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## **Cryopreservation of Equine Spermatozoa: Identification of Good and Poor Freezer Stallions and Effect of Sperm Density Per Straw**

Abed Sharqy Fahad

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Cryopreservation of equine spermatozoa: Identification of good and poor freezer stallions  
and effect of sperm density per straw

By

Abed Sharqy Fahad

A Thesis  
Submitted to the Faculty of  
Mississippi State University  
in Partial Fulfillment of the Requirements  
for the Degree of Master of Science  
in Agriculture  
in the Department of Animal and Dairy Sciences

Mississippi State, Mississippi

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2012

Cryopreservation of equine spermatozoa: Identification of good and poor freezer stallions  
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This study was carried out primarily to evaluate the cryo-tolerance of equine semen from four stallions through assessing the spermatozoa motion characteristics with Computer-Assisted Sperm Analysis (CASA). Four stallions were collected during the breeding season (summer). For each ejaculate, fresh and cryopreserved samples were taken for sperm motility characteristics evaluation. Data analysis demonstrated that sperm cells of stallions were significantly affected by ( $P < 0.05$ ) cryodamage. Stallion (A) was cryotolerant, and was classified as a good freezer, whereas stallion (D) was not and classified as a poor freezer regardless of the concentration of sperm. In addition, a concentration of  $0.4 \times 10^9$  sperm cells/ml had higher percentages of rapid sperm and velocity parameters ( $P < 0.05$ ) compared to  $0.8 \times 10^9$  sperm/ml. Further research is necessary to identify potential biomarkers for good and poor freezer stallions.

Keywords: good freezer, poor freezer, equine semen, sperm density, cryopreservation

## DEDICATION

I would like to dedicate all my work and this thesis to my mother for her love, support, prayers, and supplications and to my wife for her patience, encouragement and standing by my side to accomplish my endeavors while being away from them for almost four years. To my kids, Obaida and Ruwaida, all my love and yearning go to them, and I would say that all what I achieved is for your future in order to live in peace and having better lives away from ignorance and darkness.

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## LIST OF ABBREVIATIONS

A1	Differentiated A1 spermatogonia
A2	Differentiated A2 spermatogonia
A3	Differentiated A3 spermatogonia
A4	Differentiated A4 spermatogonia
AI	Artificial insemination
ALH	Amplitude of lateral head displacement
ART	Assisted reproductive technology
AV	Artificial vagina
B B	Spermatogonia
BCF	Beat cross frequency
CASA	Computer assisted sperm analysis
D1	INRA 82 extender
D2	INRA 82 + 2.5% glycerol extender
FSH	Follicle stimulating hormone
GLM	General linear model
GnRH	Gonadotropin releasing hormone
HPT	Hypothalamus pituitary testicular
ICSI	Intracytoplasmic sperm injection
IVF	In vitro fertility

LH	Luteinizing hormone
LIN	Linearity
LN	Liquid nitrogen
LSD	Least significant difference
PBS	Phosphate buffered saline solution
PMOT	Progressively motile sperm
PR	Pregnancy rate
RS	Rapid sperm
SAS	Statistical analysis system
STR	Straightness
VAP	Average path velocity
VCL	Curvilinear velocity
VSL	Straight line velocity
WHO	World Health Organization
ZP	Zona pellucida

# CHAPTER I

## INTRODUCTION

### **1.1 Equine Industry**

Fundamental improvements have been made since semen cryopreservation was discovered by Polge and his colleagues in 1949. Since the initial discovery, cryopreservation has become a powerful tool of the present to solve many agricultural, biomedical, and economical questions. Researchers have been trying to apply the potential of this procedure in different fields of study to have broad knowledge and adequate understanding of cryopreservation technology. The biological definition of cryopreservation is the process of cooling and storing cells, tissues, and organs at very low temperatures ( $-196^{\circ}\text{C}$ ) to decrease or even pause the metabolic process yet preserve these functions while storing. The term “Cryopreservation” has been derived from the Greek word “kryos” which means cold, so it is cold storage for the purpose of preservation for long-term future use. Indeed, it is a revolutionary tool in different domains of life in which this technique can be applied. In the Agri-food industry, for instance, it has widespread applications in preserving food as a permanent and continuous source for human beings’ demands. Similarly, in biomedical research, cryopreservation has shown significant impact and advancements in controlling animal and human diseases. The most common used methodology in semen cryopreservation which involves a collection process, extend it with extender medium, centrifuging, resuspending

in freezing extender, cooling, freezing, and storing in liquid nitrogen. Similarly, semen cryopreservation protocols have been used on several species, where some species have shown consistent results, but others are still in progress because of their cryosensitivity responsiveness.

In the dairy cattle industry, frozen-thawed semen from bulls has been successfully used for artificial insemination (AI) and has resulted in significant advancement in animal production quality (Vishwanath and Shannon, 2000). Artificial insemination has facilitated most of difficulties from which researchers struggle with, such as having an access to genetically superior sires domestically and internationally, preventing or controlling diseases that might transfer during natural mating, and finding alternative solutions for infertile male individuals. In addition, it has played an essential role increasing cows' productive capability over the past few decades. In 1951, the first calf was born by using frozen semen (Stewart, 1951), and thereafter the technique was rapidly applied on a large scale. Because of using the technique with no restrictions or limitations in cattle industry, cryopreserved spermatozoa have become the optimal and profitable substitution to increase cows' productive efficiency by selecting genetically superior sires. It is estimated that superior sires are capable of producing upwards of 60,000 doses of semen per year which is, from an economic stand point, more preferable than natural mating (Curry, 2000). Successful progress has been made in this area and, in other word, using of AI with frozen semen is even more favorable over natural mating (Bailey, 2000).

Knowledge of the fact that swine industry plays almost a parallel role as cattle does for providing meat for human consumption, however, swine production efficiency from use of cryopreserved semen is not satisfactory yet. Applying such semen

cryopreservation techniques might double the amount yield. However, the potential of utilizing cryopreservation technique is still critical for many reasons some of which are correlated with molecular compositions as well as its cryotolerance during the cryopreservation process (White, 1993). Yoshida reported that frozen boar semen has produced low farrowing rates and small litter size (Yoshida, 2000). In the same manner, it is estimated that inseminating post-thawed boar sperm leads to a reduction by 50% in farrowing rates and three piglets per litter (Johnson, 1985). One may draw a conclusion that more research should be done in this area to achieve the optimal results and appropriate cryopreservation protocols and extenders for swine.

Unlike the bull, stallion semen cryopreservation technique, which is the subject of interest, has not had the same level of success with mainly low pregnancy rates (Kuisma et al, 2006). That may be attributed to cryosensitivity of stallion sperm during freezing process. Furthermore, not all stallions are considered to be good freezers, but rather some fertile stallions might exhibit low post-thaw pregnancy rates (Kuism et al, 2006). It has been reported that (20%) of stallions produce good freezable semen, and the majority of them (60%) are negatively affected, and the rest (20%) are considered to be bad freezers (Tischner, 1979; Pickett and Amann, 1993; Vidament et al., 1997). To overcome such variation among stallions, suitable methods and new protocols should be established and introduced for cryopreservation of semen in this species.

This study was conducted and divided into two parts; the first part was to identify the good and bad freezers with respect to estimating some pertinent parameters such as sperm motility, total progressive motile sperm, rapid, and sperm velocity parameters. The second part was to evaluate the impact of sperm density / straw on sperm survivability.



Detecting some potential gene candidates would be of great interest to establish useful tools from which freezability of stallion semen can be measured in the field without engaging in the complicated and time consuming laboratory procedures.

## CHAPTER II

### LITERATURE REVIEW

#### **2.1 Cryopreservation**

The importance of sperm cryopreservation has been discovered for many decades to assist maintaining the genetic diversity of both domestic and endangered animals (Wildt, 1992; Critser and Russell, 2000). In addition, the distribution of genetically superior sires can be achieved and relatively controlled by applying such technique. Also, many studies stated that treating of iatrogenic infertility is another potential benefit of using sperm cryopreservation (Kuczynski et al., 2001; Ranganathan et al., 2002; Tash et al., 2003; Nalesnik et al., 2004; Agarwal et al., 2004). Furthermore, cryopreservation contributes, in different ways, to the genetic banking of modified animal models of human health and diseases (Knight and Abbott, 2002; Critser and Russell, 2000). Historically, Sperm cryopreservation and cryobiology date as far back as the 1600s (Sherman, 1964), but it was not potentially applied until the artificial insemination (AI) technology was rapidly conducted by the dairy industry in the early 1960s. Since then, sperm cryopreservation has become the major concern and interest of scientific studies. In 1949, Polge and his colleagues made a distinct and remarkable discovery which accidentally brought up the use of glycerol as a cryoprotectant substance to protect cells from the cold-shock at low temperatures.

