.5*	2.5/25*	30*	3:1SD*
PMI	78 (3) <sup>b</sup>	76 (5) <sup>b</sup>	75 (4) <sup>b</sup>	81 (5) <sup>a</sup>	82 (4) <sup>a</sup>
TMOT	71 (5) <sup>b</sup>	77 (5) <sup>b</sup>	73 (4) <sup>b</sup>	87 (4) <sup>a</sup>	90 (3) <sup>a</sup>
PMOT	55 (6) <sup>ab</sup>	50 (7) <sup>bc</sup>	47 (6) <sup>c</sup>	59 (6) <sup>a</sup>	62 (5) <sup>a</sup>
VCL	213 (5) <sup>c</sup>	226 (11) <sup>bc</sup>	248 (10) <sup>a</sup>	241 (6) <sup>ab</sup>	230 (9) <sup>abc</sup>
VAP	104 (2) <sup>c</sup>	121 (3) <sup>b</sup>	131 (3) <sup>a</sup>	127 (4) <sup>ab</sup>	124 (6) <sup>ab</sup>
VSL	77 (4.6) <sup>b</sup>	81 (5.0) <sup>ab</sup>	85 (5.0) <sup>a</sup>	87 (4.7) <sup>a</sup>	85 (5.9) <sup>a</sup>
STR	74 (4) <sup>a</sup>	67 (3) <sup>b</sup>	64 (3) <sup>b</sup>	66 (2) <sup>b</sup>	66 (2) <sup>b</sup>

<sup>§</sup> PMI = plasma membranes intact sperm (%)

TMOT = total sperm 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 ( $[\text{VSL}/\text{VAP}]100$ ; %)

\* 2.5/0 =  $2.5 \times 10^6$  sperm/mL with 0% seminal plasma added (n = 9)

2.5/7.5 =  $2.5 \times 10^6$  sperm/mL with 7.5% seminal plasma added (n = 9)

2.5/25 =  $2.5 \times 10^6$  sperm/mL with 25% seminal plasma added (n = 9)

30 =  $30 \times 10^6$  sperm/mL (n = 9)

3:1SD = 3:1 Simple Dilution (n = 9)

<sup>abc</sup> Within rows, rates with different superscripts differed ( $P < 0.05$ )

Table 8. Sperm motion characteristics and sperm plasma membrane integrity for 9 ejaculates (3 ejaculates per stallion) following 24 h of cooled storage (mean  $\pm$  SEM).

Laboratory Parameter <sup>§</sup>	Semen Samples					
	2.5/0*	2.5/7.5*	2.5/25*	30*	3:1SD*	3:1/30*
PMI	77 (4) <sup>ab</sup>	75 (4) <sup>b</sup>	70 (5) <sup>c</sup>	78 (5) <sup>a</sup>	78 (5) <sup>a</sup>	78 (5) <sup>a</sup>
TMOT	70 (5) <sup>c</sup>	73 (6) <sup>c</sup>	69 (4) <sup>c</sup>	82 (4) <sup>ab</sup>	83 (4) <sup>a</sup>	76 (7) <sup>b</sup>
PMOT	48 (7) <sup>a</sup>	39 (7) <sup>b</sup>	25 (4) <sup>c</sup>	42 (6) <sup>b</sup>	42 (5) <sup>b</sup>	37 (7) <sup>b</sup>
VCL	208 (9) <sup>c</sup>	248 (11) <sup>b</sup>	281 (13) <sup>a</sup>	234 (6) <sup>b</sup>	229 (8) <sup>bc</sup>	245 (7) <sup>b</sup>
VAP	97 (2.5) <sup>c</sup>	120 (3.2) <sup>b</sup>	137 (4.8) <sup>a</sup>	117 (3.3) <sup>b</sup>	117 (3.9) <sup>b</sup>	120 (3.9) <sup>b</sup>
VSL	63 (3.6) <sup>a</sup>	66 (3.5) <sup>a</sup>	63 (4.2) <sup>a</sup>	68 (4.1) <sup>a</sup>	69 (3.9) <sup>a</sup>	66 (5.0) <sup>a</sup>
STR	68 (4) <sup>a</sup>	56 (3) <sup>b</sup>	46 (3) <sup>c</sup>	56 (2) <sup>b</sup>	57 (2) <sup>b</sup>	55 (3) <sup>b</sup>

