Fall 2023

Centrifugation Cushion vs. Spermfilter™: Effects On % Recovery

Holly S. Hopkins
Missouri State University, hh5878s@login.missouristae.edu

CENTRIFUGATION CUSHION VS. SPERMFILTER™: EFFECTS ON % RECOVERY

A Master's Thesis

Presented to

The Graduate College of

Missouri State University

In Partial Fulfillment

Of the Requirements for the Degree

Master of Science, Agriculture

By

Holly Hopkins

December 2023

CENTRIFUGATION CUSHION VS. SPERMFILTER™: EFFECTS ON % RECOVERY

Agriculture

Missouri State University, December 2023

Master of Science

Holly Hopkins

ABSTRACT

It has been demonstrated that removing seminal plasma and resuspension of the sperm pellet in various diluents will improve the post-storage motility of spermatozoa from some stallions. This typically involves centrifugation. However, a sperm filtering device marketed by Botupharma (Phoenix, AZ) allows seminal plasma removal without a centrifuge. This study compared spermatozoa recovery rates using this device vs. centrifugation with and without a cushion. Thirteen ejaculates were collected from three quarter horse stallions. Prior to treatment, aliquots were diluted with Dulbecco's modified PBS at a dilution rate of 1:1. For the two centrifugation treatments, 12 ml of the diluted semen was placed in a 15 ml conical bottom tube and centrifuged at 1000g for 20 minutes with Red Cushion™, (Botupharma), or at 400g for 10 minutes without a cushion. Aliquots for the third treatment were also diluted 1:1, and 20 ml of the solution pour into the SpermFilter™ (Botupharma) for separation. The aliquots' concentration before and after dilution, filtration, or centrifugation was measured using a densimeter device (ARS, Chino, CA). In addition, following all procedures, the sperm pellet was resuspended with the same solution and again subjected to analysis for concentration with the Densimeter to determine the % of spermatozoa recovered. Data was analyzed by One-way ANOVA with Tukey's test for pairwise comparisons. Average % recovery rates were 69.4% and 60.9% for aliquots centrifuged without and with a cushion, compared to 77.6% for the SpermFilter™ treatment. In this experiment, the % recovery was lower ($P<.02$) for those aliquots centrifuged with Red Cushion™ than those separated with the SpermFilter™. However, these results could differ if a different technician compared the same procedures. Furthermore, because of the diluent used following separation, no attempt was made to compare motility following the separation procedures.

KEYWORDS: stallion, sperm filter, centrifugation, cushion, sperm recovery

CENTRIFUGATION CUSHION VS. SPERMFILTER™: EFFECTS ON % RECOVERY

By

Holly Hopkins

A Master's Thesis
Submitted to the Graduate College
Of Missouri State University
In Partial Fulfillment of the Requirements
For the Degree of Master of Science, Agriculture

December 2023

Approved:

Gary Webb, Ph.D., Thesis Committee Chair

Lacy Sukovaty, D.V.M., Committee Member

Adam McGee, Ph.D., Committee Member

Julie Masterson, Ph.D., Dean of the Graduate College

ACKNOWLEDGEMENTS

I want to thank the faculty and staff at Missouri State University for their help in getting me through the master's program, Dr. Gary Webb, Mr. Will Boyer, and my thesis committee. Coming here to complete my schooling with a master's degree with the great staff here in the animal science department has been one of my best decisions. All my great experiences and memories of my time at Missouri State University will last me a lifetime.

Thank you to Dr. Webb and Mrs. Mook for letting me find my passion in teaching students to ride, whether it was the introduction to riding classes, equestrian, or ranch horse team practices. This experience has allowed me to gain confidence in my knowledge, from starting horses to being a leader, which I never thought was possible.

I am grateful for the support given by my family with my choice to continue my learning and to be far from home. The distance was challenging; however, Natalie Mook, Will Boyer, and Sue and Gary Webb stepped up and were there for me when I needed them. I am excited to see what opportunities this institution has provided for my career goals.

I dedicate this thesis to my parents Susan and Richard Hopkins for supporting and encouraging my educational goals.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

Justification

The equine associations that allow semen to be stored and shipped have given the industry advantages. These advantages include reduced risk of disease and injury to the mare and foal, increased access to a more prominent gene pool, elimination of the cost of mare care, reduction in the possibility of sexual transmission diseases, and increased interest in breeding (McKinnon and Voss, 1993). However, the ability to store stallion semen, either cool storage or frozen, has some downsides. Cold shock is the stress of a temperature change that can result in irreversible damage (McKinnon and Voss, 1993), It was determined that stallion semen should slowly cool over 2 hours with semen extenders that have additional benefits. Benefits that were added to extenders for antibiotics, minerals, and nutrients that prolong longevity and improve physiology (Cuervo-Arango et al., 2015).

For semen from some stallions to survive storage at cool temperatures or for freezing the seminal plasma must be removed before storing semen at cold temperatures. However, the process of centrifugation can cause structural damage to the acrosome, loss of motility, and enzyme activity (Matas et al., 2007). In an effort to minimize the detrimental effects of centrifugation, the SpermFilter™ was developed and later marketed by Botu-pharma (Phoenix, AZ). The first study, led by Neto in 2012, compared stallions known as good or bad coolers and separated by sperm quality. Following cold storage, total and progressive motility were evaluated, and no significant difference was found between the treatments or the quality of the stallion's semen. The percentage of sperm recovered when filtered was 89.4% and higher than when centrifuged at 80.9% (Neto et al., 2012). A second study found the same results with the

percentage of sperm recovery and when the percentages of kinetic parameters and plasma integrity (Neto et al., 2013). A third study used centrifugation with a cushion compared to the SpermFilter™ and found that the cushion excelled in total and progressive motility compared to those separated with a filter (Schnobrich et al., 2016).

Problem Statement

There are many ways to process stallions' semen for storage. Botu-pharma produced the SpermFilter™, and a centrifugation cushion media called Red Cushion. The manufacturers claim that the products produce similar recovery rates of spermatozoa. The SpermFilter™ is cheaper and more user-friendly for the general population in the equine industry who do not have access to lab equipment. Red Cushion is an option if there is access to a centrifuge, and it makes it easier to separate the sperm pellet from the supernatant and the cushion because of the red color dye.

Objective

This study aims to analyze equine semen samples that were centrifuged with a centrifugation cushion and samples with the SpermFilter™ on the effect of the percentage of sperm recovery.

Null Hypothesis

Neither centrifugation with Red Cushion™ nor the SpermFilter™ method will influence the recovery rate of spermatozoa.

LITERATURE REVIEW

Artificial Insemination Regulations

Although rules vary for most equine breed associations, most allow artificial insemination, except the Jockey Club which is the breed registry for the Thoroughbred. For example, the American Quarter Horse Association did not allow cooled semen to be shipped until 1997 and did not allow the shipment of frozen semen until 2001 (Hopkins and Meadows, 2003). However, this association does not allow semen to used two years after the stallions' death from the birth from the birth year 2015 (American Quarter Horse Association, 2018).

According to information published in their website, the Arabian Horse developed a 1995 was the first that owners were allowed to transport cooled semen (Arabian Horse Association, 2003). American Quarter Horse Association does not allow semen to be used two years after the stallions' death from the birth year 2015 (American Quarter Horse Association, 2018).

History of Artificial Insemination

The first documented case of artificial insemination of horses was found in an Arabian veterinary textbook; the case dates back to the 14th century (Aurich, 2012). Tribes in Sudan removed the Further progress was made when Antonie Van Leeuwenhoek crafted the first microscope in 1678. This allowed humans to see spermatozoa and was thought to be the predecessor of living creatures (Figure 1) (Aurich, 2012).

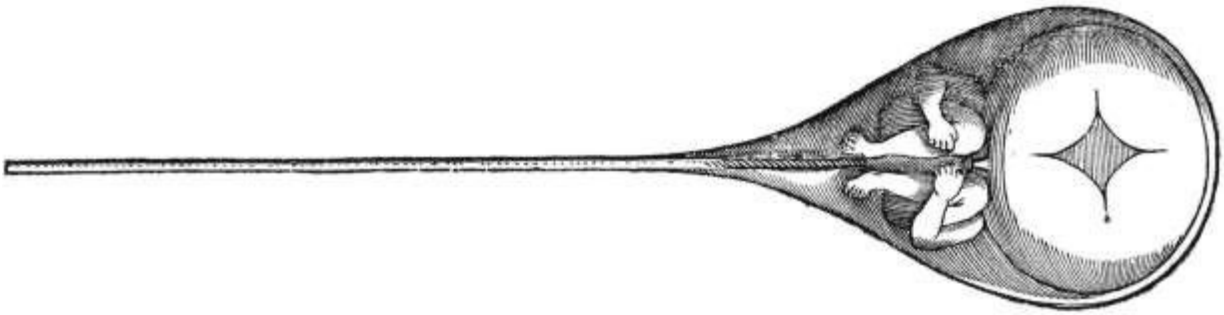

Fig. 1. Illustration of a homunculus in sperm (Lonergan, 2018)

Iwanoff conducted the first systematic study on reproductive technique in horses; the AI procedure used involved the insertion of a silk sponge in the mares' vagina before a stallion mounted to ejaculate. The sponges would be removed and placed into a designed press to extract semen (Aurich, 2012). Insemination was completed using a rubber tube and syringe; the procedure yielded high success rates. During the 1920s, research focused on methods to reduce sexually transmissible diseases which includes artificial vaginas, phantoms, and semen extenders were developed (Aurich, 2012). Semen extenders are fabricated with various ingredients for the base: milk, egg yolk, Ringer, and Locke's solutions. Ejaculations were diluted with extenders to allow the ability to be stored, and increase volume to breed more mares (Aurich, 2012). Finally in 1938, Walton succeeded with artificial insemination; semen had been centrifuged to remove seminal plasma, diluted with a glucose extender, and stored for 48 hours at 0°C.