Based upon these scientific facts, mammalian sperm cryopreservation methods were established and developed successfully. However, even with highly sophisticated equipment and technologies, only a very few mammalian species' spermatozoa can be effectively cryopreserved. Foreseen, the successful cryopreservation as estimated by post thaw sperm motility is 50% or even less than that of fresh motility. It is well-known that there is a significant variation of sperm cryosurvivability among species, individuals within same species, and even within ejaculates of individuals, which is definitely, and from biological point of view, attributed to the differences in biophysical characteristics among sperm cells (Gao et al., 1997; Thurston et al., 2002; Walters et al., 2005).

### **2.1.1 Bovine Sperm**

Cryopreservation protocols and methods for the bull to be used for AI in the cattle industry have been developed gradually since 1950s. Since then, the methods and protocols of bull sperm cryopreservation have been marginally changed. A study in field trials, for instance, concluded that bovine spermatozoa frozen to  $-79^{\circ}\text{C}$  and packed on dry ice could produce increased fertility (Bratton et al., 1955). Regarding the media formulations that usually used to freeze bull sperm cells are extenders which seem to be responsible to maintain high level of post thaw motion at low temperatures. Cholesterol has been recently added to stabilize the sperm membrane prior to freezing process. In one study Purdy and Graham (2004) showed that the addition of cholesterol to the membrane improved post thaw sperm motility. In the same manner, it was found that addition of cholesterol to membrane might have no correlation with fertility, the sperm capacitation, or acrosome reaction. Moreover, Lipids in egg-yolk are deemed as a key ingredient that primarily used to protect spermatozoa from cryo-injury (Watson and Martin, 1973;

Watson and Martin, 1975; Watson, 1975; Foote, 1998) the glycerol cryoprotective properties as well as lipids have facilitated the improvements of freezing extenders for freezing sperm cells. Such discoveries eventually established the Tris egg yolk-glycerol method for cryopreserving bovine spermatozoa which is now used widely as a standard and reliable technique (Foote, 1970a; Watson and Martin, 1973, Watson and Martin, 1975; Watson, 1975).

### **2.1.2 Porcine Sperm**

In the 1970s, preservation of boar spermatozoa was introduced with a new method instead of that for other species. A “pellet method” was first developed by Pursel and Johnson (1975) to successfully freeze boar semen. Briefly, sperm cells, in this method were cooled to 5°C at a rate of 0.22°C per minute. Then, cooled media composed of glycerol and extender was added. Sample aliquots were then placed immediately on a dry ice block (−79°C) and finally plunged into liquid nitrogen for future use. Such method was partially effective with regards to post-thaw motility, yet there were some disadvantages such as the difficulties of transporting the samples and labeling the pellets individually. Eventually, and in recent years, some researchers have developed other methods (Maxi, 5ml; mini, 0.25 and 0.5 ml) straws by which individual sperm samples can be easily identified and hence transported domestically and internationally (Bwanga et al., 1990, 1991).

### **2.1.3 Human Sperm**

According to Sherman (1978b), the first used AI in human was established by John Hunter in England around 1776. Additionally, the same author (1978a, b) stated that

1464 normal children, 11 abnormal children, and 113 spontaneous abortions were resulted from stored human semen. Because of some ethical limitations concerning the obtaining semen from donors, it has been difficult to apply cryopreservation technique in humans on a large-scale. Cryopreservation of human spermatozoa has been mainly used for treatment of infertility and for those who would undergo chemical therapy. Human sperm cells were first frozen with a survival rate of 67% in 1953 (Sherman and Bunge, 1953). Thereafter, three pregnancies (Sherman and Bunge 1953) were recorded using AI in conjunction with frozen human sperm. New technologies have been developed such as assisted reproductive technologies (ART) including intracytoplasmic sperm injection (ICSI) in azoospermic patients. Surprisingly, using *in vitro* fertility IVF/ICSI in combination with frozen sperm that was stored for 21 years yielded the birth of a live offspring (Horne et al., 2004). Moreover, it was reported (Sbracia et al., 1997) that post thaw human sperm motility range from 20-50%. Such decreased motility following cryopreservation process was probably due to cryosensitivity and thus the cryo-injury to the membranes and other intracellular compartments of human spermatozoa.

#### **2.1.4 Equine Sperm**

The equine spermatozoa are well-known by their cryosensitivity and declined cryo-tolerance. Such decreased cryo-tolerance might be attributed to the plasma membrane architecture with respect to phospholipids /cholesterol ratio. In 1957, Barker and Gandier (1957) reported the first pregnancy using post-thawed stallion spermatozoa. Since then, the frozen semen has not been used widely specifically in equine industry for breed registry limitations and low pregnancy rates (Kuisma et al., 2006). There are several factors behind unsuccessful cryopreservation technique in equine industry, such

as extenders (Heitland et al., 1996), cryoprotectants (Fiser et al., 1991), cooling and thawing rates, the variation among and within stallions (Cooter et al., 2005; Andrabi, 2007; Clulow et al., 2008), and even protocols. For stallions, several protocols should be used to evaluate each individual stallion to determine the most appropriate one.

Additionally, it is of a great value for sperm cells to possess some essential attributes after freezing and thawing through which a spermatozoon can be capable to penetrate and fertilize an oocyte. These attributes are as following: a) adequate energy for metabolism process, b) progressively motile characteristics and c) intact acrosome reaction containing the necessary lytic enzymes. Because stallion spermatozoa are known to be cryosensitive and less cryotolerant, lytic enzymes are most likely to disappear after cryopreservation process. Only a few subpopulation sperm cells that are believed to have unusually stable plasma membrane can survive cold-shock (Kuisma et al., 2006). Such subpopulation might have some distinct characteristics based on molecular levels and plasma membrane composition during the process of spermatogenesis.

## **2.2 Spermatogenesis**

Spermatogenesis is a series of regulated and complex cellular transformations through which mature sperm cells are eventually differentiated and produced. In stallion as in mammals, spermatogenesis typically occurs within the seminiferous tubules. The latter is the site where several cellular transformations take place to ultimately yield the fully developed germ cells. This process, and after consecutive events, can be generally divided into three developmental phases (Russell et al., 1993). The first phase known as proliferation phase in which several mitotic divisions of spermatogonia occur to generate an abundant number of B-spermatogonia. This phase usually lasts for approximately 19.4

days (Amann, 1993). Starting with spermatocytogenesis, spermatogonia which are localized nearest the basement membrane of seminiferous epithelium undergo several mitotic divisions to produce three types of undifferentiated A spermatogonia (A1, A2, A3). These three subtypes evolve and develop to generate (B1, B2) spermatogonia, which in turn differentiate to primary spermatocytes (Johnson, 1991) prior to the second phase.

Meiotic phase is the phase where the primary and secondary spermatocytes are generated after a couple of meiotic divisions. This phase basically takes place in the adluminal compartment of seminiferous epithelium, and it lasts for approximately 19.4 days (Amana, 1993). The third and last phase is called differentiation phase (spermiogenesis) which is localized within adluminal compartment of the seminiferous epithelium as well. The duration of this phase is around 18.6 days (Amann, 1993). During this phase, the round spermatids undergo further distinct changes and become elongated. In addition, the DNA becomes considerably condensed by which the male's genetic materials are transported while fertilizing the oocyte. Formation of the acrosome is believed to happen during this phase. The acrosome is considered as an essential and crucial site due to the presence of hydrolytic enzymes. Such enzymes are believed to specifically digest and penetrate the zona pellucida (ZP). All other compartments are likely to be developed during this phase including the mitochondria, which serve as energy houses and other metabolic functions, The flagellum (tail) is formed, and the cells finally become motile (Senger, 2005). At the end of this phase, spermatozoa become highly differentiated and potentially specialized. The mature spermatozoon consists of a head (nucleus and acrosome), mid-piece (Mitochondrial helix), and flagellum (tail). The total duration of spermatogenesis in stallions is approximately 57-58 days (Amann,

1993). Consequently, the mature and motile sperm cells proceed toward the seminiferous epithelium lumen and are ready to be ejaculated in combination with seminal fluids secreted from accessory sex glands, especially during breeding season.

