

2008

Effects of centrifugation on equine spermatozoa immediately and after cooling for 24 hours

Jose Augusto Len

Louisiana State University and Agricultural and Mechanical College, jlen@vetmed.lsu.edu

Follow this and additional works at: https://digitalcommons.lsu.edu/gradschool_theses



Part of the [Veterinary Medicine Commons](#)

Recommended Citation

Len, Jose Augusto, "Effects of centrifugation on equine spermatozoa immediately and after cooling for 24 hours" (2008). *LSU Master's Theses*. 4302.

https://digitalcommons.lsu.edu/gradschool_theses/4302

This Thesis is brought to you for free and open access by the Graduate School at LSU Digital Commons. It has been accepted for inclusion in LSU Master's Theses by an authorized graduate school editor of LSU Digital Commons. For more information, please contact gradetd@lsu.edu.

**EFFECTS OF CENTRIFUGATION ON EQUINE SPERMATOZOA IMMEDIATELY AND
AFTER COOLING FOR 24 HOURS**

A Thesis

Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
In Partial fulfillment of the
Requirements for the degree of
Master of Science

In

The Interdepartmental Program in
Veterinary Medical Sciences
Through the Department of Veterinary Clinical Sciences

by

Jose Augusto Len
Veterinarian, University of Guadalajara, 1994
August 2008

ACKNOWLEDGEMENTS

The author wants to thank Drs. Bruce Eilts, Jill Jenkins, Dale Paccamonti and Robert Godke for their assistance and support conducting the research and preparing the manuscript. Special acknowledge to Dr. Jill Jenkins, for her invaluable help and ideas, motivation and sparkling personality during the conduction of the study. To Dr. Sara Lyle who provided great ideas and helped designing and conducting the study. The help provided by the LSU veterinary and animal sciences students and the personnel of the LSU Animal Science Department is gratefully acknowledged. To Eric Theall and Alexis Parrish from the National Wetlands Research Center, your help is also gratefully acknowledged. Dr. Giselle Hosgood collaborated with the statistical analysis of the data. The author also would like to thank his family for their unconditional love and support, specially his son Joshua Emmanuel as the source of inspiration. And last but not least, the author acknowledges the LSU Equine Health Studies Program for providing the funds for this study.

TABLE OF CONTENTS

ACKNOWLEDGEMENTS.....	ii
LIST OF TABLES.....	v
LIST OF FIGURES.....	vi
ABSTRACT.....	vii
CHAPTER 1: INTRODUCTION.....	1
CHAPTER 2: LITERATURE REVIEW.....	3
2.1. Stallion Spermatozoa.....	3
2.1.1. Spermatozoal Structure and Function.....	3
2.1.2. Spermatozoal Metabolism.....	6
2.1.3. Reactive Oxygen Species (ROS).....	7
2.2. Processing of Fresh Cooled Semen.....	9
2.2.1. Seminal Plasma.....	9
2.2.2. Seminal Plasma Dilution.....	10
2.2.3. Centrifugation and Sperm Recovery Rate.....	11
2.2.4. Passive Cooling.....	14
2.3. Semen Evaluation.....	14
2.3.1. Motility.....	16
2.3.2. Concentration.....	17
2.3.3. Morphology.....	17
2.3.4. Fluorescent Probes.....	18
CHAPTER 3: CENTRIFUGATION HAS MINIMAL EFFECTS ON MOTILITY, VIABILITY AND ACROSOME INTEGRITY OF EQUINE SPERMATOOZOA IMMEDIATELY AND AFTER COOLING FOR 24 HOURS	
3.1. Introduction.....	23
3.2. Materials and Methods.....	24
3.2.1. Semen Collection.....	24
3.2.2. Semen Processing Overview.....	24
3.2.3. Centrifugation.....	25
3.2.4. Concentration.....	26
3.2.5. Motility.....	26
3.2.6. Plasma Membrane Integrity (viability).....	27
3.2.7. Acrosome Reactivity.....	28
3.2.8. Recovery Rate After Centrifugation.....	29
3.2.9. Statistical Analyses.....	30

3.3. Results.....	31
3.4. Discussion.....	33
CHAPTER 4: CONCLUSIONS.....	40
REFERENCES.....	43
VITA.....	52

LIST OF TABLES

1. Effect of centrifugation force and time, and dilution rate of equine semen on sperm motility and recovery rates.....	15
2. Mean (\pm SD) equine (n = 6) spermatozoal total motility percentage (%) immediately (D0) after centrifugation and after cooling for 24 h (D1).....	31
3. Mean (\pm SD) equine (n = 6) spermatozoal progressive motility percentage (%) immediately (D0) after centrifugation and after cooling for 24 h (D1).....	31
4. Mean (\pm SD) equine (n = 6) spermatozoal viability percentage (%) immediately (D0) after centrifugation and after cooling for 24 h (D1).....	32
5. Mean (\pm SD) equine (n = 6) viable spermatozoa non-reacted acrosome percentage (%) immediately (D0) after centrifugation and after cooling for 24 h (D1).....	32
6. Mean (\pm SD) equine (n = 6) spermatozoal recovery rate percentage (%) after centrifugation at different centrifugation forces.....	33

LIST OF FIGURES

1. Drawing of an equine spermatozoon. Structural divisions: a) head, b) neck, c) mid-piece, d) principal piece, and e) end piece (Adapted from Varner, D.D.: From a Sperm's Eye View-Revisting Our Perception of This Intriguing Cell. Milne Lecture. AAEP Proceedings, 2007).....	4
2. Drawing of an equine spermatozoon head. Structural divisions: a) plasma membrane, b) outer acrosomal membrane, c) acrosome, d) inner acrosomal membrane, e) nuclear envelope, f) nucleus, g) post-acrosomal lamina, h) proximal centriole, i) axoneme, and j) mitochondria (Adapted from Varner, D.D.: From a Sperm's Eye View-Revisting Our Perception of This Intriguing Cell. Milne Lecture. AAEP Proceedings, 2007).....	5
3. Generation of hydrogen peroxide (H ₂ O ₂) by fresh equine spermatozoa during a 30-minute incubation at 38°C under aerobic conditions. Cell concentrations of 4 x 10 ⁶ (▲), 2 x 10 ⁶ (■), and 0.5 x 10 ⁶ (◆). (Adapted from Ball et al.: Generation of reactive oxygen species by equine spermatozoa. American Journal of Veterinary Research, 2001).....	8
4. Stallion spermatozoa morphology analyzed with phase contrast microscope (Olympus BX51, DP Controller Software).....	18
5. Stallion spermatozoa morphology and viability stained with eosin-nigrosin. Dead sperm stained red (arrow) (Olympus BX45, SPOT Software).....	19
6. Stallion spermatozoa stained with SYBR-14 and PI, imaged by epifluorescent microscopy. Viable sperm stained green (SYBR-14) and dead sperm stained red (PI) (Leitz DIAPLAN microscope, SPOT Diagnostic Instrument Inc. software).....	20
7. Stallion spermatozoa stained with fluorescence isothiocynate –PNA and PI, imaged by epifluorescent microscopy. Intact acrosome stained green (FITC-PNA) and dead spermatozoa red (PI) (Leitz DIAPLAN microscope, SPOT Diagnostic Instruments Inc. software).....	21
8. Flow chart presenting steps used in processing equine semen (n=6).....	27
9. Spermatozoa viability analysis by flow cytometry. Spermatozoal populations are gated. R2 viable spermatozoa (green) and R3 non-viable spermatozoa (red) (FACSCalibur®, CellQuest software).....	28
10. Spermatozoal acrosome integrity analysis by flow cytometry. Quadratic gating indentifies four spermatozoal populations. Upper left (UL) = dead sperm non-reacted	

acrosome, upper right (UR) = dead sperm reacted acrosome, lower left (LL) = live sperm non-reacted acrosome and lower right (LR) = live sperm reacted acrosome (FACSCalibur®, CellQuest software).....30

11. Schematic of the total number of motile, viable and non-reacted acrosomes spermatozoa immediately after centrifugation (D0) and after cooling for 24 h (D1), at different centrifugal forces for 10 min.....38

ABSTRACT

The objectives of this study were to determine the effects of centrifugation on equine sperm progressive motility, plasma membrane integrity (viability), and acrosome integrity. We hypothesized that high centrifugation forces would be detrimental to equine sperm, yet recovery rates would increase. Ejaculates from six stallions were collected, extended (INRA96) to a concentration of 25×10^6 cells/mL, and subjected for 10 min to 1) no centrifugation (NC); 2) 400 x g (400); 3) 900 x g (900); and 4) 4500 x g (4500). Before and after centrifugation (Day 0), and after 24 h of cooling (Day 1), sperm motility was assessed by computer assisted semen analysis, and samples were stained with SYBR-14/propidium iodide (PI) for viability, and with PI/fluorescent isothiocyanate-PNA (*Arachis Hypogaea*) for acrosome integrity and assessed by flow cytometry. Data were analyzed using Shapiro-Wilk's statistics; using a mixed linear model, effects of treatment and day were assessed. Compared with the other treatment groups the 4500 treatment group showed reduced motility, viability, and intact acrosomes ($P < 0.05$). The 400 and 900 treatment groups yielded lower recovery rates than the 4500 treatment group (NC = $100.0 \pm 0.0\%$, 400 = $54.4 \pm 8.6\%$, 900 = $75.0 \pm 7.1\%$ and 4500 = $97.9 \pm 2.8\%$) ($P < 0.05$). Centrifugation at 400 or 900 x g did not damage equine sperm. Further studies of centrifugal forces between 900 and 4500 x g are warranted to find optimal forces that maximize recovery rate, minimize sperm damage, and do not affect fertility.

CHAPTER 1: INTRODUCTION

The first work performed in artificial insemination (A.I.) was in horses. The Arabs are considered the first to practiced A.I. in horses. In 1322 the first recorded A.I. took place after an Arab chief stole semen from the stallion of a rival to inseminate his mares. The next recorded work was performed in Italy by Lazzaro Spalanzani in 1776. He showed that equine semen placed in snow did not die, but simply inactivated the sperm and that motility returned after warming.

Initially, A.I. in horses was used to overcome infertility problems in the United States by Pearson (1880) and in Europe by a French veterinarian named Repiquet (1890). The impact of A.I. on the horse breeding industry started when in two Danes, Sand and Stribolt postulated that A.I. should be used as a tool for breed improvement rather than for treating infertility. Consequently, the Russian royal stud in 1912 asked Ivanov to investigate A.I. on stud farms.

The interest for using of A.I. grew after the Russian's work and spread to different countries including Spain, Hungary, Greece, Australia, China and Japan. By far the country that embraced A.I. in horses the most was China. By 1959, the number of mares bred in China by A.I. was estimated to be approximately 600,000 (Bowen, 1969).

The use of A.I. is commonplace in today's equine breeding industry. Commonly, mares are inseminated with semen from a desired stallion that lives far away and the semen needs to be stored and transported. Equine semen for transportation can be fresh cooled or frozen. The most commonly used is fresh cooled semen. The preference for using fresh cooled semen is based on the higher pregnancy rates compared to rates with frozen-thawed semen insemination (Jasko D.J. et al., 1992b).

In equine, sperm motion characteristics have been lowered after storage with high concentrations of seminal plasma, whereas lowering concentrations has shown beneficial effects on motility (Jasko D.J. et al., 1991). Hence, the main goal during equine semen processing of either fresh cooled or frozen semen has been the dilution of seminal plasma. Seminal plasma dilution is performed by the addition of a semen

extender to the raw semen, or by centrifugation of extended semen, partial removal of the seminal plasma followed by sperm pellet re-suspension with fresh semen extender. Centrifugation of equine extended semen, partial removal of seminal plasma and sperm pellet re-suspension have been shown to be beneficial for sperm fresh cooled storage (Brinsko et al., 2000a).

However, the effects of centrifugation on equine spermatozoa have not been fully elucidated. Some studies have shown a detrimental effect on equine sperm motility (Cochran et al., 1984; Heitland et al., 1996; Jasko D.J. et al., 1991), whereas others have shown no detrimental effects on either equine sperm motility or viability (Ferrer et al., 2004; Jasko D.J. et al., 1992a). Because of the inadequate understanding of the effects of centrifugation on equine sperm, the equine breeding industry uses conservative centrifugation forces (400 to 650 x g) for semen processing. However, these commonly used centrifugation forces cause a loss of 20 – 30% of the total number of spermatozoa. Thus, the processing of equine semen could be better optimized.

The objective of this study was to evaluate the effect of different centrifugal forces on equine sperm total and progressive motility, viability, and acrosomal integrity immediately after centrifugation and after cooling for 24 hours. Additionally, the sperm recovery rates at these different centrifugal forces were recorded.

CHAPTER 2:

LITERATURE REVIEW

2.1. Stallion Spermatozoa

Spermatozoa are unique among cells as they possess specialized functions and limited repair capacity (Amann and Graham, 1993). The production of spermatozoa (spermatogenesis) takes place in the seminiferous tubules of the testes; thereafter they are released into the rete testis and enter the epididymus. During their transit through the epididymus, spermatozoa obtain fertilizing capacity and are stored at the cauda epididymus until ejaculation.

2.1.1. Spermatozoal Structure and Function

Stallion spermatozoa are divided into five structural regions (Fig. 1): head, neck, middle piece, principal piece, and end piece (Barth and Oko, 1989). Similar structures are described for bull, ram, boar, dog and human spermatozoa. Overlying these structures is the dynamic plasma membrane (Amann and Graham, 1993).

The spermatozoal head is elliptical, flattened dorsoventrally and thicker in the posterior portion (Johnson et al., 1978; Varner et al., 2000). It can be subdivided into the nucleus, nuclear envelope, acrosome, equatorial segment, post acrosomal region, and posterior ring (Varner and Johnson, 2007). The nucleus occupies most of the head, and contains highly condensed chromatin in association with protamines. Compaction and stabilization of the DNA is a result of intra- and inter-molecular disulfide linkages of the cysteine residues from protamines (spermatozoal proteins). The nucleus is separated from the surrounding cytoplasm by the double-layered nuclear envelope (Fig. 2) (Varner and Johnson, 2007).