Semen Physiology

The physical quality of how spermatozoa are built is known as morphology. Some defects include detached heads, knobbed acrosomes, proximal and distal protoplasmic droplets, bent midpieces, and coil tails. These affect fertility since the spermatozoon cannot fertilize the

ovum (Brinsko et al., 2011). Parts of the spermatozoon are labeled based on function and anatomy (Figure 2).

The plasma membrane layer comprises lipids, proteins, and carbohydrates covering the whole cell. Under the plasma membrane layer around the head, two-thirds of the head is covered by the acrosome, which contains enzymes that break down the layer of the zona pellucida once the head is attached to the ovum. Under the acrosome membrane, the chromosomes are contained in the nucleus (Samper, 2009).

The midpiece contains multiple mitochondria containing enzymes and other organelles necessary to produce ATP. Which are necessary for the tail's movement to propel the cell toward the ovum (Samper, 2009). Energy is produced from carbohydrates and sugars. Stallions utilize glucose as the primary source of ATP. While oxygen is present, the cell uses an aerobic metabolism (McKinnon and Voss, 1993). Energy is broken down by the Krebs cycle. However, this process produces compounds that are harmful to the spermatozoa. This includes reactive oxygen species such as H2O2 or hydrogen peroxide (Pena et al., 2022). Reactive oxygen species are harmful because of the effects on motility, integrity, and viability of sperm, as well as the permeability of the plasma membrane (McKinnon and Voss, 1993). High levels can cause damage which affects the functionality of the membrane by peroxidation of the lipids contained in the membrane (Pena et al., 2022). Damage like that resulting from centrifugation increases the amount of reactive oxygen species produced.

To complete fertilization of the ovum, spermatozoa must undergo a process called capacitation. This occurs once the spermatozoa are in the uterus and allows the sperm head to bind to the zona pellucida. (Samper, 2009). After binding, the acrosome membranes fuse with plasma, which allows the enzymes in the acrosome to break down the zona pellucida, permitting

the spermatozoa to penetrate the zona. Finally, the spermatozoon's nucleus can access the oocyte's nucleus to fertilize (Brinsko et al., 2011).

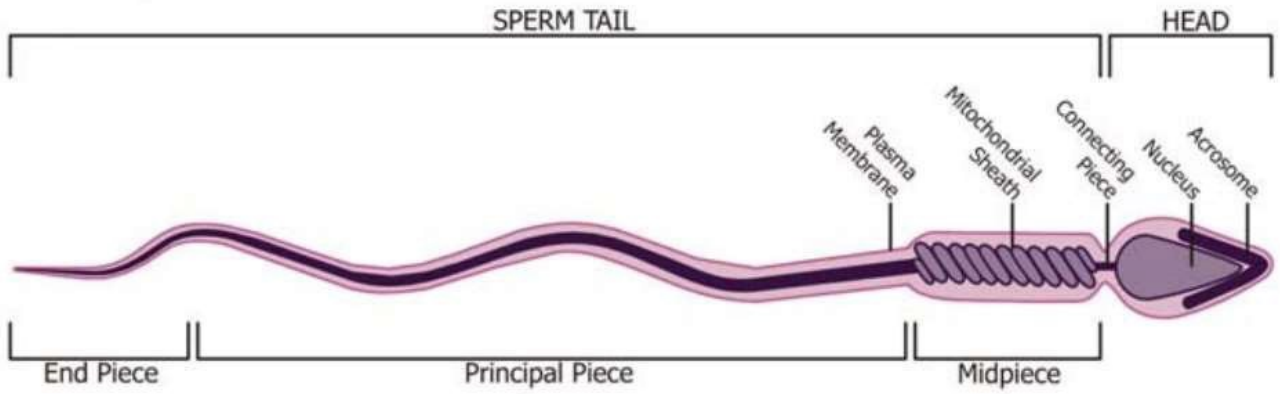

Fig. 2. Anatomical structure of a spermatozoa (Borg et al., 2010)

Response to Temperature Change

In order to prolong fertility of spermatozoa it is necessary to slow their metabolic process by lowering the temperature. However, the cooling process causes stress on the cell and damage (Marshall et al., 1984). In order to maximize the retainment of fertilizing capacity and motility, the cooling-down process is usually slow between temperatures of 68° F or 20 º C and 0º C because rapid cooling is known to cause irreversible damage. This includes abnormal swimming patterns, loss of motility, injury to plasma or acrosomal membrane, loss of intracellular components, and reduced metabolism (McKinnon and Voss, 1993). The term used for this type of damage is cold shock (McKinnon and Voss, 1993). The sperm membrane is designed with a bilayer of hydrophilic lipids and proteins surrounded by phospholipids and cholesterol (McKinnon and Voss, 1993). Each lipid has a different characteristic that makes up the membrane and makes the composition important (Figure 3). When enduring the cooling process, the lipid's natural state changes to a lipid-crystalized state (McKinnon and Voss, 1993). This is

because fatty acyl chains are disrupted to the gel state and transformed, causing the chains to become increasingly rigid and parallel. Lipids change and clump up in the microdomain of the lipid gel, causing the lipids not to be with their designated counterpart (McKinnon and Voss, 1993). Alternatively, borders between the microdomains and fluid portion create unstable ion-permeable gaps, which can cause a rupture of the membrane or membrane fusion.

Protein's function is transporting matter across the membrane, but activity decreases when temperature decreases. When both enzyme and transport activities have decreased can be an effect on the components and functions of the spermatozoa, like altered combinations and irreversible denaturation of proteins (McKinnon and Voss, 1993). Furthermore, the proteins associated with those specific lipids stopped functioning correctly because their interaction had been disrupted (McKinnon and Voss, 1993).

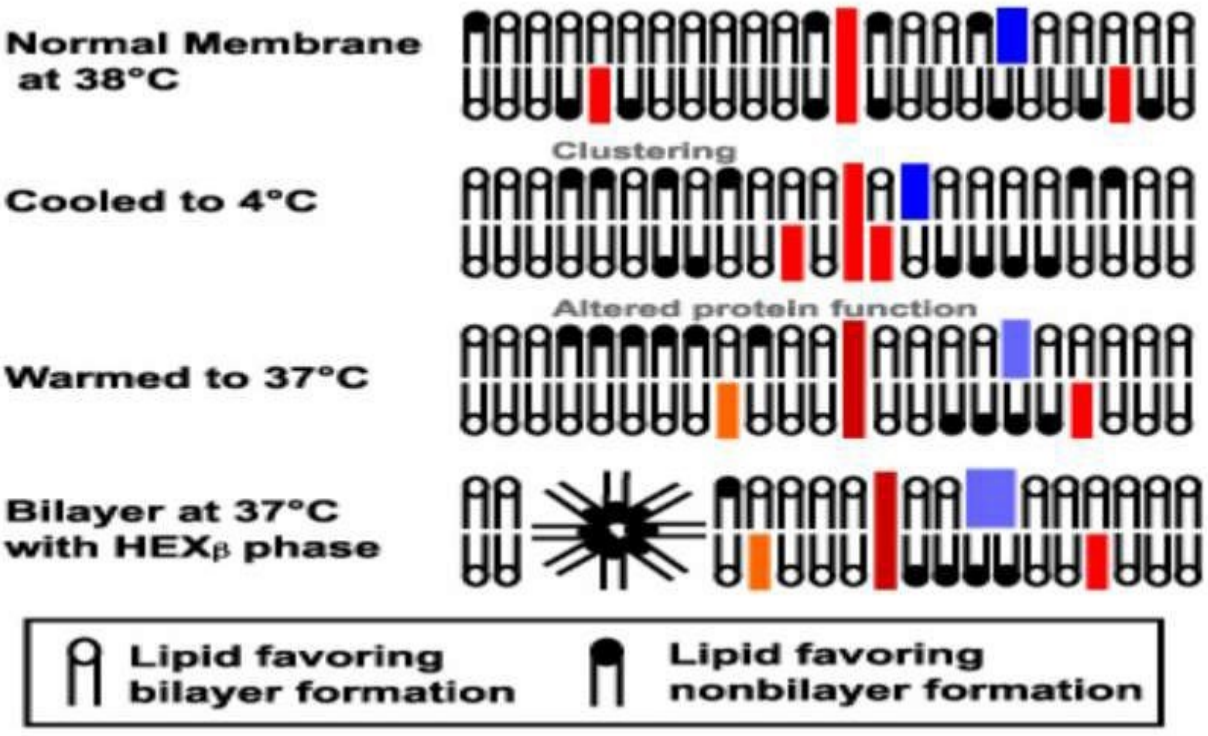

Fig 3. Representation of how thermal reaction affects the plasma membrane on the spermatozoa (McKinnon and Voss, 1993).