### **2.3 Seasonal Breeders**

To the best of our knowledge mammals and non-mammals are classified into three categories based on their mating behaviors as following: Continuous, opportunistic, and seasonal breeders. The continuous breeders include humans that mate throughout the year. Opportunistic breeders, however, mate whenever their environment permits and are influenced by so called “short-term” factors such as rainfall, food abundance, and ambient temperature. Some birds and small rodents fall under this category. Conversely, some species mate and give births during certain times of the year. These species including (horses, hamsters, sheep, goats, etc.) are named seasonal breeders. In the stallion, for instance, sperm production varies markedly due to age (Amann et al., 1979; Johnson et al., 1981; Johnson and Thompson, 1983) and season (Berndtson et al., 1983; Johnson and Thompson, 1987). Additionally, season seems to have a significant effect on the stallion physiological and endocrinological functions (Hoffman et al., 1999). As a “long-day” breeder, the mating and fertility in equine males are usually regulated by the day length “photoperiod”. Photoperiod is more likely to play a key role to induce the central control of reproduction “hypothalamus” and hence releasing proper hormones such as GnRH (Lehman et al., 1997). During breeding season (spring and summer), for example, concentrations of gonadotropins, testosterone, and estrogens are increased in stallions (Johnson, 1985; Thompson et al., 1986; Roser and Hughes, 1991). On the contrary, during non-breeding season (winter), stallions, unlike mares, remain fertile but



with some decrease in sexual behavior and spermatozoa output (Amann, 1987; Magistrini et al., 1987; Janett et al., 2003). There is evidence that exposing stallions to artificial photoperiod might maximize the reproductive capacity of the stallions as well as its sexual behavior during non-breeding season (Clay et al., 1987). That could probably be attributed to the influence of light on the central control of reproduction to regulate releasing hormones and testicular functions.

#### **2.4 Testicular Regulation**

It is well-documented that the Hypothalamus-Pituitary-Testicular (HPT) axis controls reproductive and other physiological and endocrinological functions via releasing variety of hormones (Matsumoto, 1989; Skinner, 1991; Spiteri-Grech and Nieschlag, 1993; Amann, 1993; Weinbauer and Nieschlag, 1993; Huhtaniemi and Toppari, 1995; Gnessi et al., 1997). The hypothalamus is induced to release gonadotropin releasing hormone (GnRH) which in turn stimulates the productions by the anterior pituitary gland of other functional hormones. GnRH then is stimulated to target the testis through secreting the gonadotropins, follicle stimulating hormone (FSH) and luteinizing hormone (LH; Irvine, 1984; Irvine and Alexander, 1987). FSH targets mainly the Sertoli cells, whereas LH works on the Leydig cells. Sertoli cells are found within the seminiferous epithelium and act supportively in generating sperm cells. Similarly, Sertoli cells regulate the amount of FSH secretion from pituitary gland. FSH, and with aim of testosterone, is responsible to complete the spermatogenesis process (Davies Morel, 1999).

On the other hand, Leydig cells are located within the intertubular of seminiferous tubules and supporting Sertoli cells by synthesizing testosterone hormone to terminate

spermatogenesis. Furthermore, Leydig cells are governed directly by pituitary gland via LH releasing (Amann, 1981a). Besides testosterone production, Leydig cells release estrogens as well (Eisenhauer and Roser, 1995). In fact, testosterone is responsible for sexual behavior and testicular functions (Davies Morel, 2003). It has negative effect on pituitary gland to reduce the amounts of LH and FSH and subsequently its production as well (Irvine et al., 1986; Plant, 1986; Flink, 1988, Tilbrook et al., 1991). There are other hormones that are believed to have a key role in controlling the male reproductive system. Inhibin and activin both are secreted from the Sertoli cells to help with completion of spermatogenesis process. Inhibin has a negative feedback somehow on the performans of hypothalamus and pituitary glands, whereas activin acts positively to support them (Roser, 1997). The complete role of these two hormones in stallion has yet to be elucidated. In addition, prolactin and estrogens are known to enhance, in some way, the physiological and testicular functions, yet the importance of such hormones is still not completely understood in horses (Raeside, 1969; Seamens et al., 1991; Landeck, 1997).

## **2.5 Artificial Insemination**

Artificial insemination (AI) can be defined as the process of collecting semen and depositing it into a female reproductive tract. The history of AI dates as far back as 1700s when the Italian scientist “Spallanzani” practiced his first AI attempt on a bitch using fresh semen to successfully yield three healthy pups (Herman, 1981; Foote, 1982). Thereafter, and specifically in 1799, John Hunter reported a human pregnancy with the use of AI technique (Herman, 1981). During 1880s, the improvement of fertility and increasing the pregnancy rates were relatively accomplished with the aim of AI. In Russia (Herman, 1981), the AI technique has potentially exceeded the expectations by

inseminating 15 million sheep, 40000 mares, and 1.2 million cows since 1938. At the same time, AI technology was just introduced to the US to predominantly be applied in different fields (Salisbury et al., 1978, Foote, 2001). It was reported that the number of inseminated cows surprisingly increased from 34,000 cows in 1940 to 8,500,000 in 1980 in the United States (Foote, 1982). Because of the optimal application of AI, cattle industries have successfully developed such technology to maximize the production efficiency. In the US, the use of AI has been growing rapidly in both dairy and beef cattle industries, but has yet to be widely used in swine and horses.

There are several objectives behind the idea of using AI that might probably answer many controversial questions. First, improvement of livestock animal is, from genetic standpoint, a great of importance including selection and crossbreeding of superior sires (Zafracas, 1994). Second, the availability of genetically merit sires would allow breeders to have an access to their semen either domestically or internationally (Sukalic et al., 1982). Third, controlling the venereal diseases is one of the advantages of AI technology by avoiding natural mating, and hence preventing such diseases to be transferred (Tischner, 1992; Clement et al., 1993). Additionally, it can particularly prevent or at least reduce the bad and aggressive sexual behavior of the sire (McDonnell et al., 1991; Love, 1992). Forth, and from economic perspective, AI would be considerably used to reduce costs of management and maintenance of the sire. Furthermore, many doses can be obtained per ejaculate, which in turn increases the profits. In a few recent years, AI technique has been utilized to preserve endangered species (Wildt et al., 1995). There are some applications which are involved with the use of AI such as packaging systems and spermatozoa density methods.

## 2.6 Packaging Systems

The urgent need of storing and transporting of genetic materials domestically and internationally has motivated researchers to develop various packaging systems and methods. Obviously, loading spermatozoa in small straws has facilitated transporting and identifying individual male semen (Bwanga et al., 1990, 1991). The idea was first established by Pursel and Johnson (1975) when they developed so called “pellet method” to freeze boar spermatozoa and overcome the difficulty of semen identification. The mini straws (0.25 and 0.5 ml) and maxi straws (5ml) methods were developed mainly for packaging sperm cells for long-term storage (Bwanga et al., 1990, 1991). The optimal spermatozoa number per straw definitely depends on species, glycerol concentration, post thaw quality, and AI method. Concerning the AI method, depositing semen into the cervix, for example, requires higher number of spermatozoa than the one deposited into uterus or oviduct for potential fertility. The short distance between the sperm and ova is the less number of motile sperm cells (Senger, 2005). In the same manner, the AI breeding dose with chilled semen requires less motile spermatozoa compared to frozen semen. With chilled semen, for instance, breeding dose is about 500 million progressively motile spermatozoa. On the contrary, cryopreserved semen dose is either from 300 million to 1 billion total sperm cells including dead sperm or 250 to 600 million progressively motile sperm to achieve the optimal level of pregnancy and foaling rates. However, foaling rate was practically achieved with  $80 \times 10^6$  sperm cells instead of  $40 \times 10^6$  sperm cells (Amann, 1987). The pregnancy rate, however, there was no variation due to the number of spermatozoa inseminated with either  $320 \times 10^6$  or  $800 \times 10^6$  sperm cells using mares (Leipold et al., 1998). Palmer (1984) concluded that a concentration of more

than  $100 \times 10^6$  sperm cells/ml were lethal to cryopreserved spermatozoa. In contrast, spermatozoa loaded in 0.5 ml straws at a concentration of  $400 \times 10^6$  cells/ml (Heitland et al., 1996) or  $1600 \times 10^6$  cells/ml (Leipold et al., 1998) showed no differences and the results were positively acceptable. Packaging spermatozoa in 0.5 ml straws has become the most common and convenient method in almost all species and provided preferable freezing uniformity. Packaging systems have become an essential economic subject for owners and producers because increasing the number of straws per ejaculate would be of a great value and increase the input.