The acrosome is a Golgi-derived membrane-bound vesicle formed during an early stage of spermiogenesis. Located in the rostral portion of the nucleus (bou-Haila and Tulsiani, 2000; Toshimori and Ito, 2003) it is subdivided into inner and outer acrosomal membranes, enclosing the acrosomal matrix. Composition of the inner- and

outer acrosomal membranes includes phospholipids, proteins and cholesterol. The ratios of protein to phospholipids are higher, and cholesterol to phospholipids similar

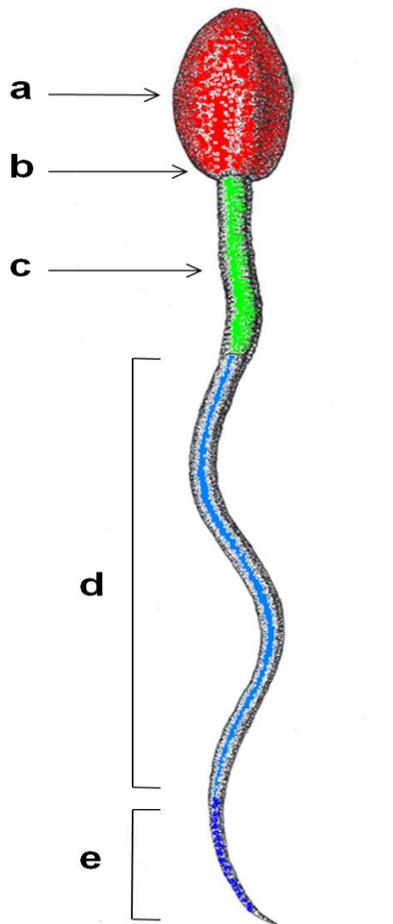


Figure 1. Drawing of an equine spermatozoon. Structural divisions: a) head, b) neck, c) mid-piece, d) principal piece, and e) end piece. (Adapted from Varner, D.D.: From a Sperm's Eye View-Revisting Our Perception of This Intriguing Cell. Milne Lecture. AAEP Proceedings, 2007).

when compared to that of the plasma membrane (Parks et al., 1987). The outer acrosomal membrane at the equatorial segment is highly fusogenic, and fuses with the overlying plasma membrane during the acrosome reaction. After the sperm-oocyte fusion, the spermatozoa undergoes the acrosome reaction, releasing hydrolytic enzymes (hyaluronidase, proacrosin/acrosin and lipases) from the acrosome matrix. These enzymes are necessary to penetrate the oocyte zona pellucida during fertilization (Abou-Haila and Tulsiani, 2000).

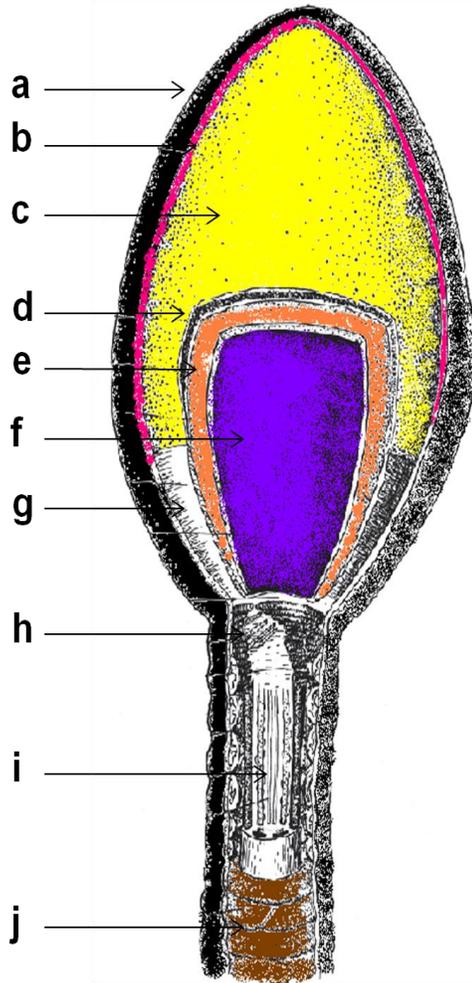


Figure 2. Drawing of an equine spermatozoon head. Structural divisions: a) plasma membrane, b) outer acrosomal membrane, c) acrosome, d) inner acrosomal membrane, e) nuclear envelope, f) nucleus, g) post-acrosomal lamina, h) proximal centriole, i) axoneme, and j) mitochondria. (Adapted from Varner, D.D.: From a Sperm's Eye View-Revisting Our Perception of This Intriguing Cell. Milne Lecture. AAEP Proceedings, 2007).

The neck is the connecting piece between the head and the middle piece and it consists of the capitulum, segmented columns and the proximal and distal centrioles (Varner and Johnson, 2007). The head is articulated by the capitulum at the implantation fossa. The segmented columns anchor the flagellum. The proximal centriole remains in the mature spermatozoa and is the site where the tail beat is initiated (Amann and Graham, 1993). The distal centriole disappears in the mature spermatozoa and gives rise to the axoneme.

The middle piece is characterized by the presence of numerous mitochondria overlying the dense fibers and the axoneme. Mitochondria are helicoidally arranged in a continuous double spiral. Stallion spermatozoa have about 40 - 50 gyres. They are the

production plant for ATP necessary for spermatozoal function and motility (Varner and Johnson, 2007).

The principal piece is composed of the fibrous sheath and axoneme of the middle piece, which continues from the annulus (demarcates the end of the middle piece) and extends along the entire length of the principal piece and terminates in the caudal principal piece. The fibrous sheath provides structural support and flexibility to the flagellum (Amann and Graham, 1993; Varner and Johnson, 2007).

The plasma membrane overlies all of the spermatozoal structures. Its main function is to fuse with the oocyte oolemma after it undergoes transformation during capacitation (Bearer and Friend, 1990; Flesch and Gadella, 2000). At the equatorial segment the plasma membrane fuses with the oocyte (Amann and Graham, 1993; Bearer and Friend, 1990). The stallion spermatozoal plasma membrane components are similar to other species (bull, ram, rooster), consisting of lipids, proteins and cholesterol. Lipids, the major component, include; phospholipids, neutral lipids and glycolipids (Flesch and Gadella, 2000; Parks and Lynch, 1992) with phospholipids being the predominant class. The phospholipids are arranged in a bilayer; within the inner layer are the aminophospholipids phosphatidylserine (PS) and phosphatidylethanolamine (PE), and located within the outer layer are the choline phospholipids sphingomyelin (SM) and phosphatidylcholine (PC) (Flesch and Gadella, 2000; Gadella and Harrison, 2000). The molar ratio of cholesterol to phospholipids in stallions (0.36) is intermediate between the bull (0.45) and boar (0.26) (Parks and Lynch, 1992). The protein to phospholipid ratio in the stallion is low (0.80) (Parks and Lynch, 1992), which confers a lower sensitivity to cold shock, compared to the boar having a highest protein to phospholipid ratio (1.26) and a higher cold shock sensitivity. Cholesterol is a structural component and maintains plasma membrane stability.

2.1.2. Spermatozoal Metabolism

Spermatozoa function by fertilizing oocytes, and spreading genetic material distant from they were produced. Spermatozoa are one of the smallest cells in the body, and do not possess energy storage (Varner and Johnson, 2007). Spermatozoa use

adenosine triphosphate (ATP) as the basic energy source and require a constant supply for cell function and survival. Energy demands vary depending on spermatozoa activity, such as; hyperactivation, acrosome reaction, sperm-oocyte fusion, and motility (Miki, 2007). To supply energy demands, spermatozoa rely on an extracellular substrate. Spermatozoa principally utilize carbohydrates to meet their energy requirements. Metabolizable carbohydrates cross the plasma membrane using protein transporters (ATP-dependent process). Spermatozoa are capable of producing ATP through oxidative phosphorylation, however glycolysis can be considered the central metabolic pathway for energy supply (Miki, 2007; Varner and Johnson, 2007). Glucose metabolized through glycolysis produces 2 molecules of pyruvate (ATP and NADH). Pyruvate is then oxidized by mitochondria to produce 36 ATP molecules (Miki, 2007). Glycolysis operates under both aerobic and anaerobic modes (Storey and Kayne, 1975), whereas oxidative phosphorylation occurs strictly under aerobic conditions. Other substrates can be utilized for energy metabolism, including pyruvic acid, lactic acid, fatty acids and amino acids (Varner and Johnson, 2007). Bull and ram spermatozoa readily metabolize fructose using protein transporters other than the glucose protein transporter (Hiipakka and Hammerstedt, 1978). Conversely, stallion spermatozoa have only a limited capacity to metabolize fructose, possibly due to the absence of a fructose transport protein (Amann and Graham, 1993). The majority of ATP produced by equine spermatozoa is used to maintain motility (Amann and Graham, 1993; Williams and Ford, 2001).

2.1.3. Reactive Oxygen Species (ROS)

Spermatozoa under aerobic conditions will produce reactive oxygen species (ROS) as sub-products of aerobic respiration (Aitken, 1995; Twigg et al., 1998), causing defects in spermatozoal structure and function. In stallion spermatozoa the primary ROS is the superoxide anion (O_2^-) which is rapidly catalyzed to hydrogen peroxide (H_2O_2). The amount of ROS produced in stallion semen is dependent on spermatozoal concentration (Fig. 3). Morphologically abnormal spermatozoa produce greater amounts of ROS than morphologically normal spermatozoa, and this may account for the

reduced fertility after storage of semen with high percentage of morphological abnormal spermatozoa (Ball et al., 2001).

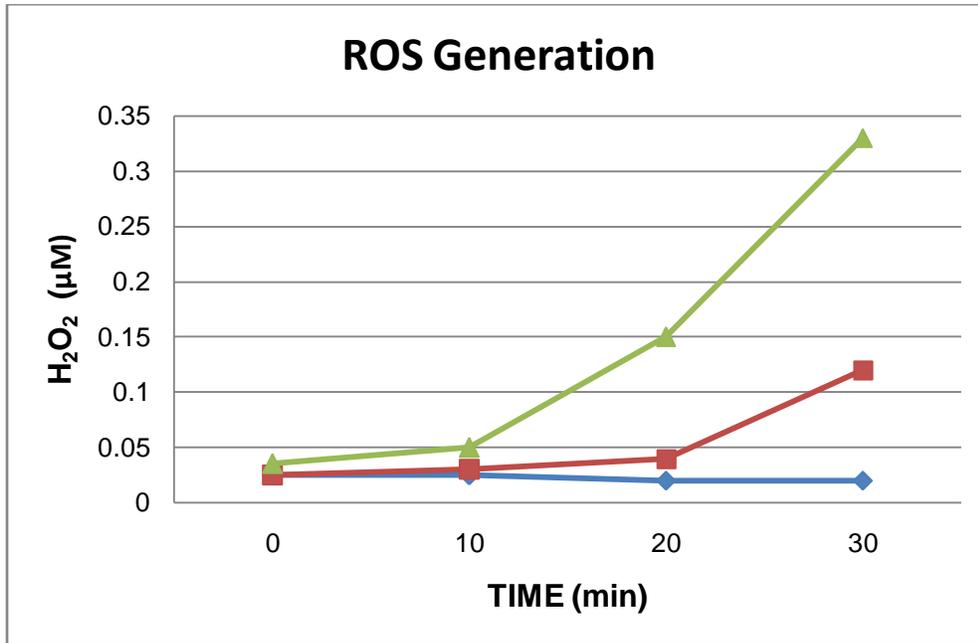


Figure 3. Generation of hydrogen peroxide (H₂O₂) by fresh equine spermatozoa during a 30-minute incubation at 38°C under aerobic conditions. Cell concentrations of 4 x 10⁶ (▲), 2 x 10⁶ (■), and 0.5 x 10⁶ (◆). (Adapted from Ball et al.: Generation of reactive oxygen species by equine spermatozoa. American Journal of Veterinary Research, 2001).

Effects of ROS on spermatozoal function include decreased motility (Aitken et al., 1993; Baumber et al., 2000; de Lamirande and Gagnon, 1992), lipid peroxidation (Aitken, 1995), and reduced spermatozoal viability and acrosome integrity (Aitken et al., 1993; de Lamirande and Gagnon, 1992), and increased DNA fragmentation (Baumber et al., 2003a; Lopes et al., 1998). Reduced viability and acrosome integrity have been shown in mouse and ram spermatozoa (Baiardi et al., 1997; Peris et al., 2007). In humans, ROS caused spermatozoa DNA fragmentation resulting in reduced fertility or infertility (Hughes et al., 1996). The viability and acrosome integrity of fresh stallion spermatozoa was not affected by ROS after incubation for 30 min in a xantine-xantine oxidase system (X-XO) that generates both O₂⁻ and H₂O₂. However, ROS caused a detrimental effect on motility (Baumber et al., 2000). Perhaps the incubation of stallion

spermatozoa for a longer period in ROS will affect the viability and acrosome integrity, although this has not been investigated. The plasma membrane composition makes it very susceptible to lipid peroxidation by ROS, causing a reduction in plasma membrane fluidity needed for sperm motility and sperm-oocyte fusion (Aitken, 1995; Storey, 1997). Stallion spermatozoa are very resistant to lipid peroxidation and only when they are exposed to high concentrations of ROS, lipid peroxidation occurs (Neild et al., 2005). Stallion spermatozoa incubated for 1 hr in a X-XO system resulted in DNA fragmentation (Baumber et al., 2003a) that may lead to reduced fertility (Love and Kenney, 1998).

Paradoxically, spermatozoa are vulnerable to oxidative stress, but at the same time, low concentrations of ROS are required for sperm maturation, capacitation and binding to the zona pellucida (Baker and Aitken, 2004; Ford, 2004; O'Flaherty et al., 2006; Parinaud et al., 1997). Reactive oxygen species promote and regulate the protein tyrosine phosphorylation in equine (Baumber et al., 2003b), bull (Rivlin et al., 2004) and human (O'Flaherty et al., 2006) spermatozoa, which is associated with sperm capacitation.

2.2. Processing of Fresh Cooled Semen

2.2.1. Seminal Plasma

Seminal plasma is a mixture of epididymal and accessory sex gland secretions in which sperm cells become suspended during ejaculation. The pH of raw stallion semen is approximately 7.6 ± 0.3 (Dowsett and Knott, 1996), and the osmolality ranges from 310 to 320 mOsm (Griggers et al., 2001). Seminal plasma is composed of minerals, proteins (Amann et al., 1987), enzymes (Pesch et al., 2005) carbohydrates (Mann, 1975), hormones (Troedsson et al., 1998) and antioxidants (Potts et al., 1999).

Seminal plasma plays an important role in spermatozoal transport and survival within the mare's uterus, as well as elimination of spermatozoa from the mare's uterus (Troedsson et al., 2005). During ejaculation, spermatozoa are coated with proteins from the seminal plasma which suppress uterine polymorphonuclear neutrophils (PMNs) from binding and phagocytosing them (Alghamdi et al., 2004; Katila, 2001; Troedsson

et al., 2005). Seminal plasma also appears to regulate spermatozoal transport into the oviducts (Troedsson et al., 2005), by decreasing uterine contractions following insemination and then increasing uterine contractions four hours post-insemination (Portus et al., 2005). The beneficial effects of seminal plasma are accomplished after contact with spermatozoa for a short time, and seminal plasma may not be needed later, because its absence in an insemination dose does not appear to have a negative effect on pregnancy rates (Portus et al., 2005).