Semen Factors

Seasonal. The sexual behavior of stallions will correspond with the mares, which makes them seasonal breeders. During spring and summer, mares experience estrus and ovulation compared to fall and winter (Pickett et al., 2010). In a study conducted at CSU, stallions were exposed to extended photoperiods using schedules similar to those used to hasten the onset of the breeding season in mares. The study occurred over ten months, starting in October 1984 and ending in July 1985. The control group was exposed to natural light compared to groups exposed to 16 hours of light to mimic the amount of sunlight (Pickett et al., 2010). Three months with various photoperiods occurred during different seasons: October, February, and June; seminal

and behavioral characteristics' yields were significantly different (Pickett et al., 2010). In another study from May to May of the following year, stallions were collected twice a day when they were collected. Pickett found that in January, the first ejaculate averaged a total volume of 45 ml, compared to the average total volume increased in June to 104 ml (Pickett et al., 2010). The average total volume decreased with the second ejaculation to 52 ml; during March, the total volume of 42 ml increased to 68 ml during June. Photoperiod also impacts hormones. From January to October, testosterone levels ranged from 3.2 ng to 1.5 ng per ml (Pickett et al., 2010). The time it took stallions to respond to sexual stimuli was lower due to the fluctuation in the endocrine system. Characteristics evaluated were total sperm, concentration, motility, and gel vs. no gel volume, while the behavior was time to complete erection, first mounting, ejaculation, and the number of mounts (Pickett et al., 2010). Progressive motile sperm ranged from 70 to 75% and was found to decrease in winter. The only significant difference found with most characteristics was during the season.

Testis Size. The amount of testicular tissue affected is the critical factor in how much sperm is produced daily (DSP) and output (DSO). Stallions with large testis produced more spermatozoa because the scrotal width contains more tissue (Pickett et al., 2010). When using artificial insemination, stallions with average scrotal width can cover 12 mares on collection days.

Age. Forty-eight stallions were divided into three different age groups, and their testis was measured before castration. The average sizes were 96 mm, 100 mm, and 109 mm (Pickett et al., 2010). When the three groups were compared, 4-6 and 9-16 years old were not significantly different. A slight difference was found between 2-3 and 9-16 years old; however, the two groups with the most significant difference were 2-3 and 4-6 years old (Pickett et al., 2010).

Collection Frequency. In another study by Pickett in 1989, stallions were collected once, thrice, and six times a week with a week of rest between switching schedules. Once a week, collections produced the highest total volume, concentration per ml, and quickest reaction time (Pickett et al., 2010). When stallions were collected six times a week produced semen at the lowest levels. Three times a week, parameter values fell between 1x and 6x; however, the amount of sperm harvested was the same at 3x and 6x days of collection (Pickett et al., 2010).

Sexual Behavior. Signs of abnormal behavior can be a struggle to perform, low libido, no erection reaction, cannot maintain erection, and inability to ejaculate. Sexual behavior is measured by the number of mounts per collection and reaction time after the stallion has entered the breeding shed. Pickett found that the reaction time changed through the season, which could be 3.4 minutes during August as long as 14 minutes in January (Pickett et al., 2010). The number of times the stallions had to mount the phantom until successful ejaculation was low in spring

was 1.4 mounts, and during fall increased to 2.3 mounts. Another study evaluated how teased and teasing affects semen and behavior characteristics. Those who were teased had half the volume compared to the stallions who were not teased. Unteased stallions were found to have longer reaction times (Pickett et al., 2010).

Semen Extenders

In order to improve results, stallion semen is diluted with liquids known as semen extenders. The benefits of extenders are to aid the proper evaluation of sperm and improve longevity, viability, and motility in unfavorable environments (McKinnon and Voss, 1993). The dilution rate of semen extender to semen varies based on concentration levels and use of semen; the ratio can range from 1 to 10 ml of extender per 1 ml of semen after the gel portion is caught in the gel filter of the AV. Formulas of extenders differ because of the various ingredients based and how well the semen from different stallions respond to the extender. Components of the extender contain lipoproteins, glucose, and antibiotics to assist with protection from cold shock, provide a source of energy, and kill bacteria (Brinsko et al., 2011).

Processing Time

During the development of the artificial insemination technique (AI), multiple studies using various animals, and the yield had mixed results were conducted (Aurich, 2012). In 1799, several successful cases of the AI were accomplished in humans; however, Pope Leo XIII banned the use of artificial insemination method on humans in 1897. A study evaluated the different processing techniques and their effect on motility and acrosomal integrity after storage at 37C. Eighteen stallions were collected in one part of the study, and the ejaculation was divided

into aliquots (Dawson et al., 2000). Aliquots were processed later by 5, 15, and 30 minutes. Approximately 25 million sperm per ml were packaged in each aliquot after they had been processed (Dawson et al., 2000). After 24-hour of storage, samples that had a delay of 5 minutes produced had the highest percentage of progressively motile sperm and intact acrosomes. No significant were found between samples that were delayed for 15 and 30 minutes during the processing; levels of post-motility declined (Dawson et al., 2000).

Various Types of Extenders

INRA96 was first compared in 1998 to the standard way of diluting equine semen with INRA82 and Kenney's (Batellier et al., 1998). INRA82 contains superheated skim milk, glucose, lactose, raffinose, sodium citrate, potassium citrate, and distilled water, compared to INRA96 is made up of various salt solutions, glucose, lactose, and casein. In experiment 1, semen was stored for 24 hours, and the pregnancy rate was 40% with INRA82 and 57% with INRA96 (Batellier et al., 1998). Experiment 2 evaluated semen after 0 and 72 hours of storage and found that INRA96 had a higher percentage of pregnancy rate.

Another group studied the effect of motility with extenders and seminal plasma (Rigby et al., 2001). The extenders used were skim milk-glucose (CST) and skim milk glucose with Tyrode's media (KMT). Each extender had an aliquot which wasuncentrifuged with a dilution ratio of 1:4 or 25 million sperm per ml and centrifuged to contain 0% or 20% seminal plasma (Rigby et al., 2001). Aliquots that would be centrifuged were diluted to a 1:3 ratio with the designated extender. To accomplish this the centrifugated had to be setfor ten minutes at 1000g. Seminal plasma was collected from raw semen and added to the aliquots as needed for the desired percentage (Rigby et al., 2001). Samples extended with CST and 0% seminal plasma had

lower motility than those with 20% SP. KMT with 0% seminal plasma was better than CST at either percentage overall. KMT with 20% seminal plasma had better motility than either CST samples (Rigby et al., 2001). The study concluded that KMT had better results when no seminal plasma was present, and parameters decreased when there was seminal plasma (Rigby et al., 2001).

In another study, two stallions in an active breeding program known not to have good-quality semen after storage were collected every other day and extended with Phosphate Buffer Saline (Webb and Arns, 2004). Ejaculations were divided into 5ml of raw semen aliquots to be centrifuged at 300-400g for 5 minutes. The control group was extended with a skim milk extender; others had skim milk and Phosphate Buffer Saline (Webb and Arns, 2004). Samples were resuspended with their same extenders at the concentrated of 50 million progressively motile sperm per ml, and two samples contained 1 billion. After the samples were stored for 24 and 48 hours at 5°C, the percentages of motile sperm were evaluated. After 24 hours of storage, the control group had 17%, and skim milk with PBS had 45% (Webb and Arns, 2004). Percentages of PMS after 48 hours were 1% for the control and 14% for skim milk with PBS.

Love et al. also evaluated how sperm motility is affected by the type of extender and seminal plasma levels. Extenders that were used were Kenney's (CST) and Kenney's mixed with potassium and Tyrode's media (KMT) (Love et al., 2005). Semen was diluted to contain 20% and 0% of seminal plasma and then centrifuged for nine minutes at 500g. After 24 hours of storage, the samples were centrifuged, and a higher percentage of total and progressive motility when seminal plasma was at 0% with KMT overall. CST had a higher percentage of motility when seminal plasma was at 20% (Love et al., 2005).

The effects of seminal plasma with three different extenders were compared with INRA96, VMD-Z, and Kenney's SKMG extenders had aliquots that had seminal plasma or were removed. Samples that contained seminal plasma were diluted to a 1:4 ratio to result in 25% seminal plasma (Webb et al., 2009). Aliquots followed the same process, but seminal plasma was removed and aliquots were resuspended. Samples were divided into 2 ml, stored into Equine Express II, and stored for 72 hours (Webb et al., 2009). All three extenders with seminal plasma experienced a significant difference in total motility at 2 hours after collection with no difference in progressive. Total motility presented higher percentages with INRA96 with seminal plasma than without seminal plasma (Webb et al., 2009). SKMG had the lowest percentages of total and progressive motility compared to the other two extenders. Samples were centrifuged to separate the damages after storage when motilities were evaluated.

In a study to evaluate storage of epididymal sperm; ten stallions were castrated; the collected testis and epididymides were dissected. The collection of spermatozoa happened by a retrograde flush of epididymis using PBS with calcium and magnesium (Neuhauser and Handler, 2017). Concentration levels contained 25 million sperm per ml after being diluted with skim milk, milk proteins, egg yolk, and INRA96. After storage, the extenders affected sperm characteristics (Neuhauser and Handler, 2017). INRA96 and milk protein extender produced the highest motility levels, and egg yolk had the lowest percentages in kinetic parameters.

Another study used on the semen extender INRA96, and some aliquots were extended with the addition of antibiotics. Addition antibiotics were combinations of amikacin disulfate and potassium penicillin (Hernandez-Avila et al., 2019). Samples were evaluated 30 minutes after mixing and 24 hours after storage on quality and bacteria growth. A significant difference was

found between progressive motile sperm and straightness with INRA96 and additional antibiotics, as well as minimum bacteria growth (Hernandez-Avila et al., 2019).