## **2.7 Computer-Assisted Sperm Analysis (CASA)**

Determining the fertilizing capacity of the sperm cells of an individual male is usually done by measuring sperm motility. However, there are several methods to evaluate the sperm motility characteristics. The light microscope is a subjective method that primarily employed for estimating sperm movement. The accuracy of this method is relatively low, and it is susceptible to human errors. Because of the variation between and within technicians (Dunphy et al., 1989), there would be a restricted correlation when assessing the fertilization rates either in vitro or vivo (Zaini et al., 1985). Due to the need of more accurate and precise results, several objective and reliable systems have been developed so far. In 1980s, Computer-assisted sperm analysis (CASA), such as CellTrack (Motion analysis corporation, Sanata Rosa, CA), CellSoft (Cryo Resources, Montgomery, NY), and HTM-2000 (Hamilton-thorn Research, Beverly, MA) were introduced and developed mainly for human laboratories (Aitken et al., 1985; Jeulin et al., 1986; Katz et al., 1986). Most CASA systems, however, work almost under the same principles, which are initially established to identify and track sperm cells and movement patterns.

Nonetheless, the fact that CASA equipments are recently the most reliable methods, they give very detailed information on different sperm motion including progressively motile sperm, rapid cells, and other velocity parameters that perhaps help evaluating post-thaw sperm quality (Verstegen et al., 2002; Rijsselaere et al., 2005; Peña et al., 2005). Up to date, CASA systems have been employed for many mammalian species (Holt et al., 1994; Holt et al, 1996; Rijsselaere et al., 2003; Wilson-leedy and Ingermann, 2007) which are available for use in commercial AI centres and veterinary practice. Mortimer (1997) has demonstrated how other sperm motility parameters can be measured by CASA (Figure 2.1) as following:

### **2.7.1 Total Sperm Motility Parameters**

- Total number of spermatozoa (%).
- Percentage of motile sperm (%).
- Percentage of progressively motile sperm (%).
- Number of rapid sperm cells ( $\geq 45 \mu\text{m/s}$ ).

### **2.7.2 Velocity Parameters (Figure 2.1)**

- VAP (Average path velocity,  $\mu\text{m/s}$ ) -the velocity of smoothed cell path.
- VCL (Curvilinear velocity,  $\mu\text{m/s}$ ) - measured over the real point to point trajectory followed by the cell.
- VSL (Straight line velocity,  $\mu\text{m/s}$ ) - measured in a straight line from the first to the last points of track.

### **2.7.3 Progression Ratios (Figure 2.1)**

- STR (Straightness, %) - measures the departure of the cell path from a straight line =  $VSL/VAP \times 100$ .
- LIN (Linearity, %) - measures the departure of the cell track from a straight line =  $VSL/VCL \times 100$ .

### **2.7.4 Head Movements (Figure 2.1)**

- BCF (Beat cross frequency, HZ) - frequency of the sperm head crosses the smoothed path.
- ALH (Amplitude of lateral head displacement,  $\mu\text{m}$ ) - mean lateral sperm head displacement throughout the smoothed path.

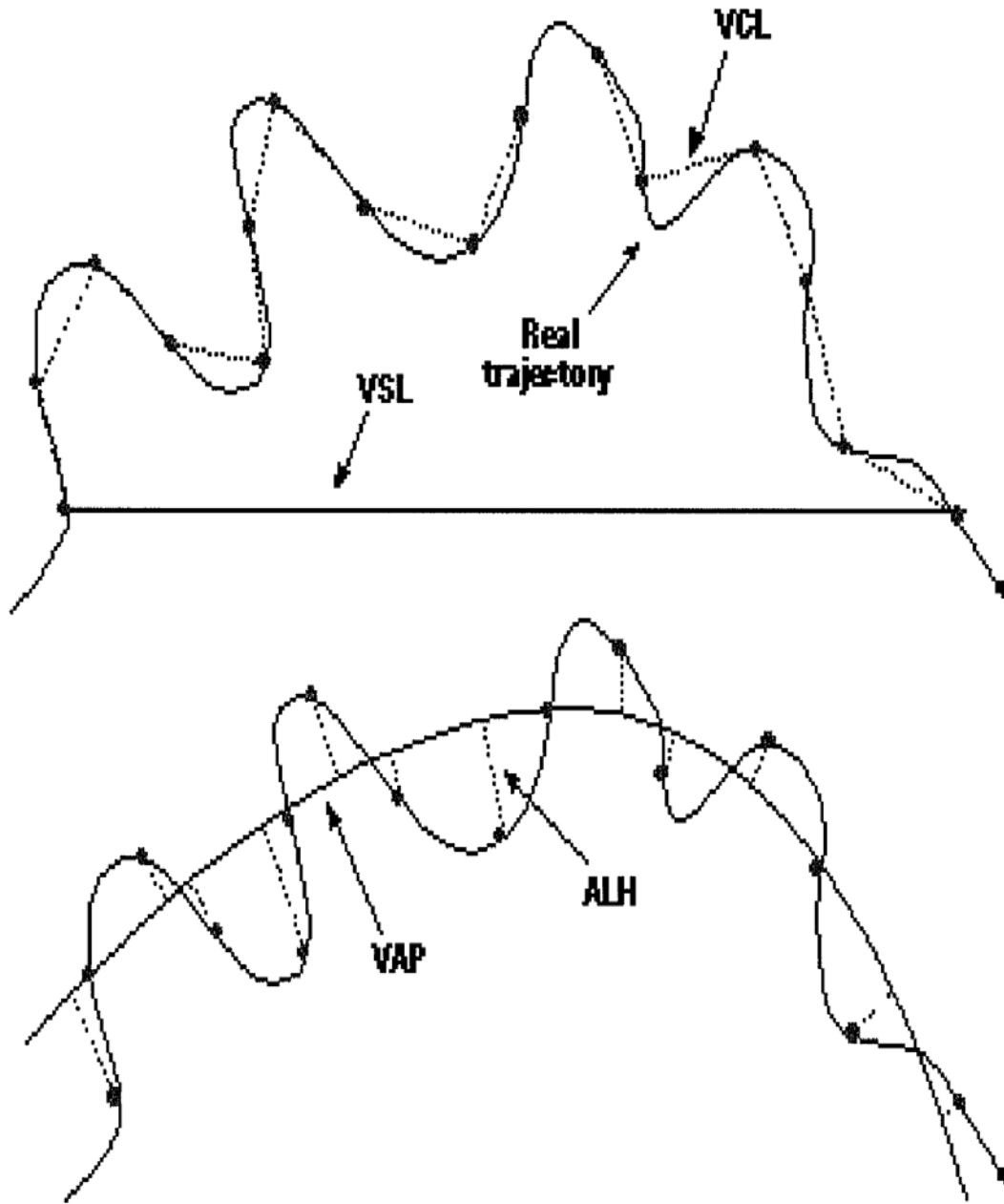


Figure 2.1 Different track speed and motility of sperm obtained by the Hamilton-Thorn Motility Analyzer.

Velocities ( $\mu\text{m/s}$ ): Curvilinear velocity (VCL); average path velocity (VAP); straight line velocity (VSL). Progression ratios (%): Linearity ( $\text{LIN} = \text{VSL}/\text{VCL} \times 100$ ); straightness ( $\text{STR} = \text{VSL}/\text{VAP} \times 100$ ). Head movements ( $\mu\text{m}$ ): Amplitude of lateral head displacement (ALH). Liu et al. (1991).