Storage of equine spermatozoa in high concentrations of seminal plasma (>20% v/v) decreased spermatozoal motility (Jasko D.J. et al., 1991; Varner et al., 1987) and caused DNA fragmentation (Love et al., 2005). Deleterious effects on spermatozoa may be due to high concentrations of ROS having accumulated in the seminal plasma from spermatozoal metabolism. During equine semen processing, the approach commonly used to overcome any deleterious effects exerted by seminal plasma is to dilute semen by adding a semen extender or by centrifuging the extended semen followed by partial removal of the seminal plasma. After seminal plasma dilution, the extended semen is placed in a passive cooling device to decrease spermatozoal metabolism and consequently ROS production.

2.2.2. Seminal Plasma Dilution

Seminal plasma is not an ideal medium for equine spermatozoa storage (Jasko D.J. et al., 1991; Moore et al., 2005; Varner et al., 1987). To preserve the fertilizing capacity of stored spermatozoa after ejaculation, it is recommended to add a semen extender to the raw semen (Varner, 1986). Semen extenders dilute the seminal plasma concentration, control pH and osmolality, supply nutrients and antimicrobials, and protect spermatozoa against cold shock during storage. Skim milk based semen extenders are commonly used to dilute raw semen. Alternatives to skim milk based semen extenders are the purified milk fraction semen extenders. The native phosphocaseinate based semen extenders have been shown to improve in vitro spermatozoal preservation and pregnancy rates per cycle, as compared with the skim milk based semen extenders (Batellier et al., 2001).

When diluting raw semen, it is recommended that two criteria are met, at least a 1:4 dilution (one part semen : 3 parts extender) (Jasko D.J. et al., 1991; Varner, 1986) and a final cell concentration between 25 to 50 x 10⁶/mL (Varner et al., 1987). In some cases however, even after meeting these criteria, inadequate spermatozoa preservation for 24 hours is not achieved. A population of stallions known as “poor coolers” do not benefit from dilution of seminal plasma for fresh cooled semen shipment (Brinsko et al., 2000a). In other situations, dilution of stallion semen with low sperm concentration (oligospermic) using the mentioned criteria may provoke the loss of spermatozoal motility and viability (“dilution effect”).

2.2.3. Centrifugation and Sperm Recovery Rate

Partial removal of the seminal plasma after centrifugation is thought to remove ROS and other components that may cause sperm damage, providing a more balanced medium for spermatozoal survival during storage after re-suspension with a semen extender. The effects of centrifugation on spermatozoa from different species vary. After centrifugation, fertilization by bull spermatozoa was decreased as compared to that by non-centrifuged cells (Pickett et al., 1975). Human spermatozoa do not suffer an immediate decrease in motility, instead motility loss is observed after a period of time, possibly caused by sub-lethal damage induced by centrifugation (Alvarez et al., 1993). Conversely, ram spermatozoal survival was improved after centrifugation and partial removal of seminal plasma from fresh semen (Ritar and Salamon, 1982). Stallion spermatozoal motility of cooled, stored semen was greatly improved after centrifugation and partial removal of seminal plasma (Jasko D.J. et al., 1991). The “poor coolers” stallion population also benefited from centrifugation, partial removal of the seminal plasma and re-suspension in semen extender (Brinsko et al., 2000a).

The effects of centrifugation on spermatozoa, removal of seminal plasma, and re-suspension of extended semen appears to be influenced by many factors. These factors include time of centrifugation, centrifugal forces, dilution rate, semen extender type and amount of seminal plasma retained. The inherent stallion individual variability also influenced the effects of centrifugation. Due to this multitude of factors, the effect of centrifugation on equine spermatozoa has not been fully elucidated. Consequently,

common centrifugation forces used to process equine semen are usually low (400 to 650 x g) (Cochran et al., 1984).

In evaluating the effects of centrifugation, the most common parameter used is sperm motility. Pickett et al. (1975) showed that undiluted semen centrifuged at 370 or 829 x g for five min had no detrimental effects on sperm motility. Later, Martin et al. (1979) showed that centrifugation of undiluted semen at 1000 x g for 5 min caused detrimental effects on spermatozoal motility.

Equine semen diluted (1:3 dilution) in Tris aminomethane, glucose, glycerol, citric acid and egg yolk extender centrifuged at 370 or 829 x g for five minutes and re-suspended had no detrimental effect on spermatozoa motility, if the re-suspended semen contained 10% of seminal plasma. Similar results were obtained when diluted semen (50×10^6 sperm/mL) in a skim milk extender was centrifuged at 400 x g for 9 min and the re-suspended semen contained 5 to 10% seminal plasma (Jasko D.J. et al., 1992a). Conversely, Jasko et al. (1991) showed that spermatozoal motility from semen extended at 50×10^6 sperm/mL and centrifuged at 500 x g for 18 min was significantly decreased when seminal plasma was not removed. Cochran et al. (Cochran et al., 1984) centrifuged equine semen extended at 50×10^6 sperm/mL in a citrate-ethylene diamine tetraacetic acid (EDTA) extender at 400 x g for 10 min and at 650 x g for 15 min. They found that progressive motility after centrifugation was not adversely affected, and even increased over time. In the same study however, centrifugation of semen extended with glucose-EDTA at a 1:1 ratio and centrifuged at 650 x g for 15 min adversely affected spermatozoal progressive motility. In a recent study (Kareskoski et al., 2006), fractionated ejaculates were diluted with a skim-milk semen extender at a 1:1 ratio and centrifuged at 500 x g for 15 min. Centrifugation adversely affected total and progressive motility in each fraction of the ejaculate, except when sperm concentration after dilution was between 2 to 26×10^6 sperm/mL.

The possible deleterious effects of centrifugation on spermatozoa may be caused by physical forces. In addition, applying higher centrifugal forces will cause a higher compaction of the sperm pellet (Cochran et al., 1984). Methods that have been used to minimize these possible outcomes after centrifugation consist of providing a “cushion”

during centrifugation of extended semen. Initially, a glucose-EDTA cushion was used and a softer sperm pellet was obtained, however sperm motility post-centrifugation was lower compared to extended semen centrifuged without a “cushion” (Cochran et al., 1984). Later, a cushioned technique involving a dense solution of iodixanol in water was used and a softer pellet was obtained after centrifugation without causing a decrease in sperm motility (Revell SG et al., 1997). Recently, centrifugation of stallion sperm diluted into a dense, inert, isotonic solutions has been used to obtain a softer sperm pellet and increase sperm recovery rates without decreasing motility (Knop et al., 2005). These later techniques of cushioned centrifugation show promising results, however their use is still limited.

To measure the effect of various times of centrifugation, Heitland et al. (Heitland et al., 1996) assessed motility after using the same centrifugal force, dilution, and semen extender, with different centrifugation times. Results from this study showed that time of centrifugation has minimal effects when semen extended (HEPES Buffered Sugar) to a concentration of 50×10^6 sperm/mL was centrifuged at $400 \times g$ for up to 16 min. Thereafter, sperm motility was detrimentally affected.

Following centrifugation, seminal plasma removal and re-suspension, the number of sperm cells recovered are of great importance. Losses of sperm cells in the supernatant decrease the total number of cells available for processing, and possibly the number of doses available for insemination. Commonly, a 40 mL volume of extended semen is centrifuged in a 50 mL conical centrifuge tube. Centrifugation in a 20 mL centrifuge tube (Cochran et al., 1984) or a 20 mL volume in a 40 mL centrifuge tube (Ferrer et al., 2004) of extended semen yielded higher sperm recovery rates; however this may be impractical due to typically large volume, and in some instances the high sperm numbers in the ejaculated equine semen. Recovery rates reported using the commonly used centrifugation forces ($400 - 650 \times g$) yielded a loss of spermatozoa in the removed supernatant of 20 – 30 % (Cochran et al., 1984; Ferrer et al., 2004; Heitland et al., 1996) of the total number of spermatozoa before centrifugation. With the intent to increase the number of spermatozoa recovered after centrifugation, Ferrer et al. (Ferrer et al., 2004) used a centrifugation force of $900 \times g$ for 10 min and recovered

91% of the total number of cells prior to centrifugation without detrimental effects on spermatozoal motility and viability.

In summary, centrifugal forces up to 900 x g may not be detrimental to sperm motility if the semen is diluted to a concentration of 25 to 50 x 10⁶ sperm/mL and the centrifugation time does not exceed 10 minutes. A summary of the effects of centrifugation and recovery rates from the scientific literature are presented in Table 1.

2.2.4. Passive Cooling

After raw semen is prepared by adding semen extender or by centrifugation and partial removal of seminal plasma follow by re-suspension, the extended semen is placed in a passive cooling device under anaerobic conditions. Cooling of the extended semen under anaerobic conditions decreases the sperm metabolic rate and reduces the aerobic metabolic pathways (Batellier et al., 2001). In consequence a balanced medium that preserves spermatozoa viability and motility is maintained.

Stallion extended semen can be cooled rapidly from 37°C to 20°C without causing damage to the spermatozoa (Kayser et al., 1992). Slow cooling below 20°C is recommended to avoid “cold shock” on equine spermatozoa (Varner et al., 1988). Passive cooling devices are designed to slowly cool extended semen at a rate of - 0.05°C/min (Brinsko et al., 2000b), and to maintain a temperature between 4 to 6°C, the optimal temperature to preserve spermatozoa of most stallions for 24 hours (Moran et al., 1992; Varner et al., 1988).

2.3. Semen Evaluation

Conventional evaluation of raw semen includes gel-free volume, spermatozoa concentration, motility and morphology. Although these evaluated parameters provide much information, their correlation with stallion fertility has not been demonstrated. Semen evaluations are helpful to differentiate between good and poor semen quality; however a good semen quality is not a guarantee of acceptable fertility (Colenbrander et al., 2003)

Table 1. Effect of centrifugation force and time, and dilution rate of equine semen on sperm motility and recovery rates

Author	Centrifugation Force (x g)	Time (min)	Dilution	Extender	Effect on motility	Recovery Rate (%)
Pickett et al. 1975	370 or 829	5	1:3 or undiluted	EYT	None	
	956	5	Undiluted		Decreased	
Martin et al. 1979	1000	5	Diluted	EDTA	None	
		5	Undiluted		Decreased	
Cochran et al. 1984	400	10	50 x 10 ⁶ sperm/mL	Citrate-EDTA	None	67
	650	15	50 x 10 ⁶ sperm/mL	Citrate-EDTA	None	70
	650	15	1:1	Glucose-EDTA	Decreased	
Jasko et al. 1991	500	18	50 x 10 ⁶ sperm/mL	Skim-milk	Decreased	
Jasko et al. 1992	400	9	25 x 10 ⁶ sperm/mL	Skim-milk	Decreased	
Heitland et al. 1996	400	4	50 x 10 ⁶ sperm/mL	HBS	None	48
		6			None	48
		8			None	68
		10			None	81
		12			None	80
		14			None	86
		16			None	83
		20			Decreased	87
		24			Decreased	95
Ferrer et al. 2004	900	10	25 x 10 ⁶ sperm/mL	Skim-milk	None	91
Kareskoski et al. 2006	500	15	1:1	Skim-milk	Decreased	

Sperm structures and functions commonly evaluated with fluorescent stains include plasma membrane integrity or viability (Garner and Johnson, 1995), acrosome integrity (Cross and Meizel, 1989; Graham et al., 1990), mitochondrial respiration (Graham et al., 1990), and DNA integrity (Evenson and Jost, 1994). Evaluation of different sperm structures and functions may allow a better prediction of the sperm fertilizing capacity (Wilhelm et al., 1996).

2.3.1. Motility

Spermatozoal motility has been considered the major criterion to assess fertility in males. The traditional assessment of spermatozoal motility has been very subjective because of the great variability of assessment that exists among observers. Using light microscopy, a drop of raw semen is placed on a pre-warmed slide and covered with a cover slip. The observer then estimates spermatozoal motility (e.g. total motility and progressive motility). Stallion raw semen may exhibit a high sperm concentration that may lead to an overestimation of sperm motility.

To overcome the subjective assessment, application of a computerized assisted semen analyzer (CASA) offers an objective and rapid approach to assess (Malmgren, 1997). In addition to the total and progressive motility, the CASA system measures other spermatozoal motion characteristics such as straight-line velocity (VSL), curvilinear velocity (VCL), average path velocity (VAP), linearity (LIN), straightness (STR), circularly motile spermatozoa (CIR), lateral head displacement (LHD), and beat cross frequency (BCF).

Analysis of sperm motion characteristics using the CASA system aids reducing the subjectivity and variability, and offers information regarding the speed and way in which the sperm moves, compared to the traditional assessment using light microscopy; however, no correlations between total motility and speed in which the sperm moves and the way they move have been made (Love et al., 2003). In addition, the relation between sperm motion characteristics and fertility is still unclear. A French study suggests that spermatozoa with rapid motility and high VAP have greater fertility

(Vidament, 2005); however, Kirk et al. (Kirk et al., 2005) indicated that VAP was not a reliable or repeatable parameter to analyze sperm quality.

2.3.2. Concentration

Spermatozoal concentrations in raw semen can be estimated using a Neubauer hemocytometer or a spectrophotometer. To estimate concentrations using a hemocytometer, raw semen is diluted to a 1:100 dilution with formol-buffered saline (FBS). The hemocytometer is loaded with the diluted semen. Thereafter the hemocytometer is examined at 400x and all the sperm heads counted within the 25 central squares represents the sperm concentration in millions/mL.

To estimate spermatozoa concentration using a spectrophotometer, a semen sample is placed in a spectrophotometer, and the optical density (O.D.) of the sample is analyzed at the appropriate wavelength. The O.D. of the sample is compared against a pre-established hemocytometer-derived standard curve to obtain the spermatozoal concentration (Jasko, 1992). The presence of debris, white blood cells and red blood cells can increase the O.D., which may result in erroneous spermatozoa concentration (Jasko, 1992). Estimation of concentration using extended semen or hemospermic semen samples is therefore not recommended.

After the raw semen concentration is estimated, the total number of spermatozoa in the ejaculate is calculated. The total number of spermatozoa is obtained by multiplying the spermatozoa concentration times the volume of gel-free semen.