Storage Containers

A study by Novello et al. (2020) compared extenders with storage containers to find the best results. Whirl-Pak bags were filled with extended semen with INRA96 or Botu-Semen Gols and then stored in Equitainer, Equine Express II, or the Botu-Flex. Samples were at room temperature for 30 minutes before being placed into the cooling containers at 5°C for 24 hours (Novelle et al., 2020). The final concentration was 100 million sperm per ml pre-storage. Each container held 16-20 samples; no significant difference was found in velocities; both extenders had better motility in Equitainer and Equine Express II. Characteristics decreased post-storage with both extenders (Novelle et al., 2020). Aliquots diluted with Botu-Semen Gold and stored in the Equintainer had lower plasma membrane integrity and better velocity than INRA96, regardless of storage container and time (Novello et al., 2020). The parameters had no significance when the three storage containers.

Nicotinic acid was used as a supplement in semen extenders for cool and frozen storage (Bahrami et al., 2020). Semen was extended with Kenney's, then centrifuged to be resuspended with 50 million sperm per ml for cooled storage and 200 million sperm per ml for cold storage. Samples that were only extended were the control group, and treatments contained 10, 20, and 30 mM (millimole) of Nicotinic Acid (Bahrami et al., 2020). The storage time starts when the samples reach 5°C and is analyzed at 6, 18, and 48 hours. Total motility decreased with all groups and throughout the storage period (Bahrami et al., 2020). The highest percentage of total motility was at 6 and 42 hours with 40 mM of Nicotinic Acid. Fast progressive sperm had the

highest levels after 42 hours with 20 and 30 mM (Bahrami et al., 2020). Velocity parameters in all samples were significantly deceased except the straightness and average path for samples with 40 mM. Viability consisted of the same pattern, and parameters only slightly decreased after 42 hours (Bahrami et al., 2020). Plasma membrane integrity declined after 18 hours in all groups, but 40 mM had the lowest percentage after 42 hours.

The same experiment was repeated, but for cold storage, the concentration contained 200 million sperm per ml. Samples were above liquid nitrogen for 15 minutes before being placed into the liquid nitrogen for 24 hours (Bahrami et al., 2020). Samples for the control group contained 0% Nicotinic Acid had the worst motility percentages. Most of the decrease in velocity parameter occurred after 6 hours; abnormal morphology increased after 6 hours in the control group. Overall, there was no change in the intact plasma membrane.

When comparing the two INRA extenders specifically INRA82 which it's is for freezing and INRA96 used for 48 hours cool storage . Ejaculations were split into aliquots and processed through single-layer centrifugation (SLC), sperm-washing, and extension (Papin et al., 2021). A sperm-washing group was centrifuged and suspended to 30 million sperm per ml. CASA (Computer Assisted Sperm Analysis) was used to analyze samples, and the study found that the treatment, time, and extender had significant reactions between them (Papin et al., 2021). The velocity parameters were higher with INRA82 most of the time, while this extender with this extender had the highest percentage of curvilinear velcocity also. If the aliquots received sperm washing the motility was found to increase; however, not in INRA96 at 0 and 96 hours (Papin et al., 2021). Single layer centrifugation and sperm washing resulted higher velocities with INRA82 than INRA96. Many protocols use less expensive extenders for centrifugation since the semen is only exposed to it for 10 minutes or less.

A study evaluated if repeated freeze and thawing of sperm affect the sperm quality and concentration; there were eight treatments with various concentrations per milliliter ranging from 5-400 million (Morse-Wolfe et al., 2023). Semen was diluted with EDTA (ethylenediaminetetraacetic acid lactose extender) containing 20% egg yolk and 3% dimethylformamide; after samples were centrifuged, the sperm pellet was the only part left. Straws were chilled slowly for 25 minutes at 4°C and then placed into the automated freezer. Once the freezer reached -143°C, straws are submerged in liquid nitrogen (Morse-Wolfe et al., 2023). Post thaw motility of spermatozoa was not to be affected by the concentration. Other parameters like percentages of rapid sperm cells, total motility, and progressive motility were higher with a concentration of 50-400 million than those below 50 million. A concenttration of three hundred million produced the highest percentages of sperm parameters However, the differences were insignificant enough to conclude that concentration levels had no effect (Morse-Wolfe et al., 2023).

Agents Added to Extenders for Freezing of Spermatozoa

An experiment compared different semen extender formulas that varied by the addtion of the cryoprotectants dimethylacetamide, dimethylformamide, or methlyformamide at 4%. Samples were first diluted to 50 million sperm per ml with Botu-Semen™ extender, then stored in Equitainer or Max-Semen Express (Agrofarma, SP, Brazil) for 24 hours prior to freezing (Melo et al., 2007). The treatment with methlyformamide produced the highest progressive motility when cooled with both storage containers, but the best average percentages of straightness and progressive motility were when cooled with Equitainer. There were no significant differences in progressively motile sperm between dimethylacetamide and

methlyformamide when stored in Max-Semen Express. The component of methlyformamide was better than dimethylformamide in the extender. Sperm motion did not significantly differ between types of storage; however, there was a significant difference in the cryoprotectant comparison (Melo et al., 2007). Both storage containers were efficient with sperm characteristics on motility and velocity; however, glycerol and methylformamide had the best progressive motility, but no other parameter had a significant difference between cryoprotectants (Melo et al., 2007).

Different studies have studied the effects of egg yolks when used in cryopreservation. Samples of turkey, duck, chicken, and chicken with omega-3 were analyzed for fatty acid content and profile (Burris et al., 2009). EZ-Freezin™ (ARS, Chino, CA) was used in the control group. Semen was diluted with INRA96 to a 1:1 ratio; seminal plasma was removed by centrigugation , and the final concentration contained 125-150 million sperm per ml (Burris et al., 2009). Samples were resuspended with egg yolk, loaded into straws to be cooled above liquid nitrogen for 8 minutes, and then submerged in liquid nitrogen. Straws were stored for three weeks minimum and then after thawing two straws of each extender were analyzed with CASA and Spermac Stain™ kit, which evaluates the acrosome status (Burris et al., 2009). The various egg yolks was evaluated that there was no effect on velocity or intact acrosomes with the various extenders. The results of EZ-Freezin™, duck, and turkey egg yolk were highly similar; however, turkey was the dominant yolk compared to egg yolks from chickens in terms of post-thaw motility (Burris et al., 2009). The addition of specific agents to a semen extender produced significant results in improving certain sperm characteristics that are highly beneficial for post-storage purposes.

Spermatozoa Concentration

Semen can be processed differently based on the plan for the collection. Shortly after collecting the stallion, the ejaculation is diluted with a semen extender. The ratio ranges from 1:1 to 1:10 (semen: extender). Semen naturally has a short life expectancy; however, diluting semen with an extender prolongs longevity (Samper, 2009). When semen has been collected should be used within 6 hours, and the ratio can be 1 part semen and 1 or 2 parts extender (Samper, 2009).

In a second part of the study conducted by Dawson et al , aliquots were diluted to contain the concentration of 25, 50, and 75 million sperm per ml with INRA96. Another set of samples was centrifuged, resuspended, and diluted again with Skim-milk with phosphate buffer saline (PBS). Following storage some samples were centrifuged for 1 minute at 1000g, resuspended with 95% ethanol, and incubated for 30 minutes to evaluate plasma membrane integrity after storage (Dawson et al., 2000). After Twenty-four hours of storage results compared the seminal plasma present or removed to concentration levels, proving a slight difference. Concentrations of 25 million per ml resulted higher percentages of progressively motile sperm after 24 and 48 hours when seminal plasma was removed (Dawson et al., 2000). The percentage of intact plasma membrane consisted of the same pattern as concentration levels; higher concentration with lower intact membranes and seminal plasma removed had better results than when seminal plasma was present.

In Australia, the effect of the number of progressively motile sperm on the pregnancy rate was tested with one 10-year-old standardbred stallion. Mares that were insemination with lower doses of 400-700 million sperm had a pregnancy percentage than mares inseminated with 700-1,000 million sperm (Newcombe et al., 2005). Forty-one mares were inseminated with more than 1.5 billion sperm in a dose and had a good percentage of pregnancy. Pregnancy is achievable

with 400 million progressively motile sperm per straw when chilled (Newcombe et al., 2005). Slight improvements were found with doses of more than 400 and less than 700 million in low volume with high concentration.

In a different study, samples contained various ranges of seminal plasma from 20-40%, analyzed with CASA at 0, 24, and 96 hours after storage. Centrifuged samples had higher percentages of progressively motile sperm, total motility, and plasma membrane integrity after storage than the control group, which contained seminal plasma (Len et al., 2020).

Centrifugation

The effects of extender and centrifugation were investigated when semen was slowly cooled on the motility patterns. Aliquots were diluted with Kenney's extender (KE), and Kenney's extender with high potassium Tyrode's medium (KMT) added for this study the sperm concentration was 50 million per ml (Padilla and Foote, 1991). Aliquots that received centrifugation were resuspended; all samples were cooled slowly to 4°C (Padilla and Foote, 1991). The evaluation of sperm occurred 0, 24, 48, and 72 hours after storage; evaluated parameters were total motility, velocity, and linearity. In the samples without centrifugation, KMT had higher velocity, but the linearity was better with KE. The aliquots centrifugated with the KMT extender had higher percentages of velocity and linearity than KE after storage (Padilla and Foote, 1991). Aliquots stored without seminal plasma removal were observed at the different storage times with the non-centrifugation KMT samples having the lowest. However, aliquots that were centrifuged and stored with KMT had higher motility (Padilla and Foote, 1991).