## CHAPTER III

### CRYOPRESERVATION OF EQUINE SPERMATOZOA: IDENTIFICATION OF GOOD AND POOR FREEZER STALLIONS AND EFFECT OF SPERM DENSITY PER STRAW

#### 3.1 Abstract

The main objective of this study was to identify the potential characteristics of good and poor semen freezers in the equine. Four stallions (A, B, C, and D) were used and collected once a week during summer months of June-July 2011. Raw semen was transported to the laboratory and diluted with cooling extender followed by the freezing diluent. Spermatozoa from the same ejaculate were then packed into 0.5 ml plastic straws in two different concentrations ( $0.4 \times 10^9$  and  $0.8 \times 10^9$  sperm/ml). Samples were analyzed after at least one week. Motility, total progressively motile sperm (PMOT), rapid sperm (RS), and velocity parameters were evaluated using the Computer Assisted Sperm Analysis (CASA). Data were statistically analyzed using general linear model (GLM) procedure in (SAS). Least Significant Difference (LSD) was run for pair wise comparison between means, and the threshold of significance was set at ( $P < 0.05$ ). The results indicated one stallion with the highest and most significant mean of PMOT and rapid sperm in both concentrations. On the contrary, another stallion had the lowest mean of PMOT and RS at  $0.4 \times 10^9$ /ml concentration only. Consequently, the concentration of  $0.4 \times 10^9$  sperm cells/ ml would be recommended for the present study, and such

discrepancies should be further investigated. In conclusion, it may be possible to classify stallions with good and poor freezing tolerant spermatozoa based upon their post-thaw motility.

### **3.2 Introduction**

Low pregnancy rate (PR) in equine industry is recently becoming a questionable issue when post-thawed semen is used for (AI). Both stallion managers and mare owners have been looking for potential solutions since such issue has emerged. One of the key factors that affect PR was spermatozoa cryoinjury during semen handling. It is well-documented, however, that sperm motility parameters are extremely affected by the freezing process, and such variation can exist between stallions and within stallion (Gebauer et al., 1976; Magistrini et al., 1987; Amann, 1987; Braun et al., 1994; Vidament et al., 1997; Batellier et al., 2001; Alghamdi et al., 2002; Henry et al., 2002; Janett et al., 2003; Schembri et al., 2003; Neild et al., 2003; Goolsby et al., 2004). Age and breed are most common sources of variation in semen characteristics, and subsequently influencing freezability of spermatozoa (Dowsett and Knott, 1996). Measuring the effect of cryopreservation on such characteristics requires an accurate and objective method, such as CASA system. Employing this system is of great importance, because it evaluates and provides detailed information on the most relevant sperm movement parameters, such as (motility (%), progressiveness of sperm (%), rapid sperm (%), average path velocity (VAP,  $\mu\text{m/s}$ ), curvilinear velocity (VCL,  $\mu\text{m/s}$ ), straight line velocity (VSL,  $\mu\text{m/s}$ ), amplitude of lateral head displacement (ALH,  $\mu\text{m}$ ), straightness (STR, %), linearity (LIN, %), and beat cross frequency (BCF, HZ). Some of which are believed to be positively correlated with fertility as determined by *in vivo* or *in vitro* studies (Wijchman et al.,

2001, Liu et al., 1991, Holt et al., 1985; Hirano et al., 2001; Aitken et al., 1985).

Interestingly, packaging of spermatozoa in different concentrations might adversely affects post-thaw sperm survivability (Varner et al., 1987). Also, it would be better to adjust the concentration of spermatozoa accordingly to avoid any detrimental effects on sperm functions (Dong et al., 2007; Yang et al., 2012). Recently, studies have reported that stallion sperm cells can be frozen at a concentration of  $40 \times 10^6$  /ml in 0.25 ml straws with no deleterious effect on spermatozoa movement parameters (Clulow et al., 2008). From an economic point of view, dividing an ejaculate from a genetically superior stallion into many doses would multiply and increase the income as well as preserving that valuable stallion.

Overall, the aim of the present study was to measure the most common sperm motility parameters that have been used for semen evaluation before and after cryopreservation process. Also, determining whether or not the high sperm density in frozen straws affects the sperm attributes in one way or another. Finally, and based upon sperm responsiveness, identifying the selected stallions as good or poor freezers would be of great interest and allow for further studies to evaluate the sperm based on the molecular markers and membrane composition differences.

### **3.3 Materials and Methods**

#### **3.3.1 Spermatozoa Preparation before Freezing**

Semen from four stallions their ages range between (6-19 years) from different breeds (A, Quarter horse; B, Quarter horse; C, Welsh pony; and D, Thoroughbred) was collected using the artificial vagina (AV; Colorado model; Animal Reproduction System, Chino, CA). Stallions were located at the Horse Unit in Mississippi State University.

Stallions were collected once a week from June to July 2011. Ejaculates were transported to the laboratory at (37°C) with the aim of warm packs (37°C) placed in a foam box.

### **3.3.2 Semen Freezing Process**

A sample (10-15 µl) of raw semen was taken for motility analysis. Raw semen was diluted in *Institute National de la Recherche Agronomique* (INRA 82, D1) extender to a final concentration of  $20 \times 10^6$  /ml in 50 ml falcon tubes and kept at room temperature (22°C) for 10 minutes. Samples were centrifuged at  $600 \times g$  for 10 minutes at room temperature. Supernatant was discarded and the sperm pellet re-suspended in INRA 82 + 2.5% glycerol (D2) to reach the final concentrations of  $0.4 \times$  and  $0.8 \times 10^9$  sperm cells/ml. Samples were placed in cool cabinet (4°C) for 2hrs. Spermatozoa were packed into well-identified 0.5 ml straws (animal ID, date of collection, and concentration), and placed on Styrofoam float held of approximately 2-3 cm above liquid nitrogen. After 30 minutes exposure, straws were plunged in the liquid nitrogen (-196°C) and stored for further analyses.

### **3.3.3 Semen Thawing Process**

Straws were taken out from the tank and placed in a water- bath set at (37°C). After 30 seconds, straws were wiped with a paper towel, and the content was placed in tubes containing the pre-warmed D1 dilution (37°C) to reach a final concentration of approximately  $20 \times 10^6$  sperm/ml. Diluted samples were immediately transferred to the incubator (37°C) for 15 minutes. Kinematic parameters were assessed, and an aliquot of (5-10µl) diluted with pre-warmed PBS.

### **3.3.4 Semen Analysis**

Motility parameters of spermatozoa were analyzed with CASA (HTM-IVOS Hamilton-Thorne Biosciences, Version 12.3. Beverly, MA). A four chamber slide (Standard Count 4 Chamber Slide Leja®, 20 micron, Nieuw Vennep, Netherlands) was placed into the CASA and set at 37°C prior to loading a volume of extended spermatozoa. Data were collected on a range of 200-400 sperm cells per sample from three random fields. All ejaculates were analyzed, and data were recorded as percentages of motility parameters (%). CASA was conducted on both frozen and fresh semen to observe the impact of cryopreservation on semen quality.

### **3.3.5 Statistical Analyses**

Significant differences were determined by least significant difference method ( $P < 0.05$ ) and GLM procedure of SAS (SAS® 9.2, copyright © 2002-2008, SAS Institute Inc., Cary, NC). Data was presented in this study as (mean  $\pm$  SEM).

## **3.4 Results**

### **3.4.1 Fresh vs. Frozen**

After running CASA with fresh semen, sperm motility parameters (motility, progressively motile sperm, and rapid sperm) were obtained as proportions of counted cells per sample. In figure (3.1), the highest mean of motility was stallion (D;  $92 \pm 4.3$  %,  $P < 0.05$ ). On the other hand, stallions (A and C) were not significantly different ( $86 \pm 3.8$ % and  $83 \pm 3.3$ %) respectively, whereas stallion (B) had the lowest mean of motility among all stallions ( $75.5 \pm 3.3$ %).

With regards to progressive sperm, only stallion (D) showed the lowest mean of progressively motile sperm ( $23 \pm 4.4\%$ ), but other individuals did not differ.

Additionally, stallions (A, C, and D) did not differ with respect to mean of rapid spermatozoa ( $61 \pm 5.4\%$ ,  $70 \pm 4.5\%$ , and  $64 \pm 6\%$ ) respectively, whereas stallion (B) was significantly different ( $45 \pm 4.5\%$ ).