2.3.3. Morphology

An important parameter measured to assess sperm quality is cellular morphology. Abnormalities in sperm morphology affect sperm fertilizing capacity. Spermatozoal morphology can be assessed by using an unstained or stained semen sample. The method preferred by the Society for Theriogenology for sperm morphology assessment is the fixed unstained method (Jasko, 1992). For the unstained method, raw semen is fixed with FBS. A drop of the fixed semen is placed on a slide covered with a cover slip, and the sample is examined under a phase contrast microscope at

1000x total magnification (Fig. 4). For the stained method, the most common combination of stains used is eosin-nigrosin (Jasko, 1992). A drop of eosin-nigrosin is mixed with raw semen on a slide. The mixture is smeared, and the morphology is examined under a light microscope at 1000x total magnification (Fig. 5). The eosin-nigrosin stain also has been used to assess sperm viability. The eosin is a membrane-impermeant dye, thus sperm with leaky membranes will stain, thus assumed dead, and live sperm will not. The disadvantage of these methods is that staining and smearing sperm may alter their morphology (Voss et al., 1981), such as changes in sperm head size and increased detached heads (Dott, 1975).



Figure 4. Stallion spermatozoa morphology analyzed with phase contrast microscope (Olympus BX51, DP Controller Software).

2.3.4. Fluorescent Probes

Assessment of sperm morphology using light microscopy provides important information on semen samples; however, the structural integrity of sperm organelles essential for fertilization can be evaluated better by using specific fluorescent probes. The integrity of the plasma membrane, acrosome, DNA and mitochondrial activity can be assessed using fluorescent probes. Levels of staining can be assessed by

fluorescent microscopy or flow cytometry, two technologies known for sensitivity. Bull and ram sperm plasma membrane integrity were initially assessed using a combination of carboxyfluorescein diacetate (CFDA) and PI (Harrison and Vickers, 1990). Later, Garner et al. (Garner and Johnson, 1995) used a combination of nucleic acid stains



Figure 5. Stallion spermatozoa morphology and viability stained with eosin-nigrosin. Dead sperm stained red (arrow) (Olympus BX45, SPOT Software).

(SYBR-14 and PI), to assess the viability of bull, boar, ram, rabbit, mouse and human spermatozoa. The SYBR-14 is a membrane-permeant nucleic acid stain, whereas PI that also stains nucleic acids cannot penetrate intact plasma membranes, similar to the eosin stain. Sperm cells that stain with SYBR-14 are viable (green) and the ones that stain with PI have a damaged plasma membrane or are non viable (red). The proposed working mechanism of the dual staining is that all cells stain with SYBR-14, as they die they permit the entrance of the membrane-impermeant dye (PI), which replaces the SYBR-14 dye from the nuclear DNA (Fig 6).

Approaches that evaluate sperm acrosomes using fluorescent microscopy require tedious staining and results have been subjective. Plant lectins are proteins that bind to specific sequences of carbohydrates. *Pisum sativum* (PSA) and *Arachis hypogea* (PNA) lectins have been used to evaluate human (Cross and Meizel, 1989),

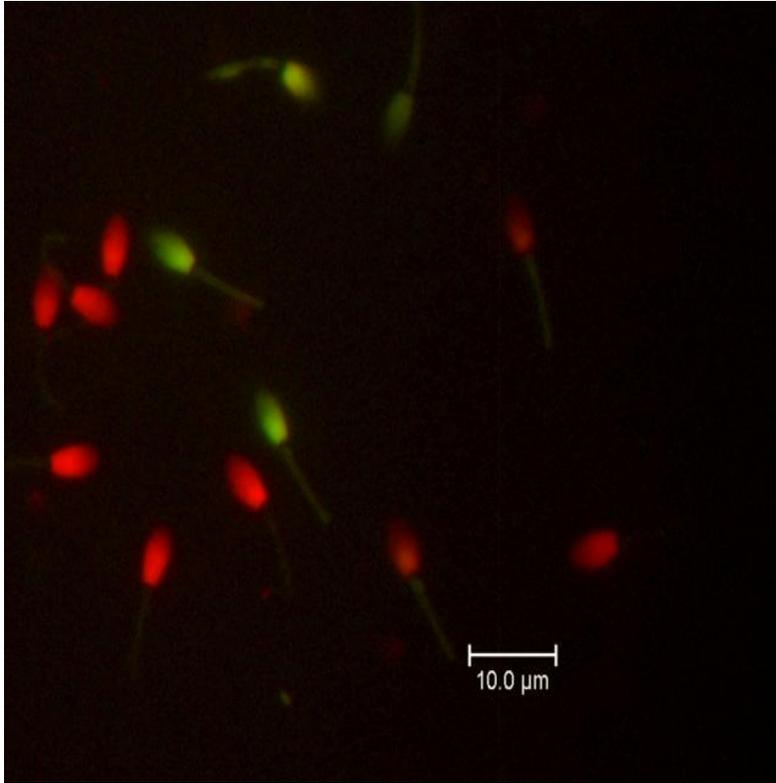


Figure 6. Stallion spermatozoa stained with SYBR-14 and PI, imaged by epifluorescent microscopy. Viable sperm stained green (SYBR-14) and dead sperm stained red (PI) (Leitz DIAPLAN microscope, SPOT Diagnostic Instrument Inc. software).

bull and ram (Graham et al., 1990), and stallion (Cheng et al., 1996; Farlin et al., 1992) acrosomes. Lectins conjugated with fluorescent probes such as fluorescein isothiocyanate (FITC), mark the target for visualization by fluorescent microscopy or flow cytometry. The PNA lectin is preferred over PSA because its binding is less non-specific and is limited to the outer acrosomal membrane (Cheng et al., 1996). The PNA lectin binds to β -galactose moieties of the outer acrosomal membrane of reacted or those undergoing the acrosome reaction; therefore, sperm with non reacted acrosome will not bind the FITC-PNA conjugate (no fluorescence), whereas sperm with reacted acrosome will bind FITC-PNA conjugate (fluorescence) (Rathi et al., 2001) (Fig. 7).

Analysis of DNA strand breakage may be performed by using several methods including gel electrophoretic assay (Comet assay) (Evenson et al., 2007; Singh et al., 1989) or flow cytometry (Tunel, Sperm Chromatin Structure Assay and Acridine orange)

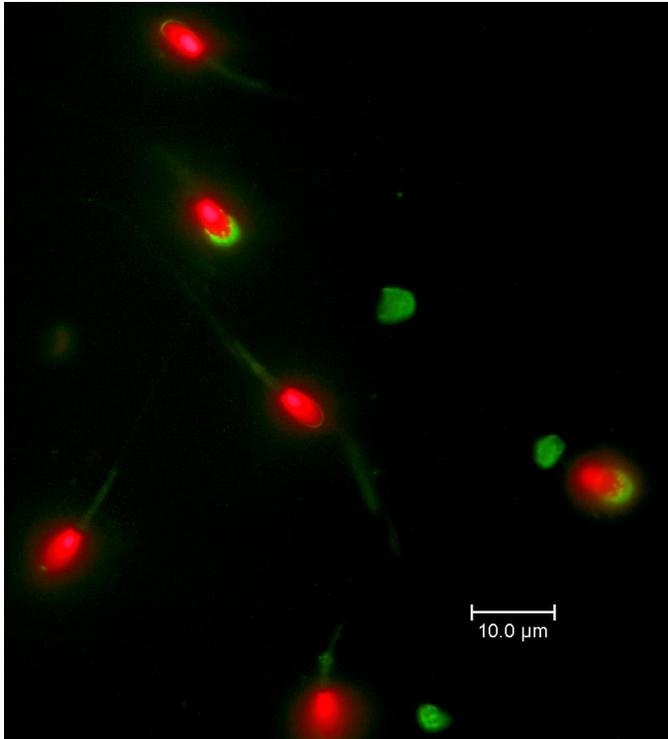


Figure 7. Stallion spermatozoa stained with fluorescence isothiocyanate –PNA and PI, imaged by epifluorescent microscopy. Intact acrosome stained green (FITC-PNA) and dead spermatozoa red (PI) (Leitz DIAPLAN microscope, SPOT Diagnostic Instruments Inc. software).

(Evenson and Wixon, 2006). In the Comet assay, sperm cells are lysed and subjected to horizontal electrophoresis. The unbroken DNA remains in the sperm head, while the broken DNA migrates to take a comet form. With the TUNEL assay, the intensity of fluorescence of each sperm is considered positive or negative to DNA fragmentation. Acridine Orange (AO) staining and the Sperm Chromatin Structure Assay (SCSA) (Evenson and Jost, 1994) share the same principle of physical DNA denaturation. Both methods use the metachromatic dye AO, the difference in the assays is the method used to cause DNA denaturation. Heat is used for the AO and acid is used for the SCSA. The AO dye intercalates into the DNA and fluoresces green when binding to double stranded DNA, and red when binding to single stranded DNA. The most commonly used method for stallion DNA breakage is the SCSA. The grade of DNA denaturation is determined by the ratio of green fluorescence (native, double-stranded DNA) or red (denatured, single-stranded DNA).

Flow cytometry can analyze one or more fluorescent probes to evaluate different attributes of cells. To analyze the cells, a stream of fluid containing individual cells is exposed to a laser beam that excites the fluorescent bound probe. Levels of fluorescence emitted are detected by specific photomultiplier tubes. Flow cytometric analyses are objective, precise, rapid, and measure a statistically relevant number of cells (Cordelli et al., 2005). Evaluation of the integrity of different sperm organelles should provide a better estimation of the percentage of sperm cells in a sperm population able to fertilize an oocyte (Wilhelm et al., 1996).

CHAPTER 3:

CENTRIFUGATION HAS MINIMAL EFFECTS ON MOTILITY, VIABILITY AND ACROSOME INTEGRITY OF EQUINE SPERMATOZOA IMMEDIATELY AND AFTER COOLING FOR 24 HOURS

3.1. Introduction

Most of the equine breed registries allow the use of A.I. for the production of foals. This has resulted in the request from many mare owners to breed them with fresh cooled or frozen-thawed semen.

From these methods, A.I. with fresh cooled semen is preferred over AI with frozen-thawed semen, because pregnancy rates are higher (Jasko D.J. et al., 1992b). The main objective in processing fresh cooled or frozen equine semen is to dilute the concentration of seminal plasma from the ejaculate. Seminal plasma is not an ideal medium for storing equine spermatozoa (Jasko D.J. et al., 1991; Moore et al., 2005; Varner et al., 1987). Dilution of seminal plasma is commonly achieved by the addition of a semen extender or by centrifugation of extended semen, partial removal of seminal plasma, followed by re-suspension of the sperm pellet with fresh semen extender.

Centrifugation effects on equine spermatozoa have been conflicting. Evaluation of the effect of centrifugation has been based on spermatozoa motility post-centrifugation. Centrifugation has been shown by some studies to be detrimental to sperm motility (Cochran et al., 1984; Jasko D.J. et al., 1991; Kareskoski et al., 2006; Pickett et al., 1975). Conversely, some other studies have shown that centrifugation does not cause a detrimental effect on sperm motility (Ferrer et al., 2004; Jasko D.J. et al., 1992a; Martin et al., 1979), and is in fact, beneficial to sperm motility of fresh cooled semen (Brinsko et al., 2000a).

Because the effects of centrifugation on equine spermatozoa have not been fully elucidated, recommended centrifugal forces used for equine semen processing are conservatively low (400 to 600 x g). Unfortunately, centrifugation of extended semen and partial removal of seminal plasma using the recommended centrifugal forces results

in a loss of 20 to 30% of the total number of spermatozoa in the supernatant (Cochran et al., 1984; Ferrer et al., 2004; Heitland et al., 1996).

The objectives of this study were to evaluate the effects of different centrifugal forces on equine sperm motility, plasma membrane (viability), and acrosome integrity. Additionally, the spermatozoal recovery rates at the different centrifugal forces were also determined.

3.2. Material and Methods

Six stallions were used in the study; five were from the LSU School of Animal Sciences and one was from the LSU School of Veterinary Medicine teaching herd. They were kept in a ½-acre pasture during the entire study and were fed a 12% protein commercial ration and free choice Alicia Bermuda hay. The stallions ranged from seven to 25 years old.

3.2.1. Semen Collection

Six stallions had semen collected at least 5 times in an every-other-day collection schedule to deplete the extra-gonadal sperm reserves (Amann et al., 1979). After the five collections it was assumed that the extra-gonadal sperm reserves were depleted and the stallions had semen collected once for the study. Before each collection, the stallion's penis was washed with cotton soaked in warm water to remove dirt and smegma. Semen was obtained by having the stallion mount a phantom mare, and then collected into a Hannover or Missouri artificial vagina. The artificial vagina was equipped with an in-line nylon filter to obtain a gel-free semen sample. After each stallion was collected, the filter containing the gel from the ejaculate was removed and the semen was transported to the laboratory within 10 minutes.

3.2.2. Semen Processing Overview

Processing of a fresh cooled semen shipment is a dynamic process. In this section the different steps of processing are listed and the details of each step are presented individually.

Upon arrival at the laboratory, the semen volume was measured and raw semen motility and sperm concentration were determined. The raw semen was then diluted 1:1 with a pre-warmed semen extender (INRA96, IMV Technologies, USA). After the semen was initially diluted, the semen was extended to a final concentration of $\sim 25 \times 10^6/\text{mL}$ (Varner et al., 1987) in a 4 x 8.5" Nasco Whirl-Pak[®] bag, by adding INRA96 at room temperature ($\sim 24^\circ\text{C}$). Extended semen motility and concentration were then evaluated. Two 25 μL samples of extended semen were obtained to assess plasma membrane and acrosome integrity (sperm cell analysis). An aliquot of 40 mL of the extended semen was then placed in each of four 50 mL conical centrifuge tubes (Corning[®], NY, USA). Each 50 mL conical centrifuge tube was centrifuged at a different centrifugation force. The treatment groups were non-centrifuged (NC), centrifuged at 400 x g (400), centrifuged at 900 x g (900) and centrifuged at 4500 x g (4500). After centrifugation, the supernatant was partially removed and the sperm pellet was re-suspended with INRA96. Concentration and motility of the re-suspended post-centrifugation semen was assessed (see below). Two 25 μL samples of the re-suspended semen were obtained to assess plasma membrane, and acrosome integrity (see below). The re-suspended semen was packaged in a Nasco Whirl-Pak[®] bag and most of the air removed from the bag. The packaged semen was placed in a passive cooling device (Equitainer, Hamilton Thorn Research, Danver, MA) and cooled for 24 hours. Next, spermatozoal motility was assessed (see below). Two 25 μL samples of this cooled semen were used to assess plasma membrane and acrosome integrity (see below). A flow chart of the semen processing is presented in Fig. 8.

3.2.3. Centrifugation

Extended semen placed in the conical centrifuge tubes was subjected to one of four centrifugation treatments for 10 min; NC, 400, 900, and 4500. An Eppendorf 5804 (Hamburg, Germany) centrifuge was used for centrifugation.

After centrifugation, the supernatant was partially removed (~ 37 mL) to the point where the centrifugation tube angled and became conical. The sperm pellet was re-suspended with INRA96 to its original volume. After 24 hours of cooling (4 to 6°C), a 25

µl aliquot from each treatment group was obtained and placed in a micro-centrifuge tube. The cooled semen in the micro-centrifuge was warmed for 5 min at 37.5°C prior to motility analysis.