A study used ten stallions that were assigned into two groups based upon how their semen survived the cooling procedure and were classified as "good" or "bad" (Brinsko et al.,

2000). Two aliquots were diluted to a 1:3 ratio of extender to semen resulting in a final percentage of 25% seminal plasma. Following samples were stored in Whirl-Pak bag and then packed in an Equintainer. The remaining aliquots was extended 1:1 and centrifuged for 12 minutes at 400g then the supernatant was removed with around 12% seminal plasma (Brinsko et al., 2000). The resuspended sperm pellet were stored, placed in an Equitainer and evaluated following 24 and 48 hours of storage. Before storage, CASA was used to analyze the samples. At 0 hours, there was significant difference between centrifuged, and non-centrifuged samples (Brinsko et al., 2000). Non-centrifugated samples from the good cooler group had higher percentage of motile sperm in storage than the bad cooler group. When the centrifuged groups were compared, there was no significant difference between the groups (Brinsko et al., 2000). After 24 hours of storage, "Good coolers" had lower total motility when centrifuged than non-centrifugation that is indicating possible damage due to centrifugation.

At Sao Paulo State University in Brazil, a study looked at cryopreserve for 24 hours in an Equitainer and various cryoprotectants with two storage systems (Melo et al., 2007). All stallions used in this were labeled "good coolers". Ejaculates were split into aliquots. One aliquot contained Botu-Crio; the other was diluted to contain 50 million sperm per ml (Melo et al., 2007). Samples were centrifuged at 600g for 10 minutes, and the supernatant was removed and replaced with Botu-Crio™ to be stored in an Equitainer for 24 hours. The samples packed into straws contained 100 million ml. The straws were placed above liquid nitrogen for 20 minutes at 5°C and then submerged in the nitrogen (Melo et al., 2007). There was no effect of cold storage of semen before freezing on total and progressive motility (Melo et al., 2007).

In a later study, the effects of centrifugation techniques on post-storage motility were compared using two centrifugation methods and two sets of speed and duration. One

centrifugation method involved diluting 5 ml of raw semen to a 1:1 ratio, followed by centrifugation at 400g for 7 minutes in a 15 ml tube with a conical bottom. The second method involved dilution of a final concentration of 50 million per ml, followed by centrifugation at 700g for 15 minutes (Webb and Dean, 2009). Aliquots from each treatment were then extended with INRA96 with 4% chicken egg yolk and 4% glycerol and frozen. A second part of the experiment used different centrifugation speends and times were compared. An estimated 85-90% of the supernatant was removed by centrifugation. Aliquots centrifuged at a lower speed of 400g, had higher percentages of total and progressive motility compared to those centrifugated at 600g and 700g.

Percentages of Seminal Plasma

The composition of the seminal plasma can vary individual to the stallion and can be affected by environmental factors (Usuga et al., 2017). Seminal plasma has specific proteins that can be helpful or harmful for particular effects on sperm quality (Jobim et al., 2011). Parameters of sperm motility and physical charaterisits decrease when severe stress occurs, including thermal, osmotic, and oxidative (Oldenhof et al., 2013). In a study by Pickett in 1975, two samples containing 2% seminal plasma were compared to two other aliquots with 10% SP. Each sample was centrifuged at 370 and 829 g force for five minutes. The results found that the percentage of seminal plasma significantly differed in the motility rate before and after freezing compared to the centrifugation (Pickett et al., 1975). No significant difference was found when the centrifugation force was compared; however, there was a slight pattern with the higher centrifugation force producing lower motility rate. The second experiment examined the effects of seminal plasma from 0-10% before and after storage (Pickett et al., 1975). The samples had 0-

10 percent of seminal plasma and were centrifuged at 956 g forces; aliquots were diluted to a 1:9 ratio.

Dawson's study found that the removal of seminal plasma was beneficial for spermatozoa of some stallions with post-storage of 24 and 48 hours. The progressive motility significantly decreased when the concentration of sperm increased, and seminal plasma was present. No significant difference occurred when the various concentration levels did not contain the seminal plasma (Dawson et al., 2000). A concentration of 75 million sperm prolonged storage because the concentration increased progressive motile sperm decreased when seminal plasma was present.

In addition to freezing in liquid nitrogen, separate aliquots of each treatment were placed into a 2 ml tube for analysis after 24 and 48 hours of storage at 5°C. Frozen centrifuged aliquots had the best total and progressive motility at 600g, but the lowest was 400g. Samples centrifuged at 700g for 15 minutes had lower levels of total motility than the control group of non-centrifugation samples. There was no significant difference in motility values when 50 ml tubes were centrifuged at 600g compared to non-centrifugation samples (Webb et al., 2009). The sperm recovery rate resulted in no differences with samples centrifuged at 700g; however, semen centrifuged in 15 ml tubes had a higher percentage of total motility than the 50 ml. The study of centrifugation techniques concluded that there was no effect on the percentage of intact acrosomes after storage (Webb et al., 2009).

A study by Barrier-Battut et al. centrifuged aliquots for 10 minutes at 600g; the supernatant was removed and replaced with INRA96 labeled, or seminal plasma was added back into the resuspended semen. Aliquots were stored for 48 hours and analyzed with CASA (Barrier-Battut et al., 2010). CASA had found significant improvements in the plasma membrane

integrity with no seminal plasma. This study determined that when seminal plasma is less than 33%, the dosage does not affect seminal plasma. The results agreed with the Dawson study of 2000.

Seminal plasma was collected through centrifugation at 600g for 15 minutes and stored at -18°C (Heise et al., 2011). Ejaculates from four stallions were divided into six aliquots; fresh ejaculate, fresh epididymal with seminal plasma, frozen-thawed ejaculated spermatozoa, frozen-thawed ejaculated spermatozoa with and without seminal plasma (Heise et al., 2011). When evaluating the effect on sperm motility, fresh with seminal plasma had the best progressively motile sperm. The frozen and thawed samples had no significant difference; however, the thawed aliquots with and without seminal plasma resulted in lower percentages of progressively motile sperm (Heise et al., 2011).

A study analyzed seminal plasma components, including the biochemical components, proteins, vitamins, and ion concentration (Usuga et al., 2017). The collected semen was centrifuged at 800g for 15 minutes then semen was diluted to a 1:1 ratio to separate the seminal plasma. Seminal plasma was centrifuged again and stored at -20°C for 24 hours before being placed into a lyophilization cycle or dehydration with extreme freezing temperatures for 30 hours (Usuga et al., 2017). Lyophilization made evaluation easier because it meets the criteria of storage and preservation conditions of seminal plasma, additionally more precise supplementation of plasma components. Samples contained high, medium, or low amounts of seminal plasma and found the percentages of parameters decreased with higher amounts of seminal plasma. There was no significant difference in seminal plasma that was fresh, frozen/thawed, or lyophilized on the quality of sperm (Usuga et al., 2017). Various studies

concluded that seminal plasma has more of a negative response to stored equine semen (Usuga et al., 2017).

Centrifugation Cushion

Another experiment investigated the effect of an extender and type of centrifugation tube; the study analyzed sperm quality and sperm recovery rate by comparing the use of INRA96 vs. a balanced salt solution as a centrifugation media and also NIPPLE tubes vs . the commonly used conical bottom tubes (Waite et al., 2008). Instead of being shaped like a conical where the bottom gradually goes to a point, the NIPPLE tube has around the bottom in the center of a small cylinder where the cushion is put. Control treatment consisted of thirty-five milliliters of extended semen as a centrifuge cushion and using Eqcellsire® as a centrifuge cushion. After the samples were resuspended, 1 ml was used to assess the quality with Computer-assisted sperm analysis (CASA), and the rest of the sample was cool stored for 24 hours (Waite et al., 2008). Progressive motility aliquots extended in INRA 96 than aliquots in the salt solution before and after storage. However, the movement velocity was not different between extender treatments (Waite et al., 2008). When the motilities were compared due to the type of centrifugation tube, values were better with the NIPPLE tube than with conical bottom tubes (Waite et al., 2008). Curvilinear and average path velocity was also higher with the NIPPLE tube. The control group had the highest average of progressive sperm motility (Waite et al., 2008). There was no difference between extenders by type of tube; however, the recovery rate of sperm was higher in the conical tubes.

A second experiment compared the effect of different centrifugation cushions and the NIPPLE tube on semen quality and the recovery rate of sperm (Waite et al., 2008). INRA96 was

used to dilute semen; the various centrifugation cushions used were Eqcellsire®, Cushion Fluid™, and Optiprep™. The centrifuge was set to run for 20 minutes at 400 or 600g (Waite et al., 2008). This experiment followed the same procedure as the first experiment. No difference was found between the two centrifugation forces, and the cushion treatments were similar in total and progressive motility (Waite et al., 2008). Post storage, all cushions received similar results on the velocity parameters and progressive sperm motility. As discussed, centrifugation of stallion semen to remove the seminal plasma can cause damage to the spermatozoa. In order to minimize the adverse effects, the centrifugation cushion was designed to have a higher density than the diluted semen and typically move to the bottom of the tube during the centrifugation process. A study by Bliss et al. (2012) evaluated semen quality and the effect of the protocols of cushioned centrifugation. Control group 1 was extended with INRA96 to a concentration of 25 million sperm per milliliter in order to decrease seminal plasma to 8-20%. The other control group was diluted to a 4:1 ratio with INRA96; neither was centrifuged. The control was evaluated after collection, and both controls were analyzed after 24 hours of storage (Bliss et al., 2012). Other groups contained 1 or 3 billion sperm with 1 or 3.5 ml of Cushion Fluid™ and centrifuged for 20 minutes at 1,000g. Afterward, the pellets were resuspended with an extender and fresh seminal plasma from centrifuged raw semen (Bliss et al., 2012). Aliquots of each treatment were stored in an Equitainer for 24 hours. No significant difference was found between the two cushion volumes, and the interaction between cushion volume and sperm number did not affect the recovery rate of sperm. However, total sperm motility and curvilinear velocity were higher with centrifugation (Bliss et al., 2012). The control group was found to have higher levels of straightness and intact plasma membranes.