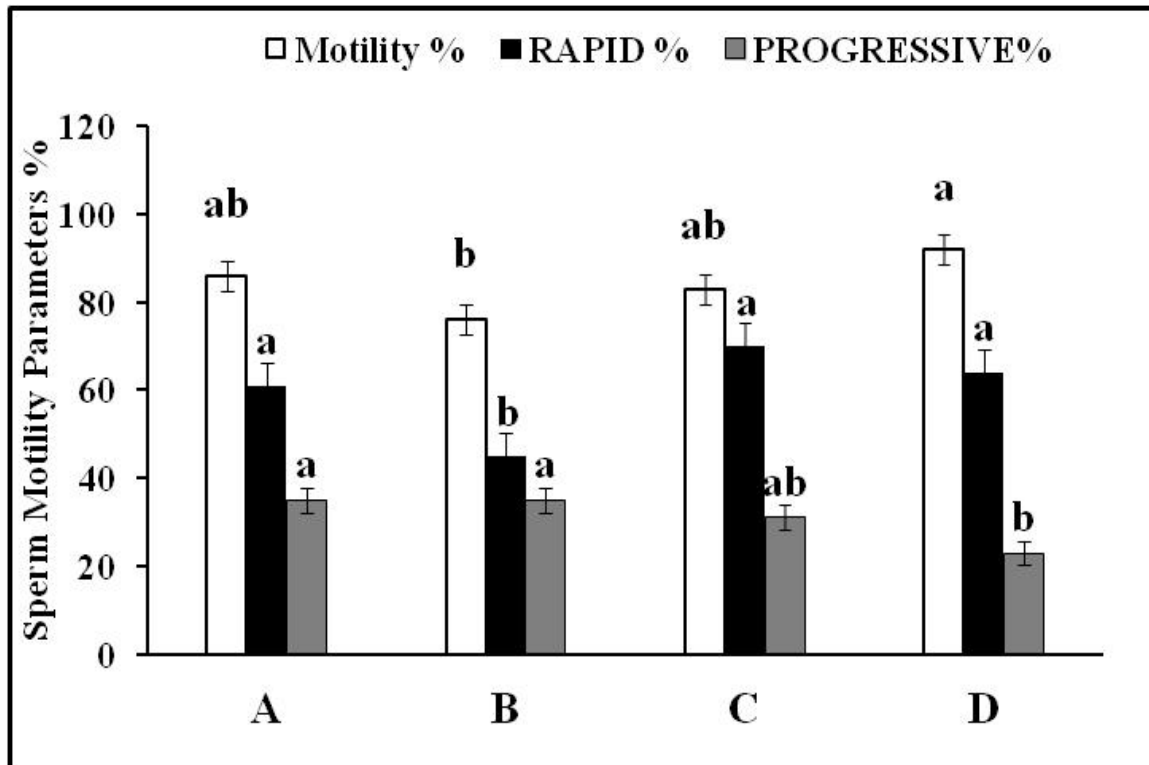


Figure 3.1 Motility parameters of four stallions (A, B, C, and D) measured by CASA immediately after collection.

Data are shown as mean percentage  $\pm$  SEM of 4-7 ejaculations per stallion. Letters (<sup>a, b, c</sup>) indicate significant differences within the same column ( $P < 0.05$ ).

It was observed, however, that all parameters mentioned above were significantly declined when spermatozoa were cryopreserved (Figure 3.2). In terms of motility, stallion (D) was severely affected by freezing process ( $92 \pm 4.3\%$  vs.  $38 \pm 6.4\%$ ), and it was the

lowest compared with other stallions although it was not significantly different from stallion (C). Similarly, for both rapid and progressive sperm parameters, stallions (A and B) were so close and there was no difference, and (C and D) did as well, but they had the lowest means ( $16 \pm 2.2\%$ ,  $7 \pm 1.4\%$ ,  $16 \pm 2.4\%$ , and  $8 \pm 1.6\%$ ) respectively, compared with stallion (A).

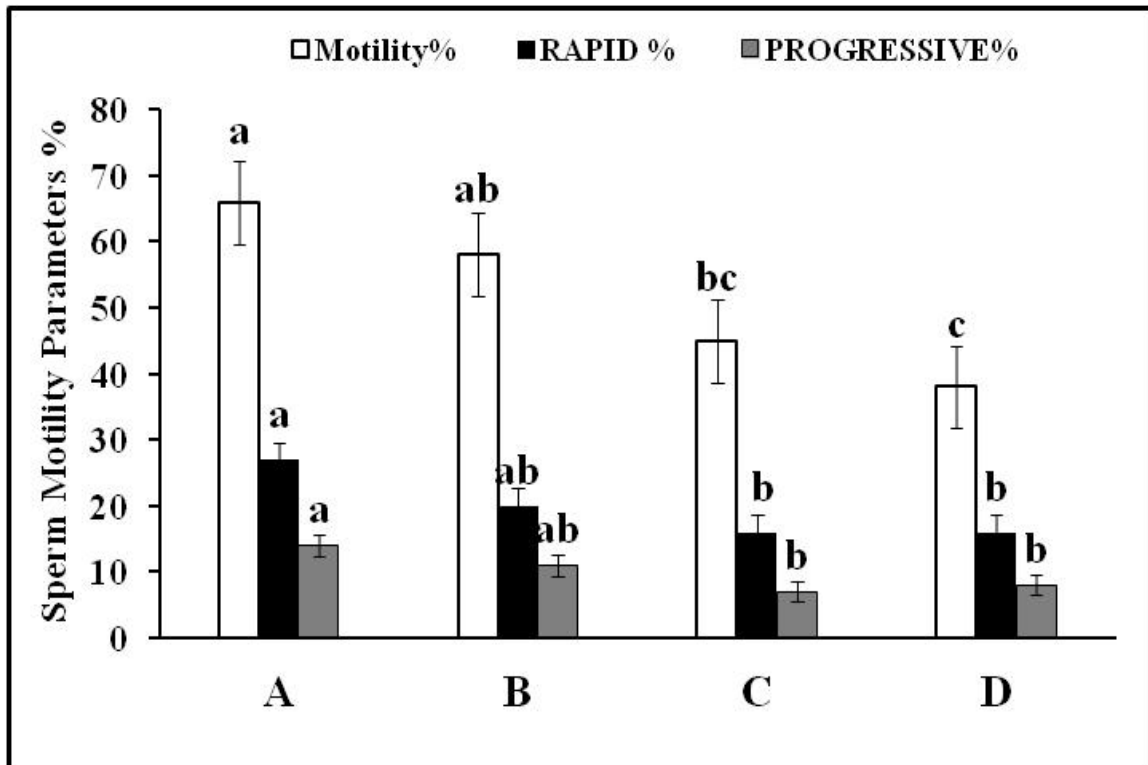


Figure 3.2 Cryopreserved spermatozoa motility parameters of four stallions (A, B, C, and D) measured by CASA.

Data are shown as mean percentage  $\pm$  SEM of 4-7 ejaculations per stallion. 4-7 ejaculations per stallion. Letters (<sup>a, b, c</sup>) indicate significant differences within the same column ( $P < 0.05$ ).

### 3.4.2 Individual Variations

Table 3.1 summarizes the individual variations and subsequently identifying the good and poor freezers by measuring post-thaw spermatozoa motility parameters using

CASA at  $0.4 \times 10^9$  sperm cells/ml. Significant variation were observed between stallions (A and D) with respect to the mean of progressive sperm cells (%) and rapid cells (%) but not for motility. Furthermore, curvilinear velocity (VCL,  $\mu\text{m/s}$ ) was the only variable under which stallions (A and D) differed significantly. A significant variation between stallions (A and D) was detected with respect to the mean of linearity (LIN, %). Similarly for BCF (Beat cross frequency, HZ) was much lower in stallion (D) than in stallion (A).

Table 3.1 Mean values ( $\pm$  SEM) of post-thawed spermatozoa motility characteristics of four individual stallions at  $0.4 \times 10^9$  sperm cells/ml.