<sup>§</sup> PMI = plasma membranes intact sperm (%)  
 TMOT = total sperm 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 ( $[\text{VSL}/\text{VAP}]100$ ; %)

\* 2.5/0 =  $2.5 \times 10^6$  sperm/mL with 0% seminal plasma added (n = 9)  
 2.5/7.5 =  $2.5 \times 10^6$  sperm/mL with 7.5% seminal plasma added (n = 9)  
 2.5/25 =  $2.5 \times 10^6$  sperm/mL with 25% seminal plasma added (n = 9)  
 30 =  $30 \times 10^6$  sperm/mL (n = 9)  
 3:1SD = 3:1 Simple Dilution (n = 9)  
 3:1/30 = 3:1 Simple Dilution Samples diluted to  $30 \times 10^6$  sperm/mL with INRA-T with 25% SP (n = 7)

<sup>abc</sup> Within rows, rates with different superscripts differed ( $P < 0.05$ )

Table 9. Sperm motion characteristics and sperm plasma membrane integrity for 9 ejaculates (3 ejaculates per stallion) following 48 h of cooled storage (mean ± SEM).

Laboratory Parameter <sup>§</sup>	Semen Samples					
	2.5/0 <sup>*</sup>	2.5/7.5 <sup>*</sup>	2.5/25 <sup>*</sup>	30 <sup>*</sup>	3:1SD <sup>*</sup>	3:1/30 <sup>*</sup>
PMI	78 (4) <sup>ab</sup>	75 (4) <sup>b</sup>	71 (5) <sup>c</sup>	79 (6) <sup>a</sup>	80 (4) <sup>a</sup>	74 (6) <sup>b</sup>
TMOT	69 (6) <sup>ab</sup>	71 (5) <sup>a</sup>	64 (5) <sup>b</sup>	75 (5) <sup>a</sup>	69 (4) <sup>ab</sup>	63 (6) <sup>b</sup>
PMOT	47 (7) <sup>a</sup>	37 (5) <sup>ab</sup>	18 (2) <sup>d</sup>	35 (6) <sup>bc</sup>	25 (3) <sup>cd</sup>	24 (6) <sup>d</sup>
VCL	197 (13) <sup>c</sup>	257 (14) <sup>b</sup>	285 (9) <sup>a</sup>	242 (11) <sup>bc</sup>	219 (12) <sup>de</sup>	230 (12) <sup>cd</sup>
VAP	90 (5.2) <sup>d</sup>	120 (4.3) <sup>b</sup>	137 (3.4) <sup>a</sup>	119 (5.6) <sup>bc</sup>	108 (5.9) <sup>c</sup>	111 (4.7) <sup>bc</sup>
VSL	59 (3.5) <sup>b</sup>	65 (3.9) <sup>ab</sup>	56 (2.4) <sup>b</sup>	68 (5.2) <sup>a</sup>	58 (3.4) <sup>b</sup>	59 (4.9) <sup>b</sup>
STR	69 (4) <sup>a</sup>	56 (4) <sup>b</sup>	41 (1) <sup>c</sup>	55 (2) <sup>b</sup>	52 (2) <sup>b</sup>	53 (3) <sup>b</sup>

<sup>§</sup> PMI = plasma membranes intact (%);  
 TMOT = total sperm motility (%); PMOT = progressive sperm motility (%);  
 VCL = curvilinear velocity (µm/s); VAP = average-path velocity (µm/s);  
 VSL = straight-line velocity (µm/s); STR = straightness ([VSL/VAP]100; %)