In a study by Len et al. (2013), all aliquots were diluted to 20 ml with a 1:1 ratio for a total concentration of twenty-five million sperm per ml. Aliquots were processed with or without 3 ml of a cushion; both were centrifuged for 10 minutes. Each conical tube was centrifugated either at 900g or 1,800g (Len et al., 2013). After the supernatant was removed and concentrated, semen was extended and evaluated after 24 hours of storage at 5°C. Total and progressive motility was similar for all aliquots except the aliquot centrifuged at 1,800 with no cushion, which received lower percentages. The sperm recovery rate for aliquots with the cushion was worse than in the control group. When no cushion was used, sperm recovery rates post-centrifugation were similar (Len et al., 2013). After storage, aliquots centrifuged at 900g had the highest total and progressive motility percentages. Aliquots centrifuged at 1,800g had higher percentages than the control group with no cushion.

SpermFilter™ for Seminal Plasma Separation

The process of centrifugation can cause damage to the spermatozoa and increase the production of reactive oxygen species to help allievate this problem. Botu-pharma developed the SpermFilter™. Three studies examined the success rate of the Sperm Filter™ compared to other methods. In March 2012, the ejaculations of thirty-one stallions were divided into three treatments. Semen that will still contain seminal plasma was labeled as CN. The other aliquots had the seminal plasma removed with the Sperm Filter™ (FLT) or by centrifugation (CT). Those aliquots were diluted to a 1:1 ratio for seminal plasma removal (Neto et al., 2012). After seminal plasma was removed, SpermFilter™ and centrifugation aliquots were resuspended with 5 ml of Botu-Crio to contain 200 million sperm per ml. The process was repeated for two experiments; all stallions were examined together, and the stallions were labeled "bad coolers." The total

concentration pre-treatment was 50 million sperm per ml; the data were analyzed by CASA. Before storage and after aliquots had been stored for 24 hours at 15°C; no significance differences were found ($P>0.05$).

Neto et al. completed another study with 31 stallions collected every other day for three weeks (Neto et al., 2013). Treatment one was the control group on fresh semen extended with a skim-milk extender; the collections processed through the Sperm Filter™ were labeled treatment two, and treatment three was centrifuged with no cushion (Neto et al., 2013). Total motility of the control group was 71.4%, while the filter group had 76% and 50.8% total motility rates. Cushion Fluid™ had a total motility rate of 72.6% and 47.5% (Neto et al., 2013). No significant difference was found when treatment one compared to treatment three in PMI and kinetic parameters.

A third study used fifteen stallions to compare SpermFilter™ and Centrifugation. The whole ejaculation was extended to a 1:1 ratio; sample groups were separated by the control group, SpermFilter™, or Cushion Fluid™ (Schnobrich et al., 2016). The control group samples were diluted to a 1:1 ratio with Botu-Semen for the final concentation of 25 million sperm per ml with seminal plasma. The 24 ml of extended semen after filtered with the SpermFilter™, and 7.5 ml remained in the filter (Figure 4). The centrifugation with Cushion Fluid™ used 50 ml and 1 ml of the cushion. The setting of the centrifuge for RFC was 1000xg and ran for 20 minutes. After removing the cushion and supernatant, the milliliters left were 7.5 and a final concentration of 25 million sperm/ml (Schnobrich et al., 2016). The samples were stored in the Equitainer for 24 hours after the aliquots were refrigerated at 5°C for 72 hours. The evaluated post-storage parametes were total sperm motility, progressive motility, and morphology. The control group produced 70.7% normal cells and 1.34% detached heads. The percentage of detached heads

increased when semen is processed with the filter (Schnobrich et al., 2016). The cushion resulted in highest percentage of normal cells and the lowest percentage of the detached head of the treatments. The control group ranged from 70% to 53% total motility, and the progressive motility rate was 61.3% to 43.3% (Schnobrich et al., 2016). The total motility rate for Sperm Filter™ treatment varied from 67% to 51.6%, and the progressive motility rate was 67% to 51.6%. The aliquots that been centrifuged with Cushion Fluid™ had evaluated progressive motility fluctuated from 63.2% to 48.1% and 70.2% to 56.7% for the total motility rate. There was no significant difference between the control group and compared to the other treatments. Total and progressive motility differed significantly with the cushion treatment than the filter (Schnobrich et al., 2016).

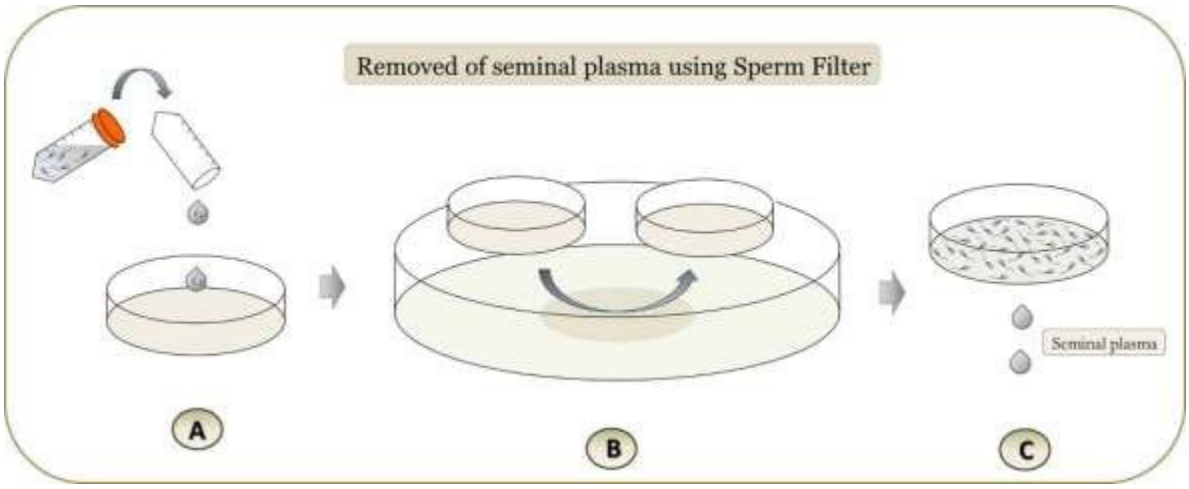

Fig. 4. The design of SpermFilter™ for operation (Neto et al., 2013)

METHODS

Animal and Collection Methods

All procedures involving the care, management, and the use of horses for this study by the Institutional Animal Care and Use Committee of Missouri State University (see Appendix). The artificial vagina (AV) used to collect semen from the stallions was a "Missouri AV." The AV was filled with water at the temperature of 45°C, lubricated with One Shot® (HR Pharmaceuticals Inc., York, PA), non-spermicidal gel, and the collection bottle with gel filter was pre-warmed. A gel filter was used to remove unwanted components like smegma, dirt, and debris while letting semen pass through the fabrication of the filter.

In this study, ejaculates from three stallions were collected with an age from 20 ± 8 years old and weighed 1050-1100 lbs. Ejaculates utilized included nine from stallion 1, two from stallion 2, and two from stallion 3. The unbalanced number of collections occurred because stallion three was put down due to colic, and the owner put stallion two back. Due to the poor initial motility of semen collected from stallion one, samples were not subject to evaluation after cooling as initially planned.

All ejaculates were evaluated for concentrations with a 590a Densimeter (ARS, Chino, CA). A cuvette tube filled with sterile water was placed inside the 590a Densimeter and zeroed to account for the optical density of the individual cuvettes. A pipette dispensed 180 ul of semen into the cuvette tube. The dilution ratio was .18mls to 3 to obtain 5% semen per cuvette. Each sample was evaluated multiple times until three readings within ± 2% were achieved. Similar concentrations were then averaged to estimate the concentration per millimeter. This procedure was followed for the pre-treatment and post-treatment samples.

Treatments

A phosphate-buffered saline solution (PBS) was used to dilute all aliquots, which allowed measurement of the concentration with the densimeter post-processing. In treatment 1, the control group, fifteen ml conical tubes with 12 ml of extended semen of a 1:1 ratio, were placed into a Variseal centrifuge (Vulcon Technologies, San Jose, CA). The Variseal ran for ten minutes at RCF 450g with a conical tube filled with 12 ml of water for counterbalance. The supernatant was removed from the condensed pellet by a 10 ml pipette. The sperm pellet was resuspended to a 1:3 ratio (semen: PBS) following the centrifugation and prior to the reading of concentration with the Densimeter. The percentage of sperm recovered was calculated by dividing the post-treatment concentration by the pre-treatment concentration.