3.2.4. Concentration

A 100 µL semen aliquot was diluted into 900 µL of FBS (1:10 dilution). Then, 100 µL of the 1:10 dilution was diluted into 900 µL of FBS (1:100 dilution). A Neubauer hemocytometer was loaded with the 1:100 dilution, and spermatozoal concentration was determined by counting all the cells within the 25 squares surrounded by the triple line from both sides of the hemocytometer, using a phase contrast microscope at 400x (Olympus BH-2, Olympus America Inc., USA). The cells counted from both sides of the hemocytometer should not have more than 10% difference and the final cell concentration was the average. If the number of cells counted from both sides had a difference greater than 10%, the hemocytometer was reloaded with the 1:100 dilution and cells were counted as described above. This procedure was performed until the difference between the cells counted from both sides of the hemocytometer was $\leq 10\%$.

3.2.5. Motility

The spermatozoal motility was analyzed by placing 2 µL of raw semen in a 20 micron height, four chamber slide (Leja Products B.V., The Netherlands) over a slide warmer at 37.5°C. The slide was then placed on an optical microscope warmed stage at 37.5°C (Olympus BX41, Olympus America Inc., USA). Using the 20x phase contrast magnification objective, spermatozoa motility was analyzed with a computer assisted sperm analysis (CASA, Sperm Vision®, Minitube, Verona, WI, USA). Mean percentages of total and progressive motility were assessed from 7 fields with at least 100 spermatozoa in each field. After 24 hours of cooling, a 25 µl sample of cooled semen from each treatment group was obtained and placed in a micro-centrifuge tube. The cooled semen in the micro-centrifuge was warmed for 5 min at 37.5°C prior to motility analysis.

3.2.6. Plasma Membrane Integrity (viability)

Fluorescent dyes were purchased from Molecular Probes Inc. (Eugene, OR, USA) unless otherwise indicated. The SYBR-14 and propidium iodide (PI) (LIVE/DEAD® Sperm Viability Kit) were used to assess plasma membrane integrity. The SYBR-14 is a permeable nucleic acid fluorescent dye that stains spermatozoa with an intact plasma membrane green (viable). The PI is an impermeable nucleic acid fluorescent dye that stains spermatozoa with disrupted plasma membrane red (dead). A stock solution SYBR-14 was prepared. One 1 μ L of the SYBR-14 solution (1 mM) was diluted into 99 μ L of phosphate buffered saline (PBS) to obtain a 0.01 mM working solution.

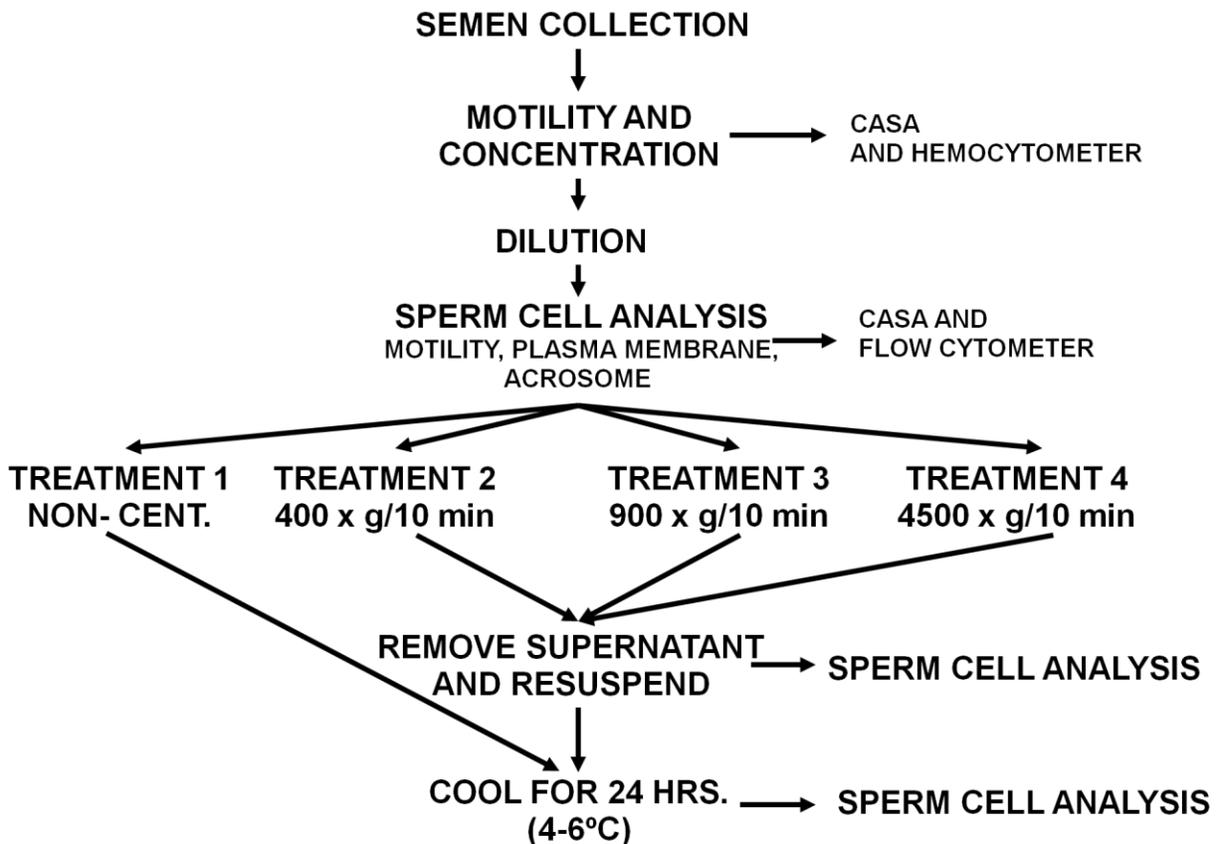


Figure 8. Flow chart presenting steps used in processing equine semen (n=6).

A 25 μ L aliquot from each of the treatment groups was stained to assess the plasma membrane integrity. After centrifugation and re-suspension of the sperm pellet, and after cooling for 24 h the samples were diluted with 250 μ L of PBS (pH 7.4 and 320

mOsm) to a concentration of 1×10^6 /mL; cells were stained by adding 2.5 μ L of SYBR-14 working solution and incubated at 37.5°C in the dark for 10 min. After incubation 2.5 μ L of PI (2.4 Mm) was added and the sample incubated in the dark for another 10 min, prior to analysis by flow cytometry.

The stained samples were analyzed with a flow cytometer (FACSCalibur®, Becton Dickinson Immunocytometry, San Jose, CA) in triplicate. Cells were gated to exclude debris using a dot plot, and the fluorescence analysis done on the ten thousand cells outside the gate. Viable and non-viable spermatozoal populations were gated and analyzed using the CellQuest (BD Bioscience, Immunocytometry Systems, San Jose, CA, USA) software as shown in Fig. 9.

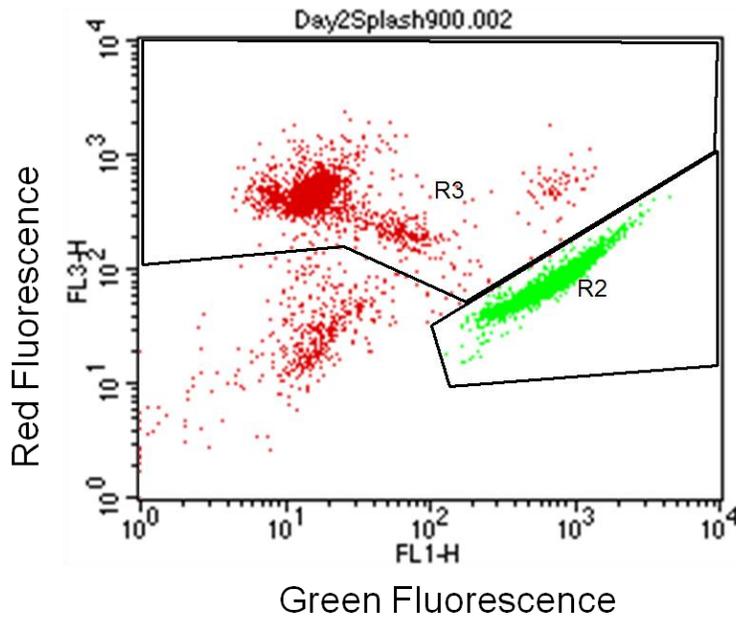


Figure 9. Spermatozoa viability analysis by flow cytometry. Spermatozoal populations are gated. R2 viable spermatozoa (green) and R3 non-viable spermatozoa (red) (FACSCalibur®, CellQuest software).

3.2.7. Acrosome Reactivity

A 25 μ L aliquot from each of the treatment groups was stained to assess the acrosome integrity after centrifugation and re-suspension of the sperm pellet, and after cooling for 24 h. To estimate the acrosome reactivity, spermatozoa were dual stained

with FITC-PNA/PI. The fluorescein-conjugated *Arachis Hypogaea* agglutinin (FITC-PNA) binds to the β -galactose moieties of the outer acrosomal membrane and fluoresce green (Cheng et al., 1996). The PI served to differentiate between viable and non viable spermatozoa. The semen sample (25 μ L) was diluted with 250 μ L PBS to a concentration of 1×10^6 /mL; sperm cells were stained with 2.0 μ L of the FITC-PNA solution (1 mg/mL) and incubated at 37.5°C in the dark for 10 min. After incubation 2.5 μ L of PI was added and incubated in the dark for another 10 min, prior to analysis by flow cytometry.

The stained samples were analyzed by flow cytometry (FACSCalibur®, Becton Dickinson Immunocytometry, San Jose, CA) using a quadratic gating. Viable sperm non-reacted acrosome, viable sperm reacted acrosome, dead sperm non-reacted acrosome, and dead sperm reacted acrosome populations were analyzed using the CellQuest (BD Bioscience, Immunocytometry Systems, San Jose, CA, USA) software as shown in Fig. 10.

3.2.8. Recovery Rate After Centrifugation

To determine sperm recovery rate, sperm concentration was measured two times. First it was measured on the extended semen before centrifugation, and then after centrifugation, partial removal of seminal plasma and sperm pellet re-suspension. The number of sperm cells counted from the post-centrifugation re-suspended semen was divided by the initial number of sperm cells prior to centrifugation then multiplied by 100, to obtain the percentage of recovered sperm cells.

$$\text{SCR (\%)} = (\text{SSR}/\text{TSC}) \times 100$$

SCR: sperm cells recovered

TSC: total number of sperm cells

SSR: sperm cells in re-suspended semen

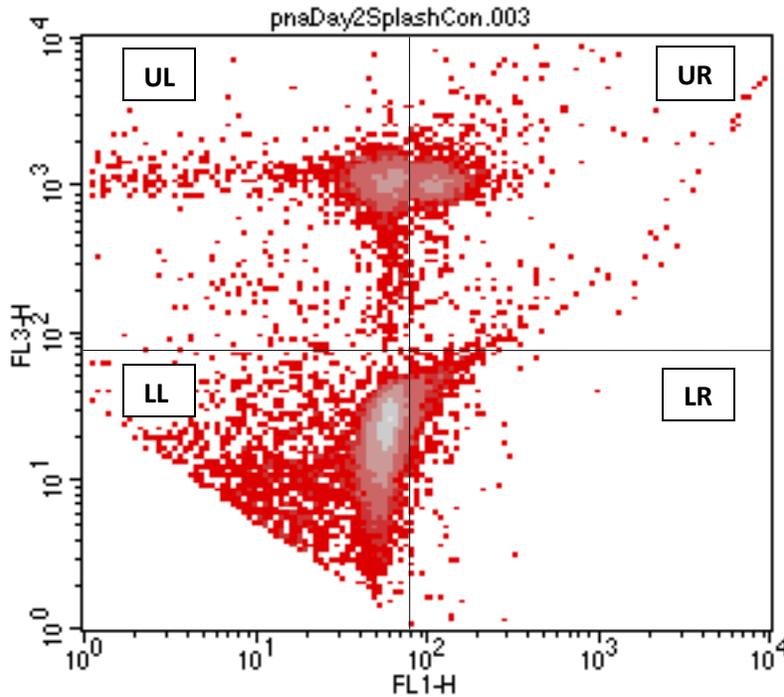


Figure 10. Spermatozoal acrosome integrity analysis by flow cytometry. Quadratic gating identifies four spermatozoal populations. Upper left (UL) = dead sperm non-reacted acrosome, upper right (UR) = dead sperm reacted acrosome, lower left (LL) = live sperm non-reacted acrosome and lower right (LR) = live sperm reacted acrosome (FACSCalibur®, CellQuest software).

3.2.9. Statistical Analyses

The mean (\pm SD) percentage total motility, progressive motility, viability, acrosome reactive status and recovery rates were analyzed using Shapiro Wilk's to evaluate if the data followed a normal distribution.

The mean (\pm SD) percentage total motility, progressive motility, viability, and acrosome integrity were compared for an effect of treatment and time, using a mixed linear model including the random variance of stallion across treatments. Where there was significant interaction of treatment and day at $p < 0.05$, ad hoc comparisons were made using Scheffe's adjustment to maintain type I error at 0.05.

The mean (\pm SD) percentage recovery rates were compared for an effect of treatment using a mixed linear model including the random variance of stallion across treatments. Where there was significant effect of treatment at $p < 0.05$, ad hoc comparisons were made between treatment groups to non-centrifuged treatment group using Dunnet's adjustment to maintain type I error at 0.05.

3.3. Results

Total and progressive motility of sperm cells was not affected immediately or after cooling for 24 h, when extended semen was centrifuged at the 400 or 900 treatment group, compared to the NC treatment group. Conversely, total and progressive motility was affected immediately and after cooling for 24 h when the extended semen was centrifuged at the 4500 treatment group (Tables 2 and 3).

Table 2. Mean (\pm SD) equine (n = 6) spermatozoal total motility (TM) percentage (%) immediately (D0) after centrifugation and after cooling for 24 h (D1).

Treatment	D0 TM (%)	D1 TM (%)
NC	86.4 \pm 6.4 ^a	80.1 \pm 6.4 ^a
400 x g	87.9 \pm 7.7 ^a	78.2 \pm 11.3 ^a
900 x g	86.5 \pm 7.2 ^a	76.0 \pm 9.9 ^a
4500 x g	78.4 \pm 6.6 ^b	70.2 \pm 11.8 ^b

*Values within column with different subscripts are different.

Table 3. Mean (\pm SD) equine (n = 6) spermatozoal progressive motility (PM) percentage (%) immediately (D0) after centrifugation and after cooling for 24 h (D1).