<sup>\*</sup> 2.5/0 = 2.5 x 10<sup>6</sup> sperm/mL with 0% seminal plasma added (n = 9)  
 2.5/7.5 = 2.5 x 10<sup>6</sup> sperm/mL with 7.5% seminal plasma added (n = 9)  
 2.5/25 = 2.5 x 10<sup>6</sup> sperm/mL with 25% seminal plasma added (n = 9)  
 30 = 30 x 10<sup>6</sup> sperm/mL (n = 9)  
 3:1SD = 3:1 Simple Dilution (n = 9)  
 3:1/30 = 3:1 Simple Dilution Samples diluted to 30 x 10<sup>6</sup> sperm/mL with INRA-T with 25% SP (n = 7)

<sup>abc</sup> Within rows, rates with different superscripts differed (P < 0.05)

At T24 (Table 8), the PMI for 30, 3:1SD, 3:1/30 was higher than 2.5/7.5 and 2.5/25 ( $P < 0.05$ ), but similar to 2.5/0 ( $P \geq 0.05$ ); and 2.5/7.5 was higher than 2.5/25 ( $P < 0.05$ ). The TMOT for 30, 3:1SD, and 3:1/30 was higher than for all of the  $2.5 \times 10^6$  treatments ( $P < 0.05$ ); 3:1SD was higher than 3:1/30 ( $P < 0.05$ ), but similar to 30 ( $P \geq 0.05$ ). The PMOT for 2.5/0 was higher than all other treatments ( $P < 0.05$ ), and 2.5/25 was lower than all other treatments ( $P < 0.05$ ). The VCL and VAP for 2.5/25 was higher than all other treatments ( $P < 0.05$ ); and 2.5/0 was lower than all other treatments ( $P < 0.05$ ) except for the VCL of the 3:1SD treatment ( $P \geq 0.05$ ). No difference was seen for VSL among all treatments ( $P \geq 0.05$ ). The STR for 2.5/0 was higher and for 2.5/25 was lower than all other treatments ( $P < 0.05$ ).

At T48 (Table 9), the PMI for 30 and 3:1SD was higher than all other treatments ( $P < 0.05$ ), but not different than 2.5/0 ( $P \geq 0.05$ ); and 2.5/25 was lower than all other treatments ( $P < 0.05$ ). The TMOT at 2.5/7.5 and 30 was higher than 2.5/25 and 3:1/30 ( $P < 0.05$ ), but was not different than 2.5/0 and 3:1SD ( $P \geq 0.05$ ). The PMOT for 2.5/0 was higher than all other treatments ( $P < 0.05$ ) except 2.5/7.5 ( $P \geq 0.05$ ); and 2.5/25 and 3:1/30 were lower than all other treatments ( $P < 0.05$ ) except 3:1SD ( $P \geq 0.05$ ). The VCL and VAP for 2.5/25 were higher than all other treatments ( $P < 0.05$ ). The VSL at  $30 \times 10^6$  was higher than all other treatments ( $P < 0.05$ ) except 2.5/7.5 ( $P \geq 0.05$ ). The STR for 2.5/0 was higher and for 2.5/25 was lower than all other treatments ( $P < 0.05$ ). Values for TMOT at T0 ranged from 71 to 90%. After 48 h of cool-storage, values for the  $2.5 \times 10^6$  sperm/mL treatments declined less than the 30 and 3:1SD treatments.

### III.3. Discussion

The “dilution effect” refers to the detrimental effect on sperm quality (typically sperm motility) following the addition of a high volume of a diluent to neat semen, resulting in a sperm concentration less than  $20 \times 10^6$  sperm/mL [51-60,65,66,70].

Maintenance of sperm quality is important to the success of commercial equine artificial insemination programs. Semen dilution with milk-based extenders is a key component to maintaining sperm quality especially as storage time increases. In stallions, sperm and seminal plasma concentrations typically accepted to optimize sperm quality in skim milk-glucose extender are  $25 \times 10^6$  sperm/mL [90] and 5 to 20% seminal plasma [75,90]. The optimal sperm concentration is based on a study by Varner et al. [70] where the highest total and progressive motility was maintained in semen diluted to  $25 \times 10^6$  sperm/mL compared to  $50 \times 10^6$  and  $100 \times 10^6$  sperm/mL over 24 h of room-temperature (25°C) storage. Sperm concentration less than  $25 \times 10^6$  sperm/mL were not evaluated due concerns regarding the “dilution effect” demonstrated in other species [51-59, 61-69].