Parameters	Stallions			
	A	B	C	D
<u>Motility</u>				
Motility (%)	64.2 $\pm$ 8.4 <sup>a</sup>	54.0 $\pm$ 7.6 <sup>a</sup>	42.7 $\pm$ 7.6 <sup>a</sup>	41.3 $\pm$ 9.4 <sup>a</sup>
Progressive (%)	16.8 $\pm$ 2.2 <sup>a</sup>	12.0 $\pm$ 2.0 <sup>ab</sup>	9.5 $\pm$ 2.0 <sup>b</sup>	9.0 $\pm$ 2.5 <sup>b</sup>
Rapid (%)	29.0 $\pm$ 3.5 <sup>a</sup>	21.2 $\pm$ 3.2 <sup>ab</sup>	19.5 $\pm$ 3.2 <sup>ab</sup>	18.0 $\pm$ 3.9 <sup>b</sup>
<u>Velocities</u>				
VAP ( $\mu\text{m/s}$ )	59.6 $\pm$ 5.1 <sup>a</sup>	57.2 $\pm$ 4.6 <sup>a</sup>	52.0 $\pm$ 4.6 <sup>a</sup>	45.7 $\pm$ 5.7 <sup>a</sup>
VCL ( $\mu\text{m/s}$ )	112.2 $\pm$ 7.4 <sup>a</sup>	109.0 $\pm$ 6.8 <sup>a</sup>	91.2 $\pm$ 8.3 <sup>ab</sup>	86.0 $\pm$ 6.8 <sup>b</sup>
VSL ( $\mu\text{m/s}$ )	47.0 $\pm$ 4.9 <sup>a</sup>	42.2 $\pm$ 4.5 <sup>a</sup>	41.4 $\pm$ 4.5 <sup>a</sup>	33.0 $\pm$ 5.5 <sup>a</sup>
<u>Progression Ratios</u>				
STR (%)	72.4 $\pm$ 2.9 <sup>a</sup>	70.3 $\pm$ 2.7 <sup>a</sup>	69.8 $\pm$ 2.7 <sup>a</sup>	66.5 $\pm$ 3.3 <sup>a</sup>
LIN (%)	45.0 $\pm$ 2.0 <sup>a</sup>	42.2 $\pm$ 2.2 <sup>ab</sup>	39.5 $\pm$ 2.0 <sup>ab</sup>	36.5 $\pm$ 2.4 <sup>b</sup>
<u>Head Movement</u>				
BCF (HZ)	38.8 $\pm$ 2.6 <sup>a</sup>	35.6 $\pm$ 2.9 <sup>ab</sup>	34.5 $\pm$ 2.3 <sup>ab</sup>	30.2 $\pm$ 2.3 <sup>b</sup>
ALH ( $\mu\text{m}$ )	4.9 $\pm$ 1.7 <sup>a</sup>	4.8 $\pm$ 1.7 <sup>a</sup>	4.6 $\pm$ 2.1 <sup>a</sup>	4.3 $\pm$ 2.1 <sup>a</sup>

Data are mean ( $\pm$ SEM) of 4-6 ejaculates from four stallions (A, B, C, and D). Significant differences ( $P < 0.05$ ) are indicated by different letters (<sup>a, b</sup>) on the same line across stallions.

Even with high spermatozoa density ( $0.8 \times 10^9$  sperm cells/ ml), as anticipated, stallion (A) showed significance ( $P < 0.05$ ) over stallion (D) in most of the relevant parameters, such as motility (%), progressive sperm (%), rapid cells (%), average path



velocity (VAP,  $\mu\text{m/s}$ ), and straight line velocity (VSL,  $\mu\text{m/s}$ ), and all such differences mentioned above are summarized in Table 3.2.

Table 3.2 Mean values ( $\pm$  SEM) of post-thawed spermatozoa motility characteristics of four individual stallions at  $0.8 \times 10^9$  sperm cells/ml.

Parameters	Stallions			
	A	B	C	D
<b>Motility</b>				
Motility (%)	$67.7 \pm 8.8^a$	$62.5 \pm 8.8^a$	$50.2 \pm 8.8^{ab}$	$34.5 \pm 8.8^b$
Progressive (%)	$11.5 \pm 1.4^a$	$9.5 \pm 1.4^{ab}$	$6.2 \pm 1.4^{bc}$	$4.7 \pm 1.4^c$
Rapid (%)	$24.0 \pm 2.4^a$	$19.2 \pm 2.4^{ab}$	$13.5 \pm 2.4^b$	$11.7 \pm 2.4^b$
<b>Velocities</b>				
VAP ( $\mu\text{m/s}$ )	$52.1 \pm 4.3^a$	$50.3 \pm 4.3^{ab}$	$42.1 \pm 4.3^{ab}$	$38.3 \pm 4.3^b$
VCL ( $\mu\text{m/s}$ )	$98.7 \pm 9.6^a$	$96.4 \pm 9.6^a$	$91.2 \pm 9.6^a$	$75.9 \pm 9.6^a$
VSL ( $\mu\text{m/s}$ )	$40.3 \pm 3.4^a$	$38.4 \pm 3.4^a$	$32.3 \pm 3.4^{ab}$	$25.0 \pm 3.4^b$
<b>Progression Ratios</b>				
STR (%)	$70.5 \pm 1.7^a$	$70.0 \pm 1.7^a$	$69.7 \pm 1.7^a$	$64.0 \pm 1.7^a$
LIN (%)	$42.5 \pm 2.2^a$	$41.0 \pm 2.2^a$	$39.2 \pm 2.2^a$	$37.0 \pm 2.2^a$
<b>Head Movement</b>				
BCF (HZ)	$39.3 \pm 3.2^a$	$35.2 \pm 3.2^a$	$32.4 \pm 3.2^a$	$31.4 \pm 3.2^a$
ALH ( $\mu\text{m}$ )	$4.9 \pm 0.4^a$	$4.2 \pm 0.4^a$	$4.1 \pm 0.4^a$	$3.9 \pm 0.4^a$

Data are mean ( $\pm$ SEM) of 4-6 ejaculates from four stallions (A, B, C, and D). Significant differences ( $P < 0.05$ ) are indicated by different letters (<sup>a, b, c</sup>) on the same line across stallions.

### 3.4.3 Effect of Sperm Density Packaging

The effect of sperm density during freezing on post-thawed survival was evaluated as well by comparing two concentrations ( $0.4 \times$  and  $0.8 \times 10^9$  sperm cells/ml) used in this experiment. The experiment was carried out by measuring all sperm movement parameters and progression ratios to ensure consistency and accuracy throughout the study. Data are shown as mean ( $\pm$  SEM) in Table 3.3. As clearly stated on the table, only progressively motile sperm, average path velocity (VAP,  $\mu\text{m/s}$ ), and

straight line velocity (VSL,  $\mu\text{m/s}$ ) were significantly higher in concentration ( $0.4 \times 10^9$  sperm cells/ ml) compared to ( $0.8 \times 10^9$  sperm cells/ ml) used in this experiment. In fact, for other parameters were almost the same for both concentrations.

Table 3.3 Effects of sperm density on post-thawed sperm motion characteristics using two different concentrations ( $0.4 \times 10^9$  vs.  $0.8 \times 10^9$  cells/ ml).

Parameters	Concentrations	
	$0.4 \times 10^9$ sperm cells/ml	$0.8 \times 10^9$ sperm cells/ml
Motility (%)	$51.0 \pm 4.3^a$	$54.0 \pm 5.0^a$
Progressive (%)	$11.8 \pm 1.0^a$	$8.0 \pm 1.2^b$
Rapid (%)	$22.0 \pm 1.6^a$	$17.0 \pm 1.9^a$
VAP ( $\mu\text{m/s}$ )	$54.0 \pm 2.3^a$	$45.7 \pm 2.7^b$
VCL ( $\mu\text{m/s}$ )	$99.8 \pm 4.2^a$	$87.7 \pm 4.9^a$
VSL ( $\mu\text{m/s}$ )	$41.4 \pm 2.2^a$	$34.0 \pm 2.5^b$
STR (VSL/VAP, %)	$70.0 \pm 1.2^a$	$68.5 \pm 1.3^a$
LIN (VSL/VCL, %)	$41.1 \pm 1.1^a$	$40.8 \pm 1.3^a$
BCF (HZ)	$34.5 \pm 1.3^a$	$34.5 \pm 1.5^a$
ALH ( $\mu\text{m}$ )	$4.7 \pm 0.2^a$	$4.3 \pm 0.2^a$

Data are mean ( $\pm$ SEM) of 4-6 ejaculates from four stallions (A, B, C, and D). Significant decreases ( $P < 0.05$ ) are indicated by different letters (<sup>a, b</sup>) on the same line across concentrations ( $0.4 \times$  and  $0.8 \times 10^9$  cells/ ml).

### 3.5 Discussion

After evaluating fresh and post thawed sperm characteristics employing CASA, it was evident that cryopreservation had a significant effect on sperm movement parameters by declining their performance efficiency. All three major motility parameters (Motility, progressively motile sperm, and rapid cells, %) appeared to be extremely decreased following the cryopreservation procedure (Figure 3.2). Conversely, differences between

spermatozoa characteristics of fresh and frozen sperm seemed to vary among stallions used in the present study. Stallion (D), for instance, showed relatively higher motile sperm (92%) over other stallions when estimating its fresh semen. On the other hand and after exposing to very low temperature (-196°C), spermatozoa of the same stallion was negatively affected and had the lowest mean (42%) compared to stallion (A, 64%) in Figure 3.2. In terms of progressively motile sperm (%), all selected stallions were adversely affected by the cryopreservation procedure, specifically stallions (C and D), yet stallion (A) was more resistible to boiling temperature of (LN). On the same manner, rapid cells of frozen spermatozoa were significantly decreased for all stallions, especially stallions (C and D) compared with fresh semen. Interestingly, and supporting the suggestion that even some fertile stallions might exhibit high motility percentages, their semen survivability and cryotolerance vary considerably and consistent with previous studies (Kuisma et al., 2006).