Treatment	D0 PM (%)	D1 PM (%)
NC	82.6 \pm 6.7 ^a	76.4 \pm 7.1 ^a
400 x g	85.6 \pm 8.7 ^a	74.9 \pm 12.2 ^a
900 x g	84.2 \pm 7.4 ^a	73.4 \pm 10.8 ^a
4500 x g	74.2 \pm 6.2 ^b	66.5 \pm 11.5 ^b

*Values within column with different subscripts are different.

Spermatozoal plasma membrane integrity (viability) was not affected by centrifugation immediately or after cooling for 24 h when extended semen was centrifuged at the 400 or 900 treatment group, compared to the NC treatment group. Conversely, sperm viability was affected immediately and after cooling for 24 h when

extended semen was centrifuged at the 4500 treatment group; however, after cooling for 24 hours, sperm plasma membrane integrity was not different from the NC treatment group (Table 4).

Spermatozoa acrosomal reactivity was not affected by centrifugation immediately or after cooling for 24 h when extended semen was centrifuged at the 400 or 900 treatment group, compared to the NC treatment group. Conversely, spermatozoa acrosome integrity was affected immediately and after cooling for 24 h when extended semen was centrifuged at the 4500 treatment group; however, after 24 h of cooling, acrosome integrity was not different from the NC treatment group (Table 5).

Table 4. Mean (\pm SD) equine (n = 6) spermatozoal viability percentage (%) immediately (D0) after centrifugation and after cooling for 24 h (D1).

	D0	D1
Treatment	Viable (%)	Viable (%)
NC	81.1 \pm 6.2 ^a	76.4 \pm 5.2 ^{ab}
400 x g	85.5 \pm 5.6 ^a	81.1 \pm 2.8 ^a
900 x g	81.7 \pm 5.2 ^a	79.0 \pm 4.6 ^a
4500 x g	72.8 \pm 6.6 ^b	70.7 \pm 7.8 ^b

*Values within column with different subscripts are different.

Table 5. Mean (\pm SD) equine (n = 6) viable spermatozoa non-reacted acrosome percentage (%) immediately (D0) after centrifugation and after cooling for 24 h (D1).

	D0	D1
Treatment	Non-reacted (%)	Non-reacted (%)
NC	81.9 \pm 4.6 ^a	76.0 \pm 5.7 ^{ab}
400 x g	83.7 \pm 2.4 ^a	80.3 \pm 5.8 ^a
900 x g	81.3 \pm 5.0 ^a	79.6 \pm 5.3 ^a
4500 x g	72.5 \pm 6.1 ^b	69.5 \pm 8.5 ^b

*Values within column with different subscripts are different.

Spermatozoa recovery rate was affected by centrifugation. Spermatozoa recovery rate was decreased when extended semen was centrifuged at the 400 or 900 treatment group, compared to the NC treatment group. Conversely, spermatozoa recovery rate was similar to the NC treatment group when extended semen was centrifuged at the 4500 treatment group (Table 6).

Table 6. Mean (\pm SD) equine (n = 6) spermatozoal recovery rate percentage (%) after centrifugation at different centrifugation forces.

Treatment	Recovery Rate (%)
NC	100 ^a
400 x g	54.4 \pm 8.6 ^b
900 x g	75.0 \pm 7.1 ^b
4500 x g	97.9 \pm 1.16 ^a

*Values within column with different subscripts are different.

3.4. Discussion

Storage of spermatozoa for long periods of time in the excurrent duct system is characterized by a reduction in motility and an increase in morphological abnormalities (Amann, 1993; Varner et al., 1991). Removal of the extra-gonadal sperm reserves allows the assessment of recently produced spermatozoa. Stallions used in this study were collected at least 5 times (Amann et al., 1979) to deplete the extra-gonadal reserves prior to obtaining the ejaculate used in the study.

In this study, the major source of variability was the stallions. Initial total and progressive motility and viability varied between stallions, and their response to the different treatments also varied. However, these variations still allowed the data to follow a normal distribution.

The effect of centrifugation on equine spermatozoal motion characteristics (total and progressive motility) has not been fully elucidated. In the present study centrifugation of extended semen up to 900 x g for 10 min did not cause a reduction in mean total and progressive motility of equine spermatozoa immediately after centrifugation. Results from this study are in agreement with the results of Ferrer et al. (2004) who also centrifuged extended semen at 900 x g for 10 min without causing a reduction on sperm motility immediately after centrifugation.

Centrifugation of extended semen without partial removal of seminal plasma caused a decrease in sperm motility of stored semen (Jasko D.J. et al., 1991). The decrease in sperm motility can be attributed in part to the centrifugation-induced generation of ROS (Parinaud et al., 1997). In the present study, centrifugation of

extended semen up to 900 x g for 10 min, partial removal of seminal plasma, followed by re-suspension of the sperm pellet with fresh semen extender did not cause a decrease and maintained sperm motility after cooling for 24 hours. It can be concluded that components of seminal plasma (e.g. ROS), that would cause a detrimental effect on sperm motility of stored semen were removed with the seminal plasma after centrifugation.

Spermatozoal plasma membrane integrity is important for fertilization. Disruption or damage may impair sperm-oocyte fusion (Aitken, 1995). The equine plasma membrane can be damaged by semen centrifugation during processing of fresh cooled semen (Aurich, 2005). In the present study, centrifugation of extended semen up to 900 x g for 10 min, partial removal of seminal plasma followed by re-suspension of the sperm pellet with fresh semen extender did not cause damage to the sperm plasma membrane, immediately ($81.7 \pm 5.2\%$) or after cooling for 24 hours ($79.0 \pm 4.6\%$), assessed by LIVE/DEAD® Sperm Viability Kit. Similar to the present study, previous studies have demonstrated that extended semen centrifuged at 600 x g for 10 min (Pagl et al., 2006) or 900 x g for 10 min (Ferrer et al., 2004) did not cause damage to the sperm plasma membrane, immediately after centrifugation or after cooling for 24 h, assessed by LIVE/DEAD® Sperm Viability Kit. It may be possible that removal of ROS in the seminal plasma after centrifugation, may have reduced the plasma membrane disruption caused by the sperm capacitation process induced by ROS (Ford, 2004; O'Flaherty et al., 2006). This may explain the higher sperm viability in the 900 treatment group after cooling for 24 hours (79.0 ± 4.6), compared to the NC treatment group ($76.4 \pm 5.2\%$).

An intact or non-reacted spermatozoal acrosome is necessary to penetrate the zona pellucida and further fertilization of an oocyte (Abou-Haila and Tulsiani, 2000). Results from our study showed that centrifugation of the 900 treatment group, partial removal of seminal plasma, followed by re-suspension of the sperm pellet with fresh semen extender, did not induce the equine sperm to undergo the acrosome reaction immediately after centrifugation or after cooling for 24 hours. Compared to the NC treatment group, the 900 treatment group non-reacted acrosome percentage was

similar immediately after centrifugation ($81.9 \pm 4.6\%$ and $81.3 \pm 5.0\%$ respectively), however after cooling for 24 hours the non-reacted acrosomes percentage was greater for the 900 treatment group compared to the NC treatment group ($79.6 \pm 5.3\%$ and 76.0 ± 5.7 respectively). In agreement with these results, Dawson et al. (Dawson et al., 2000) assessed the integrity of equine sperm acrosome using FITC-PSA after extended semen was centrifuged at 400 to 600 x g for 15, and reported no damage immediately after centrifugation or after cooling for 24 hours.

Interestingly, sperm viability and non-reacted acrosomes immediately after centrifugation from the NC ($81.1 \pm 6.2\%$ viable and $81.9 \pm 4.6\%$ non-reacted) and 4500 ($72.8 \pm 6.6\%$ viable and $72.5 \pm 8.5\%$ non-reacted) treatment groups were different immediately after centrifugation; however after cooling for 24 hours, sperm viability and non-reacted acrosomes were similar for the NC ($76.4 \pm 5.2\%$ viable and $76.0 \pm 5.7\%$ non-reacted) and 4500 ($70.7 \pm 7.8\%$ viable and $69.5 \pm 8.5\%$ non-reacted) treatment groups. Perhaps, on the 4500 treatment group, centrifugation caused an immediate damage to sperm viability and acrosome integrity percentage that occurred over time in the NC treatment group. Nevertheless, harmful components from the seminal plasma were removed (in the supernatant) from the 4500 treatment group as previously mentioned, probably limiting the damage to sperm viability and acrosome after cooling for 24 hours, compared to the NC treatment group, where no seminal plasma was removed.

In our study, equine sperm recovery rates increased as centrifugation force increased. When equine extended semen was centrifuged at the 400 and 900 treatment groups, equine sperm recovery rates were $54.4 \pm 8.6\%$ and $75.0 \pm 7.1\%$ respectively, from the total number of sperm cells before centrifugation. These recovery rates are different compared to a previous study by Cochran et al. (1984), where recovery rates were 67.0% and 70% for extended semen centrifuged at 400 x g for 10 min and 650 x g for 15 min. Differences in the recovery rates obtained in that study compared to the present study may be explained by the volume of supernatant removed after centrifugation. Cochran et al. (1984) did not report the volume of supernatant removed after centrifugation. In our study, we tried to simulate the equine semen processing that

occurs in a clinical situations and removed supernatant up to the point where the centrifuged tube angled and became conical (~ 37 mL). It is possible that in the present study more supernatant was removed compared to the study of Cochran et al. (1984), consequently losing more cells in the supernatant, however this is not known. Another possible reason for the differences in recovery rates between the studies may be the cell counting method used. Cochran et al. (1984) used an automated cell counter and in our study we used a hemocytometer. A margin of error may be expected with the hemocytometer when counting low numbers of cells as was the case in our study, possibly causing under- or over-estimation of sperm concentration. Noteworthy was the fact that sperm counts using the hemocytometer, in some instances needed to be performed several times until the number of cells counted from both sides of the hemocytometer was within 10%. Ferrer et al. (2004) reported recovery rates of 74.3% and 91.0% for extended semen centrifuged at 400 x g and 900 x g for 10 min. In this study, similar to our study, semen was diluted to 25×10^6 /mL and volume of 40 mL before centrifugation; however recovery rates were calculated by subtracting the number of cells counted in the supernatant from the initial number of cells in the extended semen. Calculating sperm concentration with very low number of cells can be tedious and may result in a higher margin of error, causing under- or over-estimation of sperm concentration. In addition, sperm concentration in the supernatant was estimated using the 1:10 dilution instead of 1:100, trying to decrease the margin of error; however a high margin of error may be expected and may account for the difference between studies. Moreover, sedimentation of sperm cells can be affected by the type of extender (viscosity) used for centrifugation. In our study the type of semen extender used for centrifugation (INRA96) was different, compared to the studies by Cochran et al. (1984) (Citrate-EDTA) and Ferrer et al. (2004) (skim milk), and the viscosity between extenders may be different causing a difference in sedimentation of cells and consequently the number of cells in the pellet. Recovery rates between the studies may differ, however equine sperm recovery rates increased as centrifugal forces increased. In the present study, equine sperm recovery rate was 97.9% when extended semen was centrifuged at 4500 X g for 10 min, this is the highest recovery rate of equine sperm ever reported after centrifugation.

The sperm pellet, being difficult to re-suspend has been a problem when higher centrifugal forces are used. A centrifugation “cushion” is recommended (Knop et al., 2005; Revell SG et al., 1997) when using high centrifugation forces. In the present study re-suspension of the sperm pellet was easily performed when extended semen was centrifuged up to 900 x g for 10 min. A dense pellet, more difficult to re-suspend was found when extended semen was centrifuged at 4500 x g for 10 min. An apparent positive relation of the hardness of the pellet with the amount of debris in the ejaculate was noticed. Due to the difficulty of gently handling and re-suspending cells from a compact pellet, some deleterious effects to the sperm cells may have been produced. The deleterious effect to sperm motility, plasma membrane and acrosomes was observed immediately after re-suspension of the sperm pellet and did not increase after cooling for 24 hours.

To our knowledge, this is the first study where equine spermatozoa have been subjected to centrifugation at 4500 x g and evaluated for total motility, progressive motility, viability and acrosomal reactivity. Interestingly, although equine spermatozoa integrity (motility, viability and acrosome) was affected when extended semen was centrifuged at the 4500 treatment group in relation to the recovered cells, the total number of motile, viable and non-reacted acrosome spermatozoa recovered after centrifugation in the 4500 treatment group was greater, compared to the 400 and 900 treatments groups. In addition, after cooling for 24 h, the number of intact spermatozoa recovered at the 4500 treatment group was similar to the 900 treatment group and higher than the 400 treatment group. This can be better appreciated using a practical example. For example, a total of 1×10^9 spermatozoa are commonly packed in an insemination dose of fresh cooled semen. If all the measured parameters in this study are combined, the number of motile, viable and non-reacted acrosome spermatozoa available after centrifugation (D0) would be 575×10^6 , 342×10^6 , 430×10^6 , and 405×10^6 for the NC, 400, 900 and 4500 treatment groups respectively; and 465×10^6 , 277×10^6 , 358×10^6 and 337×10^6 after cooling for 24 h (D1) for the NC, 400, 900 and 4500 treatments groups respectively (Fig. 11). This practical example demonstrates that the total number of motile, viable and non-reacted acrosome spermatozoa immediately after centrifugation and after cooling for 24 hours is greater in the 900 and 4500 treatment

groups compared to the 400 treatment group. It was also demonstrated that the NC treatment group yields the highest number of motile, viable and non-reacted acrosome spermatozoa, and it can be concluded that centrifugation should be used during the processing of fresh cooled semen only when needed (oligospermic or poor cooler stallions).