Studies in the stallion evaluating the effect of sperm concentration less than  $20 \times 10^6$  sperm/mL on sperm quality have not apparently been previously performed. Some low-dose-deep horn insemination studies use, in addition to low sperm numbers in small volumes, low sperm concentrations. It is unclear in the Morris et al. study [40] and the DHI experiment in Chapter II of this thesis (Experiment 1) whether reduced fertility with the  $0.5 \times 10^6$  insemination dose was due to the low sperm number or low sperm concentration (i.e. “dilution effect”). Therefore, it was of interest to compare sperm

quality (motility, velocity, and plasma membrane integrity) between conventionally diluted semen (i.e.  $30 \times 10^6$  sperm/mL, 3:1 extender:semen (i.e. 25% seminal plasma)) and semen diluted to a very low sperm concentration (the concentration of the sperm used for insemination in the 0.5 million sperm group in Experiment 1; i.e.  $2.5 \times 10^6$  sperm/mL).

We found, in contrast to reported finding in other species, that dilution to very low concentrations had little effect on sperm quality. Initially at T0, PMI and TMOT for conventional semen dilution treatments (30 and 3:1SD) were higher than all  $2.5 \times 10^6$  sperm/mL treatments, suggesting that the “dilution effect” may be a factor; however, by T48, PMI was similar among the 3:1SD, 30, and 2.5/0 treatments and TMOT was similar among the 3:1SD, 30, 2.5/0 and 2.5/7.5 treatments. Notably, at T48, the mean PMOT was highest in the 2.5/0 and 2.5/7.5 treatments. These findings suggest that while the more dilute samples appear to have slightly lower sperm quality at T0 (e.g. 75-78% PMI sperm vs. 81-82% for higher concentrations), the dilute samples maintain PMI, TMOT, and PMOT better over time than do more concentrated treatments. The high sperm quality in all the  $2.5 \times 10^6$  sperm/mL samples after 48 h conflicts with the concept of the “dilution effect.”

Historically, the “dilution effect” was studied using electrolyte solutions (e.g., sodium chloride, sodium citrate), seminal plasma, or combinations of the two [52-58,62,63,66,69,73]; however, it was subsequently questioned whether the decrease in sperm quality was due to diluent components or was a true “dilution effect” [74].

This study found that component combinations containing BSA, EDTA and/or fructose improved sperm longevity; whereas, single component diluents tended to depress motility. This difference in single vs. multiple component diluents may explain the “dilution effect.”

Assessment of sperm quality following high dilution has been primarily assessed using subjective measures of sperm motility, allowing for bias as sperm density on the microscope slide may not only affect the subjective evaluation of motility but will allow identification of the dilution treatment. To eliminate this bias, some studies diluted the treatments to a uniform concentration prior to analysis [55,57,62,70], whereas other studies did not [52,53,56,63,66,69]. Semen samples with higher concentrations could have been interpreted to have elevated motility as a result of motile sperm colliding with immotile sperm and giving the impression of motility. Makler et al. [73] evaluated human sperm motility in semen samples diluted 1:1 to 1:6 (semen volume to diluent volume) objectively using multiple exposure photography, and reported no deleterious effect of dilution rate on sperm motility. In that study, the neat semen concentrations were 12 to 210 x 10<sup>6</sup> sperm/ml (mean = 73 x 10<sup>6</sup> sperm/mL) which means that the evaluated concentrations were as high as 105 x10<sup>6</sup> sperm/mL and as low as 1.7 x 10<sup>6</sup> sperm/mL. These findings agree with those of the present study and stress the importance of objective measures of sperm motility.