Aliquots from treatment two were centrifuged in 15 ml tubes with 3 ml of Red Cushion™ (Botupharma, Phoenix, Az) in the bottom of the tube. In order to place the cushion in the tube at the bottom underneath the extended semen, a 3 ml syringe with an 18g needle extracted 3 ml of Red Cushion™. Then a cannula with a 0.5°C semen straw replaced the needle to add the cushion. The cushion was slowly pushed to the tip of the straw and inserted into the bottom of the conical tube at a 45° angle. The 15 ml conical tubes contained 12 ml of extended semen diluted at 1:1 with PBS. For this treatment, tubes were centrifuged in an Eppendorf Centrifuge 5804 R 15-amp version (Thermo Fisher Scientific, Waltham, MA) RFC 1000g for twenty minutes. Following centrifugation, a 10 ml pipette removed the supernatant until it reached the sperm ring. The sperm ring was left at the tip of the conical tube and resuspended with phosphate buffer to a 1:3 (semen: extender) ratio. The 3 ml syringe with cannulas and straw removed the Red Cushion™ (Botupharma, Phoenix, AZ).

For treatment three, the SpermFilter™ (Botupharma, Phoenix, AZ) was used to separate the spermatozoa from 20 ml of extended semen, a 1:1 ratio of semen to PBS consisting of 10 ml of semen and 10 ml of PBS. Before processing, the filter was placed in the incubator for ten to fifteen minutes. The extended semen in the filter was gently swirled around while repeatedly touching the tray. Once a limited amount of liquid was left, the sperm pellet was resuspended and washed out of the filter with a 5 ml PBS extender. The filter was tilted at 45 angles to disperse 5 ml of the extender around the filter to collect any sperm that might have gotten stuck on the synthetic membrane of the filter. The extended semen was poured into a sterile tube to measure volume. Dilution rate was about 1:1 to run samples in the Densimeter for concentration levels.

During data collection, one ejaculate only contained enough semen to run treatments 1 and 2. Due to the uneven number of ejaculates per stallion and treatments per ejaculate, treatments were compared using an unbalanced one-way ANOVA (Mini-Tab 18) with a significance $P<0.05$ used to compare the recovery of spermatozoa for each of the treatments.

RESULTS

The recovery rate of spermatozoa in treatment 1 averaged 69.4%, compared to treatments 2 and 3 which averaged 60.9% and 77.62%, respectively (Table 1). When treatments were subjected to statistical analyis by one-way ANOVA, the mean sperm recoveries were significantly higher for treatment two vs. treatment and three (P=0.021). However, there was no significant difference in mean recovery rates between treatments one and two (P=0.233) or treatments one and three (P=0.195).

Table 1
The percentage of sperm recovery by centrifugation with or without a cushion or with sperm filter by stallion, ejaculate, and treatment.

Treatment 1: No Cushion			Treatment 2: Red Cushion			Treatment 3: SpermFilter		
Stallion	Ejaculate	%	Stallion	Ejaculate	%	Stallion	Ejaculate	%
1	1	88.4	1	1	93.0	1	1	86.9
1	2	66.3	1	2	69.0	1	2	90.0
1	3	42.5	1	3	44.7	1	3	94.0
1	4	72.1	1	4	93.0	1	4	66.3
1	5	97.1	1	5	75.4	1	5	97.0
1	6	73.0	1	6	66.0	1	6	73.5
1	7	71.5	1	7	57.4	1	7	94.7
1	8	62.8	1	8	65.3	1	8	65.1
1	9	81.8	1	9	66.5	1	9	66.7
2	1	86.7	2	1	57.1	2	1	53.3
2	2	50.5	2	2	62.2			
3	1	57.0	3	1	63.3	3	1	73.6
3	2	52.0	3	2	41.9	3	2	70.8
Mean		69.0[ab]			61.0[a]			78.0[b]

[ab] means with different superscripts, that percentage does not follow the pattern.

DISCUSSION

Compared Results

In this experiment, analysis by one-way ANOVA found that the percent of sperm recovered with the SpermFilter™ was not significantly higher than the two centrifuging treatments. Therefore, it can be concluded in this experiment that the SpermFilter™ worked at least as well as centrifugation with or without a cushion. The device is cheaper and may result in less damage to sperm, thus improving survival and conception rates. However, these last parameters were not assessed in the present experiment. It should be noted that further testing of this device against centrifugation procedures using different times and speeds might yield different results.

The 2012 study by Neto et al. was to determine that experiment the efficacy of the Sperm Filter in removing the seminal plasma from the semen before freezing. The control group was centrifuged in a 50 ml tube with no cushion. Like this current study, the data showed no significant difference between the SpermFilter™ and centrifugation. A 2016 study by Schnobrich et al. found slightly different data. Schnobrich analyzed the comparison of cushioned centrifugation and Sperm Filter filtration on longevity and morphology of cooled-stored equine semen. Unlike Neto's studies, Schnobrich's study found total motility of spermatozoa in centrifugation group was higher than those separated by the filter. The treatment with a cushion was established as the best fit for acquiring progressive motile sperm; the data showed that the cushion had a 100% recovery rate, while the filter received 93%. The treatment resulted in a better recovery percentage; however, there was no difference in the motility percentage. There was no significant difference between the control group that used a skim milk extender and

either treatment, like the data in the previous section. As mentioned previously, due to the low initial motility of spermatozoa from the stallion that contributed to most of the ejaculates were unable to compare the post-storage motility of aliquots concentration by the different methods.

Applications

The equine reproduction industry is looking for ways to improve the post-storage of stallion semen and prolong longevity. The essential components of this study's cost were Eppendorf™ Model 5804R and 15 RPM™, & the SpermFilter™. The centrifuge can be found on fisher scientific for $13,659.98. The Variseal centrifuge (Vulcon Technologies) is no longer produced; the new model, "Spectrofuge," goes for $1,411. Red Cushion™ by Botu-pharma can cost $67.10 for 20 ml or $229.00 for 100 ml per bottle, costing $6-10 per dose. The Botu Sperm Filter costs $80.30 and can be used up to ten times which is $8.30 per use (Table 2). In order to pay off the cost of the Eppendorf centrifuge and the fee for a single use is eight dollars and three cents; it would take 1,706 times to cancel the purchase the cost. For the same price of usage for the Variseal; it would take 176 times to break even (Table 3). The industry would have to evaluate these costs from a personal and business point of view to validate the return on investment. In that mindset, interest in breeding contacts, stallion fee pricing, treatment with the more suitable outcome, and the technician's skill are all variables. The centrifugation procedure using Red Cushion™ requires skill to successfully remove the sperm pellet without disturbing the cushion. When a stallion is in high demand, procedures that produce the highest amount of good semen quality can easily offset processing costs. The SpermFilter™ may be more appropriate. Also, further research using stallions that would be classified as bad coolers might

result in improved post-storage for spermatozoa which were concentrated with a SpermFilter™ as compared to centrifugation.

Limitations

In this study, there were multiple limitations that may have affected the results. First, the study needed to be more balanced regarding the number of collections per stallion. Secondly, as mentioned, due to the poor initial motility of the stallion that was used most, we were unable to complete the second part of the study, which was to evaluate motility after storage. Would the data have been more similar to Schnobrich' s study if stallions had improved motility as planned? Finally, the percentage of sperm recovered when using the Red Cushion™; treatment could have had a higher recovery rate if the treatment had happened more consistently. Consistency would have improved the newly learned skill of dealing with a cushion and removed any outliers to get a dependable recovery rate. The Red Cushion™ treatments are sensitive when separating the supernatant and cushion from the sperm ring.

Table 2
The number of times that each brand of centrifuge must be used to pay off the intial purchase cost as compared to the cost per use of the SpermFilter.

	$ Cost	$ per use	# of times used
Eppendorf	13,659.98	8.03	1,701
Spectofuge	1,411.00	8.03	179

n/a: not applicable

Table 3
Cost comparison per dose when a thousand doses are centrifuged with and without a cushion or concentrated with the SpermFilter.

	$ Cost	Per Dose	Total	$ Per Dose
Centrifuge With Cushion				
Eppendorf Model 5804R	$13,659.98			
Red Cushion™	$2,290.00	$2.03		
15 mL Conical Tubes	$822.00	$0.82		
			$16,711.38	$16.77
Variseal Centrifuge	$1,411.00			
15 mL Conical Tubes	$822.00	$0.82		
			$1,833.00	$1.83
SpermFilter™	$80.30	$8.03		$80.30

n/a: not applicable

REFERENCES

Arabian Horse Association. 2003. History of the Association. Arabianhorseassociation.com https://www.arabianhorses.org/export/content.export/aha-docs/History_of_the_Association.pdf

American Quarter Horse Association. 2018. Foals bred by cooled transported and frozen semen. AQHA. https://www.aqha.com/-/foals-bred-by-cooled-transported-and-frozen-semen?redirect=%2Fregistration

Aurich, J., 2012. Artificial Insemination in Horses- More than a Century of Practice and Research. J. Equine Vet. Sci. 32, 458-463.

Bahrami. A., Divar, M., Azari, M., Kafi, M., 2020. Nicotinic Acid (Niacin) Supplementation in Cooling and Freezing Extenders Enhances Stallion Semen Characteristics. J. Equine Vet. Sci. 94, 103236.

Barrier-Battut, J., Bonnet, C., Girarudo, A., Dubois, C., Caillaud, C., Vidament, M., 2010. Removal of seminal plasma by centrifugation, before cooled storage enhances membrane stability of stallion spermatozoa. J. Anim. Reprod. Sci. 121, 188-190.

Batellier, F., Duchamp, G., Vidament, M., Arnaud, G., Palmer, E., Magistrini, M., 1998. Delay insemination is successful with a new extender for storing fresh equine semen at 15°C under aerobic conditions. Theriogenology 50. 229-236.