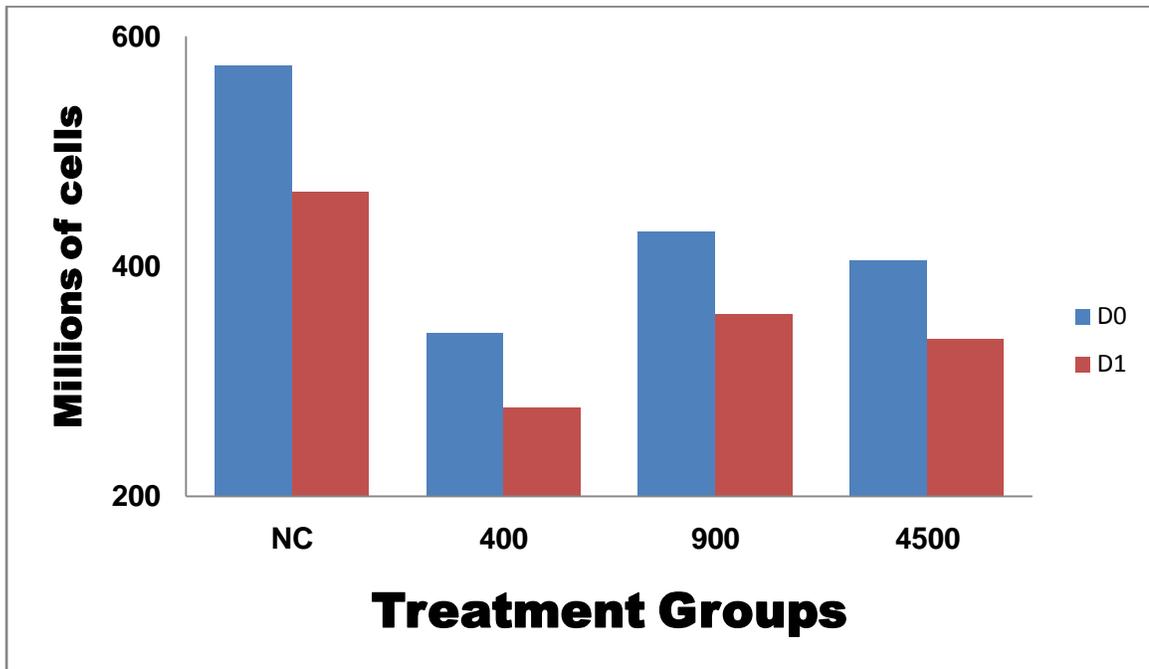


Figure 11. Scheme of the total number of motile, viable and non-reacted acrosomes spermatozoa immediately after centrifugation (D0) and after cooling for 24 h (D1), at different centrifugal forces for 10 min.

Within practitioners, it is believed that the sperm cells of the supernatant after centrifugation may be of better quality compared to the cells of the sperm pellet. During the study, sperm cells from the supernatant of some stallions ($n = 3$) were evaluated similarly as the cells in the sperm pellet. No difference in total motility, progressive motility, viability or acrosome reactive status was observed (data not shown) between sperm cells in the pellet of the NC, 400 and 900 treatment groups, compared to that of the supernatant.

New technologies for evaluating equine spermatozoa increases the knowledge of the capability of spermatozoa to undergo the processes required for fertilizing an oocyte; however, attempts to correlate laboratory evaluations of spermatozoa with fertility have yielded various results (Colenbrander et al., 2003; Kirk et al., 2005; Nie et al., 2002). Using a combination of parameters is thought to be better than using a single parameter to predict fertility; however until further studies are performed and a correlation between a parameter or combination of parameters with fertility is found, these parameters should not be used as fertility predictors.

Further investigation using centrifugation forces between 900 x g and 4500 x g are warranted to identify the optimal centrifugation force or range of forces that yields the maximum recovery rate without causing damage to equine spermatozoa. Also, further studies are warranted to evaluate the effect of centrifugation treatments on sperm integrity and fertility.

CHAPTER 4:

CONCLUSIONS

Efficient processing of equine semen for fresh cooled semen or frozen semen is essential in today's equine industry. Stallions in great demand will benefit from semen processing that cause minimal loss of spermatozoa. Use of centrifugation and partial removal of seminal plasma for equine semen processing has the advantage of decreasing the seminal plasma concentration in an ejaculate. In this study, we found that the effect of centrifugation on equine spermatozoa varied between stallions. Some stallions, but not all, with initial lower motility suffered a decrease in total and progressive motility, viability and acrosome reactivity, immediately after centrifugation, regardless of the centrifugal force used. Perhaps, spermatozoa from these stallions had an intrinsic defect and centrifugation may have exacerbated the problem. Nevertheless, the variance between stallions observed did not influence the results, and all the samples followed a normal distribution.

The major problem encountered during the study was sperm concentration determination. Spermatozoal concentration estimation from the extended semen and after removal of the supernatant and re-suspension was difficult and needed to be repeated several times until the difference between two replicate counts using a hemocytometer were within the desired range (10%). This difficulty was due to the low spermatozoal concentrations after dilution of the raw semen and much lower concentrations after removal of the supernatant. Therefore the margin of error may have increased. This fact explains the reason why Ferrer et al. (2004), trying to decrease the margin of error, used the 1:10 dilution instead of the 1:100 dilution in her study. An automated cell counter could not be used because the semen extender was turbid and concentration estimation by the spectrophotometer would have been erroneous. Sperm concentration of extended stallion semen can be performed using a flow cytometer, and possibly could be a better approach for further studies.

When seminal plasma was not removed, centrifugation has been shown to decrease sperm motility (Jasko D.J. et al., 1991; Pickett et al., 1975) or to have no

detrimental effects (Ferrer et al., 2004; Jasko D.J. et al., 1991; Jasko D.J. et al., 1992a; Padilla and Foote, 1991) when seminal plasma was partially removed. In this study, similar to previous studies (Ferrer et al., 2004; Kareskoski et al., 2006), centrifugation up to 900 x g for 10 min, partial removal of seminal plasma, followed by re-suspension of the sperm pellet had no detrimental effect on equine spermatozoal motility, viability and acrosomal integrity compared to the non-centrifuged extended semen. In conclusion, centrifugation up to 900 x g for 10 min can be used to process stallion semen without causing detrimental effects, and can be recommended to process semen from oligospermic and “poor coolers” stallions.

The toughness expressed by sperm cells was surprising. The initial purpose of centrifuging extended semen at 4500 x g for 10 min was to kill the spermatozoa, as a positive control; however, this was not the outcome. However, centrifugation of equine spermatozoa at 4500 x g for 10 min did cause statistical significant detrimental effects on total and progressive motility, viability and non-reacted acrosomes; but this damage was not extreme and the recovery rate was high in that it was not different from the non-centrifuged extended semen (100%). These results indicated that a centrifugal force between 900 x g and 4500 x g, where sperm recovery rates are maximized and sperm integrity is minimally affected, exists and warrants investigation.

Assessment of motility, viability and acrosome integrity offers valuable information with regard to the fertilizing capacity of spermatozoa. It is reasonable to expect a better fertilizing capacity with a higher number of motile, viable and non-reacted acrosomal spermatozoa in an insemination dose; however, the relation between laboratory tests and fertility has been unclear (Colenbrander et al., 2003; Kirk et al., 2005; Nie et al., 2002). If all the parameters evaluated in this study are considered, the total number of motile, viable and non-reacted acrosomes after cooling for 24 h for a commonly used insemination dose (1×10^9 spermatozoa) was greatest for the NC treatment group (465×10^6 spermatozoa), followed by the 900 treatment group (358×10^6 spermatozoa), then the 4500 treatment group (337×10^6 spermatozoa), and the least the 400 treatment group (277×10^6 spermatozoa). Showing that centrifugation should only be used when it is indicated (oligospermic or poor cooler stallions), not

because of damage to the spermatozoa, but due to losses of sperm cells in the supernatant discarded.

REFERENCES

- Aitken, R. J. 1995. Free radicals, lipid peroxidation and sperm function. *Reproduction, Fertility, and Development* 7:659-668.
- Aitken, R. J., D. Buckingham, and D. Harkiss. 1993. Use of a xanthine oxidase free radical generating system to investigate the cytotoxic effects of reactive oxygen species on human spermatozoa. *Journal of Reproduction and Fertility* 97:441-450.
- Alghamdi, A. S., D. N. Foster, and M. H. T. Troedsson. 2004. Equine seminal plasma reduces sperm binding to polymorphonuclear neutrophils (PMNs) and improves the fertility of fresh semen inseminated into inflamed uteri. *Journal of Reproduction and Fertility* 127:593-600.
- Alvarez, J. G., J. L. Lasso, L. Blasco, R. C. Nunez, S. Heyner, P. P. Caballero, and B. T. Storey. 1993. Centrifugation of human spermatozoa induces sublethal damage; separation of human spermatozoa from seminal plasma by a dextran swim-up procedure without centrifugation extends their motile lifetime. *Human Reproduction*. 8:1087-1092.
- Amann, R. P. 1993. Physiology and endocrinology. In: *Equine Reproduction*. A. O. McKinnon and J. L. Voss, eds. Lea & Febiger, Philadelphia. 658-685.
- Amann, R. P., M. J. Cristanelli, and E. L. Squires. 1987. Proteins in stallion seminal plasma. *Journal of Reproduction and Fertility*. Supplement 35:113-120.
- Amann, R. P. and J. K. Graham. 1993. Spermatozoal function. In *Equine Reproduction*. A. O. McKinnon and J. L. Voss, eds. Lea & Febiger, Philadelphia. 715-745.
- Amann, R. P., D. L. Thompson, Jr., E. L. Squires, and B. W. Pickett. 1979. Effects of age and frequency of ejaculation on sperm production and extragonadal sperm reserves in stallions. *Journal of Reproduction and Fertility* Supplement 27:1-6.
- Aurich, C. 2005. Factors affecting the plasma membrane function of cooled-stored stallion spermatozoa. *Animal Reproduction Science* 89:65-75.
- Baiardi, G., R. D. Ruiz, M. F. De Cuneo, A. A. Ponce, J. L. Lacuara, and L. Vincenti. 1997. Differential effects of pharmacologically generated reactive oxygen species upon functional activity of epididymal mouse spermatozoa. *Canadian Journal of Physiology and Pharmacology* 75:173-178.
- Baker, M. A. and R. J. Aitken. 2004. The importance of redox regulated pathways in sperm cell biology. *Molecular and Cellular Endocrinology* 216:47-54.

- Ball, B. A., A. T. Vo, and J. Baumber. 2001. Generation of reactive oxygen species by equine spermatozoa. *American Journal of Veterinary Research* 62:508-515.
- Barth, A. D. and R. J. Oko. 1989. Normal bovine spermatogenesis and sperm maturation. In: *Abnormal Morphology of Bovine Spermatozoa*. A. D. Barth and R. J. Oko, eds. Iowa State University Press, Iowa. 19-88.
- Batellier, F., M. Vidament, J. Fauquant, G. Duchamp, G. Arnaud, J. M. Yvon, and M. Magistrini. 2001. Advances in cooled semen technology. *Animal Reproduction Science* 68:181-190.
- Baumber, J., B. A. Ball, C. G. Gravance, V. Medina, and M. C. vies-Morel. 2000. The effect of reactive oxygen species on equine sperm motility, viability, acrosomal integrity, mitochondrial membrane potential, and membrane lipid peroxidation. *Journal of Andrology* 21:895-902.
- Baumber, J., B. A. Ball, J. J. Linfor, and S. A. Meyers. 2003a. Reactive oxygen species and cryopreservation promote DNA fragmentation in equine spermatozoa. *Journal of Andrology* 24:621-628.
- Baumber, J., K. Sabeur, A. Vo, and B. A. Ball. 2003b. Reactive oxygen species promote tyrosine phosphorylation and capacitation in equine spermatozoa. *Theriogenology* 60:1239-1247.
- Bearer, E. L. and D. S. Friend. 1990. Morphology of mammalian sperm membranes during differentiation, maturation, and capacitation. *Journal of Electron Microscopy Technique* 16:281-297.
- Abou-Haila, A. and D. R. P. Tulsiani. 2000. Mammalian sperm acrosome: Formation, contents, and function. *Archives of Biochemistry and Biophysics* 379:173-182.
- Bowen, J. 1969. Artificial insemination in the horse. *Equine Veterinary Journal* 1:98-110.
- Brinsko, S. P., E. C. Crockett, and E. L. Squires. 2000a. Effect of centrifugation and partial removal of seminal plasma on equine spermatozoal motility after cooling and storage. *Theriogenology* 54:129-136.
- Brinsko, S. P., K. R. Rowan, D. D. Varner, and T. L. Blanchard. 2000b. Effects of transport container and ambient storage temperature on motion characteristics of equine spermatozoa. *Theriogenology* 53:1641-1655.
- Cheng, F. P., A. Fazeli, W. F. Voorhout, A. Marks, M. M. Bevers, and B. Colenbrander. 1996. Use of peanut agglutinin to assess the acrosomal status and the zona pellucida-induced acrosome reaction in stallion spermatozoa. *Journal of Andrology* 17:674-682.

- Cochran, J. D., R. P. Amann, D. P. Froman, and B. W. Pickett. 1984. Effects of centrifugation, glycerol level, cooling to 5 C, freezing rate and thawing rate on the post-thaw motility of equine sperm. *Theriogenology* 22:25-38.
- Colenbrander, B., B. M. Gadella, and T. A. E. Stout. 2003. The predictive value of semen analysis in the evaluation of stallion fertility. *Reproduction in Domestic Animals* 38:305-311.
- Cordelli, E., P. Eleuteri, G. Leter, M. Rescia, and M. Spano. 2005. Flow cytometry applications in the evaluation of sperm quality: semen analysis, sperm function and DNA integrity. *Contraception* 72:273-279.
- Cross, N. L. and S. Meizel. 1989. Methods for evaluating the acrosomal status of mammalian sperm. *Biology of Reproduction* 41:635-641.
- Dawson, G. R., G. W. Webb, J. A. Pruitt, T. M. Loughin, and M. J. Arns. 2000. Effect of different processing techniques on motility and acrosomal integrity of cold-stored stallion spermatozoa. *Journal of Equine Veterinary Science* 20:191-194.
- de Lamirande, E. and C. Gagnon. 1992. Reactive oxygen species and human spermatozoa: I. Effects on the motility of intact spermatozoa and on sperm axonemes. *Journal of Andrology* 13:368-378.
- Dott, H. M. 1975. Morphology of stallion spermatozoa. *Journal of Reproduction and Fertility. Supplement* 23:41-46.
- Dowsett, K. F. and L. M. Knott. 1996. The influence of age and breed on stallion semen. *Theriogenology* 46:397-412.
- Evenson, D. P., K. Kasperson, and R. L. Wixon. 2007. Analysis of sperm DNA fragmentation using flow cytometry and other techniques. *Society of Reproduction and Fertility Supplement* 65:93-113.
- Evenson, D. P. and L. Jost. 1994. Sperm chromatin structure assay: DNA denaturability. *Methods in Cell Biology* 42:159-176.
- Evenson, D. P. and R. Wixon. 2006. Clinical aspects of sperm DNA fragmentation detection and male infertility. *Theriogenology* 65:979-991.
- Farlin, M. E., D. J. Jasko, J. K. Graham, and E. L. Squires. 1992. Assessment of *Pisum sativum* agglutinin in identifying acrosomal damage in stallion spermatozoa. *Molecular Reproduction and Development* 32:23-27.
- Ferrer, M. S., D. Paccamonti, B. E. Eilts, S. K. Lyle, A. H. Aljarrah, and R. V. Devireddy. 2004. Improvement of sperm recovery rates after centrifugation of stallion semen. *Proceedings of the 2004 SFT Annual Conference & SFT/ACT Symposia*. 7.