In the 2.5 x 10<sup>6</sup> sperm/mL treatments, VCL and VAP increased as seminal plasma increased. These results are similar to previous studies by Texas workers [77,80] that found VCL was higher in 20 vs. 0% seminal plasma when semen was diluted to 25



$\times 10^6$  sperm/mL in a skim milk-glucose extender. While they did not evaluate VAP, they did report a similar relationship between seminal plasma content and VSL, a finding not demonstrated in the present study. It is unclear why the 3:1SD and 3:1/30 samples (also with 25% seminal plasma) did not have higher mean VCL and VAP than did the 2.5/7.5 and 2.5/25 at T24 and T48. It is possible that the relationship between seminal plasma is dependent on sperm concentration and that the effect sperm concentration has on pH and subsequently velocity. For example, in most species seminal plasma contains  $\sim 20$  mM bicarbonate, which may lead to an increase in medium pH and subsequently, sperm velocity over time with incubation in air [91]. Higher sperm concentration ( $30 \times 10^6$  vs.  $10 \times 10^6$ /mL), however, will lower diluent pH and thus may lower sperm velocity [92].

In Experiment 1, fertility was less in semen diluted to  $2.5 \times 10^6$  compared to  $5 \times 10^6$  sperm/mL, but this treatment also had the lowest number of sperm in the inseminate (0.5 vs. 1 million sperm, respectively). While  $2.5 \times 10^6$  sperm/mL is more dilute than  $5 \times 10^6$  sperm/mL, the latter is still very dilute and resulted in good fertility which suggests that, in Experiment 1, fertility differences are due to total sperm number rather than the “dilution effect.”

Previous studies reported that fertility was lower when mares were bred in the uterine body with  $250 \times 10^6$  PMS at concentrations of either  $2.5 \times 10^6$  sperm/mL in 100 mL or  $5 \times 10^6$  PMS/mL in 50 mL compared to  $25 \times 10^6$  sperm/mL in 10 mL [67,67]. Other factors that may have affected the fertility outcome include inseminate volume and the possibility of reflux of the semen through the cervix, resulting in a lower number of sperm remaining in the uterus.

In summary, based on sperm motion characteristics and plasma membrane integrity reported in this study, the conception of a “dilution effect” in a stallion at  $2.5 \times 10^6$  sperm/mL when diluted with INRA-T is not supported . These findings further suggest that there may be other factors such as diluent components, temperature, and evaluation method that have led to the concept of the “dilution effect.”

## CHAPTER IV

### SUMMARY AND CONCLUSIONS\*

A comparison of pregnancy rates between hysteroscopic (HYS) and transrectally guided deep-horn insemination (TRG) techniques was made using a subthreshold insemination dose of  $0.5 \times 10^6$  total sperm. This subthreshold dose was determined in a preliminary experiment using one stallion comparing the fertility of conventional UBI with  $\geq 1 \times 10^9$  sperm (16/18, 89%),  $1 \times 10^6$  (6/14, 43%), and  $0.5 \times 10^6$  (3/12, 25%) sperm. A subthreshold insemination dose was used to assure that study results would not be confounded by supra-threshold levels of sperm that could overcome the effect of insemination technique. The results of this study indicate that there was no difference detected between the TRG and HYS techniques when a subthreshold insemination dose of  $0.5 \times 10^6$  sperm was inseminated. The TRG technique reduces time factors, equipment expense, labor, and risk of possible nosocomial infection.

The sperm concentration used in Experiment 1 was low (i.e.  $2.5 \times 10^6$  sperm/mL) and there was concern that the “dilution effect” may have affected sperm quality (i.e. motility). Experiment 2 (Chapter III experiment) was designed to compare differences in sperm extended to  $2.5 \times 10^6$  sperm/mL to conventional dilution at either  $30 \times 10^6$  sperm/mL or 3:1 extender:semen. Initial values for total motility ranged from 71 to 90%;

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and after 48 h of cool-storage, values for the  $2.5 \times 10^6$  sperm/mL treatments declined less than the  $30 \times 10^6$  sperm/mL and 3:1 extender:semen treatments. These results suggest that while total motility for the  $2.5 \times 10^6$  sperm/mL treatments is initially lower than conventional dilution rates, long-term storage (i.e. 48 h) indicates that samples diluted to 3:1SD and 30 have a greater rate of decline than the  $2.5 \times 10^6$  treatments. These results suggest that the reduced fertility in the 0.5 million insemination dose ( $2.5 \times 10^6$  sperm/mL) may be due to the total number of sperm inseminated rather than the low sperm concentration.

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