Bliss, S., Brinsko, S., Vogu, J., Haygen, S., Teague, S., Love, C., Blanchard, T., Varner, D., 2012. The impact of cushioned centrifugation protocols on semen quality of stallions. Theriogenology 77, 1232-1239.

Brinsko, S., Crockett, E., Squires, E., 2000. Effect of centrifugation and partial removal of seminal plasma on equine spermatozoal motility after cooling and storage. Theriogenology 54, 129-136.

Brinsko, S., Blanchard, T., Varner, D., Schumacher, J., Love, C., Hinrichs, K., Hartman, D., 2011. Manual of Equine Reproduction, 3rd ed. Mosby, St. Louis, MO

Borg, C., Wolski, K., Gibbs, G., O'Bryan, M., 2010. Phenotyping male infertility in the mouse: how to get the most out of a non-performer. Hum. Reprod. Update. 16, 205-224.

Burris, C., Webb, G., Harmon, S., Baker, R., 2009. Effects of Egg Yolk Source on the Cryoperservation of Stallion Semen. J. Equine Vet. Sci. 31, 166-173.

Cuervo-Arango, J., Nivola, K., Vanhkatnen, L., 2015. The effect of storage temperature of stallion semen on pregnancy rates. J. Equine Vet. Sci. 35, 611-616.

Dawson, G., Webb, G., Pruitt, J., Loughin, T., Arns, M., 2000. Effect of different processing technique on motility and acrosomal integrity of cold-stored stallion spermatozoa. J. Equine Vet. Sci. 20, 191-194.

Heise, A., Thompson, P., Gerber, D., 2011. Influence of seminal plasma on fresh and post-thaw parameters of stallion epididymal spermatozoa. Anim. Reprod. Sci. 123, 192-201.

Hernandez-Avila, C., Love, C., Serafini, R., Teague, S., Varner, D., 2019. Supplemental antibiotics in a commercial extender for stallion semen. J. Equine Vet. Sci. 80, 33-35.

Hopkins, F., Meadows, D., 2003. THN1002- cooled shipped horse semen. https://trace.tennessee.edu/cgi/viewcontent.cgi?article=1020&context=utk_agexani

Jobim, M., Trein, C., Zirkler, H, Gregory, R., Sieme, H., Mattos, R., 2011. Two-dimensional polyacrylamide gel electrophoresis of equine seminal plasma proteins and their relation with semen freezability. Theriogenology 76, 765-771.

Len, J., Beehan, D., Lyle, S., Eilts, B., 2013. Cushioned versus noncushioned centrifugation: sperm recovery rate integrity. Theriogenology 80, 648-553.

Len, J., Beehan, D., Ebrahimie, E, Lyle, S., 2020. Stallion sperm integrity after centrifugation to reduce seminal plasma concentration and cool storage for 4 days. J. Equine Vet. Sci. 85, 102819.

Lonergan, P., 2018. Review: historical and futuristic developments in bovine semen technology. Anim. s4-s18.

Love, C., Brinsko, S., Rigby, S., Thompson, S., Blanchard, T., Varner, D., 2005. Relationship of seminal plasma level and extender type to sperm motility and DNA integrity. Theriogenology 63, 1584-1591.

Marshall, F., Lamming, G., Parkes, A., 1984. Marshall's physiology of reproduction. 4th ed. Springer, New York, NY.

Matas, C., Decuadro, G., Martanez-mira, S., Gadea, J., 2007. Evaluation of a cushioned method for centrifugation and processing for freezing boar semen. Theriogenology 67, 1087-1091.

McKinnon, A., Voss, J., 1993. Equine reproduction. 1st ed. Lea & Febiger. Philadelphia, PA.

Melo, C., Zahn, F., Martin, I., Orlandi, C., Dell'Aqua, J., Alvarenga, M., Papa, F., 2007. Influence of semen storage and cryoprotectant on post-thaw viability and fertility of stallion spermatozoa. J. Equine Vet. Sci. 27, 171-175.

Morse-Wolfe, B., Bleach, E., Kershaw, C., 2023. An investigation of equine sperm quality following cryopreservation at low sperm concentration and repeated freeze-thawing. J. Equine Vet. Sci. 120, 104167.

Neto, C., Monteiro, G., Soares, R., Pedrazzi, C., Dell'Aqua, J., Papa, F., Alvarenga, M., 2012. Effect of removing seminal plasma using a sperm filter on the viability of refrigerated stallion semen. J. Equine Vet. Sci. 33, 40-43.

Neto, C., Monteiro, G., Soares, R., Pedrazzi, C., Dell'Aqua, J., Papa, F., Castro-Chaves, M., 2013. New seminal plasma removal method for freezing stallion semen. Theriogenology 79, 1120-1123.

Neuhauser, S., Handler, J., 2017. Pregnancy outcome using highly concentrated, cooled stored stallion semen, and different dilution protocols with autologous seminal plasma before insemination. J. Equine Vet. Sci. 56, 56-62.

Newcombe, J., Lichtwark, S., Wilson, M., 2005. Case report: the effect of sperm number, concentration, and volume of insemination dose of chilled, stored, and transported semen on pregnancy rate in standardbred mares, J. Equine Vet. Sci. 25, 525-530.

Novello, G., Podico, G., Segabinazzi, L., Canisso, I., 2020. Semen parameters and embryo recovery of cooled stallion semen extended with sodium caseinate-cholesterol (Botugold) and phosphoscaseinate (INRA96) based extenders. J. Equine Vet. Sci. 89, 103043.

Oldenhof, H., Gojowsky, M., Wang, S., Henke, S., Yu, C., Rohn, K., Walker, W., Sieme, H., 2013. Osomotic stress and membrane phase changes during freezing of stallion sperm: mode of action of cryoprotective agent 1. Biol. Reprod. 88, 68.

Padilla, A., Foote, R., 1991. Extender and centrifugation effects on the motility of slow-cooled stallion spermatozoa. J. Anim. Sci. 69, 3308-3313.

Papin. J., Stuhtmann, G., Sieme, H., Lundeheim, N., Ntallaris, T., Morrell, J., 2021. Stored stallion sperm quality depends on sperm preparation method in INRA82 or INRA96. J. Equine Vet. Sci. 98, 103367.

Pena, F., Ortiz-Rodgragues, G., Gil, M., Ortega-Ferrusola, C., Martan-Cano, F., 2022. An integrated overview on the regulation of sperm metabolism (glycolysis-krebs cycle-oxidative phosphorylation). Anim. Reprod. Sci. 246, 106805.

Pickett, B., Sullivan, J., Byers, W., Pace, M., Remmenga, E., 1975. Effect of centrifugation and seminal plasma on motility and fertility of stallion and bull spermatozoa. Fertil. Steril. 26. 167-174.

Pickett, B., Amann, R., McKinnon, A., Squires, E., Voss, J., 2010. Management of the stallion for maximum reproduction efficiency. J. Equine Vet. Sci. 14, 310.

Rigby, S., Brinsko, S., Cochran, M., Blanchard, T., Love, C., Varner, D., 2001. Advances in cooled semen technologies: seminal plasma and semen extender. Anim. Reprod. Sci. 68, 171-180.

Samper, J., 2009. Equine breeding management and artificial insemination. 2nd ed. Saunders, Phildelphia, PA.

Schnobrich, M., Roach, J., Ellerbrock, R., Bradecamp, E., Alvarenga, M., Kline, K., Canisso, I., 2016. Comparison of cushioned centrifugation and sperm-filter filtration on sperm longevity and morphology of cooled-stored equine semen. J. Equine Vet. Sci. 43, 567-568.

Usuga, A., Rojano, B., Restrepo, G., 2017. Effect of seminal plasma components on the quality of fresh and cryopreserved. J. Equine Vet. Sci. 58, 103-111.

Waite, J., Love, C., Brinsko, S., Teague, S., Salazar, J., Mancill, S., Varner, D., 2008. Factors impacting equine sperm recovery rate and quality following cushioned centrifugation. Theriogenology 70, 704-714.

Webb, G., Arns, M., 2004. Case study: use of modified phosphate buffered saline as a component of semen extenders allows the use of transported semen from two stallions. 2004. Prof Anim. Sci. 20, 278-281.

Webb, G., Dean, M., 2009. Effect of centrifugation technique on post-storage characteristics of stallion spermatozoa. J. Equine Vet. Sci. 29, 675-680.

Webb, G., Dean, M., Humes, R., Heywood, J., 2009. A comparison of the ability of three commercially available diluents to maintain the motility of cold stored stallion semen. J. Equine Vet. Sci. 29, 229-232.

APPENDIX: MISSOURI STATE IACUC APPROVAL

Missouri State
UNIVERSITY

May 2, 2023

RE: IACUC protocol 2022-03

Holly Hopkins,

IACUC protocol #2022-03 entitled "Use of Sperm Filter vs. Centrifugation to Concentrate Stallion Semen" was approved by the committee on February 25, 2022 and expires February 24, 2025.

The protocol reflects that you are approved to work with Dr. Gary Webb on this project.

Thank you and if you need anything in the future regarding this protocol please contact me either via email (johnnapedersen@missouristate.edu) or at 417-836-3737.

Sincerely,

Johnna Pedersen

Johnna Pedersen
IACUC Administrator/Member
Interim Director of Research Administration

OFFICE OF RESEARCH ADMINISTRATION
901 South National Avenue, Springfield, MO 65897 • Phone: 417-836-5972
www.missouristate.edu
An Equal Opportunity/Affirmative Action/Minority/Female/Veterans/Disability/Sexual Orientation/Gender Identity Employer and Institution