- Flesch, F. M. and B. M. Gadella. 2000. Dynamics of the mammalian sperm plasma membrane in the process of fertilization. *Biochimica et Biophysica Acta - Reviews on Biomembranes* 1469:197-235.
- Ford, W. C. L. 2004. Regulation of sperm function by reactive oxygen species. *Human Reproduction Update* 10:387-399.
- Gadella, B. M. and R. A. Harrison. 2000. The capacitating agent bicarbonate induces protein kinase A-dependent changes in phospholipid transbilayer behavior in the sperm plasma membrane. *Development* 127:2407-2420.
- Garner, D. L. and L. A. Johnson. 1995. Viability assessment of mammalian sperm using SYBR-14 and propidium iodide. *Biology of Reproduction* 53:276-284.
- Graham, J. K., E. Kunze, and R. H. Hammerstedt. 1990. Analysis of sperm cell viability, acrosomal integrity, and mitochondrial function using flow cytometry. *Biology of Reproduction* 43:55-64.
- Griggers, S., D. L. Paccamonti, R. A. Thompson, and B. E. Eilts. 2001. The effects of pH, osmolarity and urine contamination on equine spermatozoal motility. *Theriogenology* 56:613-622.
- Harrison, R. A. P. and S. E. Vickers. 1990. Use of fluorescent probes to assess membrane integrity in mammalian spermatozoa. *Journal of Reproduction and Fertility* 88:343-352.
- Heitland, A. V., Jasko D.J., E. L. Squires, J. K. Graham, B. W. Pickett, and Hamilton C. 1996. Factors affecting motion characteristics of frozen-thawed stallion spermatozoa. *Equine Veterinary Journal* 28:47-53.
- Hiipakka, R. A. and R. H. Hammerstedt. 1978. 2 Deoxyglucose transport and phosphorylation by bovine sperm. *Biology of Reproduction* 19:368-379.
- Hughes, C. M., S. E. M. Lewis, V. J. Kelvey-Martin, and W. Thompson. 1996. A comparison of baseline and induced DNA damage in human spermatozoa from fertile and infertile men, using a modified comet assay. *Molecular Human Reproduction* 2:613-619.
- Jasko D.J., J. A. Hathaway, V. L. Schaltenbrand, W. D. Simper, and E. L. Squires. 1992a. Effect of seminal plasma and egg yolk on motion characteristics of cooled stallion spermatozoa. *Theriogenology* 37:1241-1252.
- Jasko D.J., D. M. Moran, M. E. Farlin, and E. L. Squires. 1991. Effect of seminal plasma dilution or removal on spermatozoal motion characteristics of cooled stallion semen. *Theriogenology* 35:1059-1067.
- Jasko D.J., D. M. Moran, M. E. Farlin, E. L. Squires, R. P. Amann, and B. W. Pickett. 1992b. Pregnancy rates utilizing fresh cooled, and frozen thawed stallion semen.

- 38th Annual Convention of the American Association of Equine Practitioners. 649-660.
- Jasko, D. J. 1992. Evaluation of stallion semen. *The Veterinary Clinics of North America. Equine practice* 8:129-148.
- Johnson, L., R. P. Amann, and B. W. Pickett. 1978. Scanning electron and light microscopy of the equine seminiferous tubule. *Fertility and Sterility* 29:208-215.
- Kareskoski, A. M., T. Reilas, M. Andersson, and T. Katila. 2006. Motility and plasma membrane integrity of spermatozoa in fractionated stallion ejaculates after storage. *Reproduction in Domestic Animals* 41:33-38.
- Katila, T. 2001. Sperm-uterine interactions: a review. *Animal Reproduction Science* 68:267-272.
- Kayser, J. P., R. P. Amann, R. K. Shideler, E. L. Squires, D. J. Jasko, and B. W. Pickett. 1992. Effects of linear cooling rate on motion characteristics of stallion spermatozoa. *Theriogenology* 38:601-614.
- Kirk, E. S., E. L. Squires, and J. K. Graham. 2005. Comparison of in vitro laboratory analyses with the fertility of cryopreserved stallion spermatozoa. *Theriogenology* 64:1422-1439.
- Knop, K., N. Hoffmann, D. Rath, and H. Sieme. 2005. Effects of cushioned centrifugation technique on sperm recovery and sperm quality in stallions with good and poor semen freezability. *Animal Reproduction Science*. 89:294-297.
- Lopes, S., A. Jurisicova, J. G. Sun, and R. F. Casper. 1998. Reactive oxygen species: Potential cause for DNA fragmentation in human spermatozoa. *Human Reproduction* 13:896-900.
- Love, C. C., S. P. Brinsko, S. L. Rigby, J. A. Thompson, T. L. Blanchard, and D. D. Varner. 2005. Relationship of seminal plasma level and extender type to sperm motility and DNA integrity. *Theriogenology* 63:1584-1591.
- Love, C. C. and R. M. Kenney. 1998. The relationship of increased susceptibility of sperm DNA to denaturation and fertility in the stallion. *Theriogenology* 50:955-972.
- Love, C. C., J. A. Thompson, S. P. Brinsko, S. L. Rigby, T. L. Blanchard, V. K. Lowry, and D. D. Varner. 2003. Relationship between stallion sperm motility and viability as detected by two fluorescence staining techniques using flow cytometry. *Theriogenology* 60:1127-1138.
- Malmgren, L. 1997. Assessing the quality of raw semen: a review. *Theriogenology* 48:523-530.

- Mann, T. 1975. Biochemistry of stallion semen. *Journal of Reproduction and Fertility*. Supplement 23:47-52.
- Martin, J. C., E. Klug, and A. Gunzel. 1979. Centrifugation of stallion semen and its storage in large volume straws. *Journal of Reproduction and Fertility Supplement* 27:47-51.
- Miki, K. 2007. Energy metabolism and sperm function. *Society of Reproduction and Fertility Supplement* 65:309-325.
- Moore, A. I., E. L. Squires, and J. K. Graham. 2005. Effect of seminal plasma on the cryopreservation of equine spermatozoa. *Theriogenology* 63:2372-2381.
- Moran, D. M., D. J. Jasko, E. L. Squires, and R. P. Amann. 1992. Determination of temperature and cooling rate which induce cold shock in stallion spermatozoa. *Theriogenology* 38:999-1012.
- Neild, D. M., J. F. H. M. Brouwers, B. Colenbrander, A. ero, and B. M. Gadella. 2005. Lipid peroxide formation in relation to membrane stability of fresh and frozen thawed stallion spermatozoa. *Molecular Reproduction and Development* 72:230-238.
- Nie, G. J., J. G. W. Wenzel, and K. E. Johnson. 2002. Comparison of pregnancy outcome in mares among methods used to evaluate and select spermatozoa for insemination. *Animal Reproduction Science* 69:211-222.
- O'Flaherty, C., E. de Lamirande, and C. Gagnon. 2006. Positive role of reactive oxygen species in mammalian sperm capacitation: triggering and modulation of phosphorylation events. *Free Radical Biology and Medicine* 41:528-540.
- Padilla, A. W. and R. H. Foote. 1991. Extender and centrifugation effects on the motility patterns of slow-cooled stallion spermatozoa. *Journal of Animal Science*. 69:3308-3313.
- Pagl, R., J. E. Aurich, S. Iler, M. Kankofer, and C. Aurich. 2006. Comparison of an extender containing defined milk protein fractions with a skim milk-based extender for storage of equine semen at 5 °C. *Theriogenology* 66:1115-1122.
- Parinaud, J., D. Le Lannou, G. Vieitez, J. F. Griveau, P. Milhet, and G. Richoilley. 1997. Enhancement of motility by treating spermatozoa with an antioxidant solution (Sperm-Fit) following ejaculation. *Human Reproduction* 12:2434-2436.
- Parks, J. E., J. W. Arion, and R. H. Foote. 1987. Lipids of plasma membrane and outer acrosomal membrane from bovine spermatozoa. *Biology of Reproduction* 37:1249-1258.
- Parks, J. E. and D. V. Lynch. 1992. Lipid composition and thermotropic phase behavior of boar, bull, stallion, and rooster sperm membranes. *Cryobiology* 29:255-266.

- Peris, S. I., J. F. Bilodeau, M. Dufour, and J. L. Bailey. 2007. Impact of cryopreservation and reactive oxygen species on DNA integrity, lipid peroxidation, and functional parameters in ram sperm. *Molecular Reproduction and Development* 74:878-892.
- Pesch, S., M. Bergmann, and H. Bostedt. 2005. Determination of some enzymes and macro- and microelements in stallion seminal plasma and their correlations to semen quality. *Theriogenology* 66:307-313.
- Pickett, B. W., J. J. Sullivan, W. W. Byers, M. M. Pace, and E. E. Remmenga. 1975. Effect of centrifugation and seminal plasma on motility and fertility of stallion and bull spermatozoa. *Fertility and Sterility* 26:167-174.
- Portus, B., T. Reilas, and T. Katila. 2005. Effect of seminal plasma on uterine inflammation, contractility and pregnancy rates in mares. *Equine Veterinary Journal* 37:515-519.
- Potts, R. J., T. Mjefferies, and L. J. Notarianni. 1999. Antioxidant capacity of the epididymis. *Human Reproduction* 14:2513-2516.
- Rathi, R., B. Colenbrander, M. M. Bevers, and B. M. Gadella. 2001. Evaluation of In Vitro Capacitation of Stallion Spermatozoa. *Biology of Reproduction* 65:462-470.
- Revell SG, Pettit MT, and Ford TC. 1997. Use of centrifugation over iodixanol to reduce damage when processing stallion sperm for freezing. *Journals of Reproduction and Fertility*:38. (Abstr.)
- Ritar, A. J. and S. Salamon. 1982. Effects of seminal plasma and of its removal and of egg yolk in the diluent on the survival of fresh and frozen-thawed spermatozoa of the Angora goat. *Australian journal of biological sciences* 35:305-312.
- Rivlin, J., J. Mendel, S. Rubinstein, N. Etkovitz, and H. Breitbart. 2004. Role of hydrogen peroxide in sperm capacitation and acrosome reaction. *Biology of Reproduction* 70:518-522.
- Singh, N. P., D. B. Danner, R. R. Tice, M. T. McCoy, G. D. Collins, and E. L. Schneider. 1989. Abundant alkali-sensitive sites in DNA of human and mouse sperm. *Experimental Cell Research* 184:461-470.
- Storey, B. T. 1997. Biochemistry of the induction and prevention of lipoperoxidative damage in human spermatozoa. *Molecular Human Reproduction* 3:203-213.
- Storey, B. T. and F. J. Kayne. 1975. Energy metabolism of spermatozoa. V. The Embden Myerhof pathway of glycolysis: activities of pathway enzymes in hypotonically treated rabbit epididymal spermatozoa. *Fertility and Sterility* 26:1257-1265.

- Toshimori, K. and C. Ito. 2003. Formation and organization of the mammalian sperm head. *Archives of Histology and Cytology* 66:383-396.
- Troedsson, M. H. T., A. Desvovsuges, A. S. Alghamdi, B. Dahms, C. A. Dow, J. Hayna, R. Valesco, P. T. Collahan, M. L. Macpherson, M. Pozor, and W. C. Buhi. 2005. Components in seminal plasma regulating sperm transport and elimination. *Animal Reproduction Science* 89:171-186.
- Troedsson, M. H. T., I. K. M. Liu, and B. G. Crabo. 1998. Sperm transport and survival in the mare. *Theriogenology* 49:905-915.
- Twigg, J., N. Fulton, E. Gomez, D. Stewart Irvine, and R. John Aitken. 1998. Analysis of the impact of intracellular reactive oxygen species generation on the structural and functional integrity of human spermatozoa: Lipid peroxidation, DNA fragmentation and effectiveness of antioxidants. *Human Reproduction* 13:1429-1436.
- Varner, D. D. 1986. Collection and Preservation of Stallion Spermatozoa. *Proceedings of the Annual Meeting of the Society for Theriogenology*. 13-33.
- Varner, D. D., T. L. Blanchard, S. P. Brinsko, C. C. Love, T. S. Taylor, and L. Johnson. 2000. Techniques for evaluating selected reproductive disorders of stallions. *Animal Reproduction Science* 60-61:493-509.
- Varner, D. D., T. L. Blanchard, C. L. Love, M. C. Garcia, and R. M. Kenney. 1987. Effects of semen fractionation and dilution ratio on equine spermatozoal motility parameters. *Theriogenology* 28:709-723.
- Varner, D. D., T. L. Blanchard, C. L. Love, M. C. Garcia, and R. M. Kenney. 1988. Effects of cooling rate and storage temperature on equine spermatozoal motility parameters. *Theriogenology* 29:1043-1054.
- Varner, D. D. and L. Johnson. 2007. From a Sperm's Eye View - Revisiting Our Perception of This Intriguing Cell. 53th Annual Convention of the American Association of Equine Practitioners. 104-177.
- Varner, D. D., J. Schumacher, T. L. Blanchard, and L. Johnson. 1991. Diseases of the epididymis. In: *Diseases and Management of Breeding Stallions*. P. Pratt, ed. American Veterinary Publications, Goleta. 233-240.
- Vidament, M. 2005. French field results (1985-2005) on factors affecting fertility of frozen stallion semen. *Animal Reproduction Science* 89:115-136.
- Voss, J. L., B. W. Pickett, and E. L. Squires. 1981. Stallion spermatozoal morphology and motility and their relationships to fertility. *Journal of the American Veterinary Medical Association* 178:287-289.

Wilhelm, K. M., J. K. Graham, and E. L. Squires. 1996. Comparison of the fertility of cryopreserved stallion spermatozoa with sperm motion analyses, flow cytometric evaluation, and zona-free hamster oocyte penetration. *Theriogenology* 46:559-578.

Williams, A. C. and W. C. L. Ford. 2001. The role of glucose in supporting motility and capacitation in human spermatozoa. *Journal of Andrology* 22:680-695.

VITA

Jose Augusto Len was born in Panama City, Republic of Panama, in 1970. He grew up in Panama City. He studied veterinary medicine at the School of Veterinary Medicine and Zootechnics of the University of Guadalajara, Mexico, and obtained his veterinary degree in 1994. He then worked in equine reproduction at a Thoroughbred breeding farm in Volcan, Chiriqui, Republic of Panama. In 1998, he became the veterinarian for the Panamanian Racing Commission at the Hipodromo Presidente Remon race track in Panama City, Republic of Panama. From 1999 to 2005, he was the Manager and Chief Veterinarian of Cerro Punta Horse Farm, an internationally renowned Thoroughbred breeding farm in Panama, and the largest in Central America. In 2005, he started a combined residency/master of science program in theriogenology at the School of Veterinary Medicine, Louisiana State University. His main areas of interest are equine and canine reproduction.