By comparing Figures (3.1, 2), our findings indicated that stallion (A) sperm movement was remarkably higher than other individuals after freezing and thawing processes even though it did not differ significantly compared with stallion (B). The current study also shed light on the variation among stallions in how their semen responded to freezing/thawing processes when the spermatozoa were packed in different concentrations. However, we employed two different concentrations to make certain that there were sufficient viable cells to use for (AI). Indeed, when sperm cells were packed in low density ( $0.4 \times 10^9$  cells/ml), there were significant differences across stallions with respect to progressive sperm, rapid cells, VCL, LIN, and BCF.

Regarding the variation of freezability among stallions, it was evident that stallion A was apparently cryotolerance and more resistant to the cold-shock. In the present study, our findings were consistent with Cătană and his team (Cătană et al., 2008). In their study, they employed three stallions in attempt to examine two different extenders. Similarly, the two extenders were assigned for each individual stallion to assess the performance of post-thaw sperm in terms of motility and viability.

Because glycerol is considered as a penetrating cryoprotectant, it might rearrange membrane lipid and protein, which increases the fluidity and permeability for ions to be transmitted through plasma membrane during the process of cryopreservation (Holt, 2000). It is reported, however, that the presence of glycerol in the freezing extender can induce osmotic damage to the sperm cells (Purdy, 2006). Alteration of plasma membrane bilayers of spermatozoon, specifically the acrosome integrity, and interaction with bound proteins and glycoproteins are other negative effects of glycerol to decrease cryosurvivability (Buhr et al., 2001). Therefore, spermatozoa from different species and within the same species may respond significantly and differently depending upon their plasma membrane compositions. In bovine, for instance, glycerol (5-7%) has been effectively and successfully used as a cryoprotectant for centuries (Snedeker and Gaunya, 1970). On the other hand, a severe damage was observed during freezing and thawing of buffalo sperm cells in the presence of glycerol (< 5%, Rasul et al., 2007). Our study indicated that there were good and bad freezer stallions based on their post-thaw sperm motility characteristics, and that might be caused by their interactions with the levels of glycerol in the extender. The concentration of glycerol may have different effect on individual stallions, which suggest possible differences in their membrane composition.

Consequently, there should be some biomarkers (i.e. protein, lipid, etc.) on the plasma membrane that can be investigated to recognize such stallions prior to freezing process.

Nevertheless, there are many other factors that might affect the stallions to produce freezable and more resistant semen to low temperatures. Age is a key factor on semen quality and sperm characteristics such as volume of ejaculate, concentration, and viability (Dowsett and Knott, 1996). Here, we used a wide range of aged stallions that may not be ruled out in the identification of good and poor freezers. Similarly, stallions from different breeds vary significantly in terms of semen characteristics. Arabian breeds, for example, seem to have the lowest number of dead spermatozoa and more ejaculate compared to other breeds (Dowsett and Knott, 1996). It is unclear, however, whether there are some genetic differences in terms of sperm cryotolerance among or within the same breed of stallions. Regarding post-thawed sperm characteristics, both the age and the breed have a remarkable effect. In one study using three different aged stallions (5-8 years) from two different breeds (Furioso North-Star and English Pure Breed) reported that the youngest had higher post-thaw motility and viability (Cătană et al., 2008). Moreover, nutrition is another source of variation in semen quality. Deficiency of vitamin A has been reported to induce spermatogenic failures (i.e. one or more sperm parameters fall below the World Health Organization in human (WHO) criteria for normozoospermia) compared to normospermia (Reid, 1960), while deficiencies/ excesses in minerals (i.e. cadmium) might decrease or block sperm production (Gunn and Gould, 1970). Additionally, a study was done by Kentucky Equine Research (2012) showed that sperm motility and concentrations of cryopreserved semen were increased in stallions fed with grain diets and a supplement of omega-3.

We also placed an emphasis on the sperm density packaging in an effort to develop a recommended method that might be used with stallion AI. In this study, two different concentrations were examined ( $0.4 \times 10^9$  and  $0.8 \times 10^9$  sperm cells/ml). We observed that the concentration of  $0.4 \times 10^9$  sperm cells/ml was significantly preferred over the other one ( $0.8 \times 10^9$  sperm cells/ml). Our assessment was primarily based on motility parameters such as progressively motile sperm which is the most common parameter used to evaluate the post-thawed sperm quality. Table 3.3 showed that ( $0.4 \times 10^9$  sperm cells/ml) concentration was significantly different with respect to progressive (%), VAP ( $\mu\text{m/s}$ ), and VSL ( $\mu\text{m/s}$ ). Heitland et al. (1996) stated that sperm cells frozen in skim milk-egg yolk extender, which was the same extender we used in our experiment, at a concentration of  $400 \times 10^6$  /ml had higher means of total motile (%) and progressively motile sperm (%) compared to spermatozoa frozen at 800 or  $1,500 \times 10^6$  /ml. Our findings showed the same trends and were in agreement with their findings. Leipold et al. (1998), however, reported discrepant findings when they stated that packaging spermatozoa at concentrations of 400 and  $1,600 \times 10^6$  /ml had the same percentages of total motile and progressively motile spermatozoa. Consequently, in the present study, freezing equine spermatozoa at lower concentration  $0.4 \times 10^9$  /ml resulted in a higher percentage of progressively motile sperm and velocity parameters (VAP and VSL;  $\mu\text{m/s}$ ) compared to spermatozoa frozen at  $0.8 \times 10^9$  /ml.

## CHAPTER IV

### CONCLUSIONS

Cryopreservation is well-known to have a severe effect on sperm parameters and functions. Cryopreserved spermatozoa are less motile, viable, and capacitated than the fresh sperm. For the sperm to progress through a female reproductive tract and to reach the ova, it should be physiologically and functionally intact and capable to fertilize the oocyte. Employing CASA, all sperm motility and velocity parameters evaluated to determine the effect of cryopreservation process on equine semen. Total motility, progressively motile sperm, and rapid sperm percentages are significantly decreased after freezing procedures.

Nevertheless the fact that spermatozoa might show cryo-survivability, some stallions are less freezable than others. In the present study, stallion (A) exhibits to be more resistant to the low temperatures ( $-196^{\circ}\text{C}$ ), and its spermatozoa kinematics are less likely to be affected by the process of cryopreservation. Consequently, and based upon the results, stallion (A) should be classified as a good freezer among all four stallions that are assigned in this study. On the contrary, stallion (D) had the lowest percentages of progressively motile sperm, rapid cells, and even some of velocity parameters regardless the concentration of spermatozoa. Stallion (D) is therefore known as a poor freezer due to the tendency to be extremely influenced by freezing and thawing process. Practically, and to confirm the findings, AI should be practiced using the cryopreserved spermatozoa of

both stallions (A and D) to inseminate mares. Meanwhile, mares should be considerably managed to avoid any variation that might contradict or perhaps reduce the likelihood of conception.

Loading spermatozoa in high density is becoming of a great interest for equine industry. In fact, packaging sperm cells in higher concentrations would reduce the costs of labor and packaging materials as well as storage space. Similarly, it is less time-consuming for thawing and inseminating a dose on field. For example, instead of thawing two or more low concentrated straws, it would be beneficial to thaw only one highly concentrated straw as an insemination dose. The results in the present study, however, indicate that low sperm density ( $0.4 \times 10^9/\text{ml}$ ) tends to be more preferable than the high concentration specifically in terms of progressively motile sperm only. Such drawback could be overcome via examining different sorts of protocols, and the discrepancy can be further investigated.

After having stallions identified as good and poor freezers, more work should be done on the molecular levels to determine whether or not there are some notable biomarkers that may help identifying stallions on field without recourse to the timely and costly freezing process.



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APPENDIX A

EFFECT OF CRYOPRESERVATION ON MOTILITY AND STRAIGHT LINE

VELOCITY PARAMETERS OF STALLION SPERMATOZOA

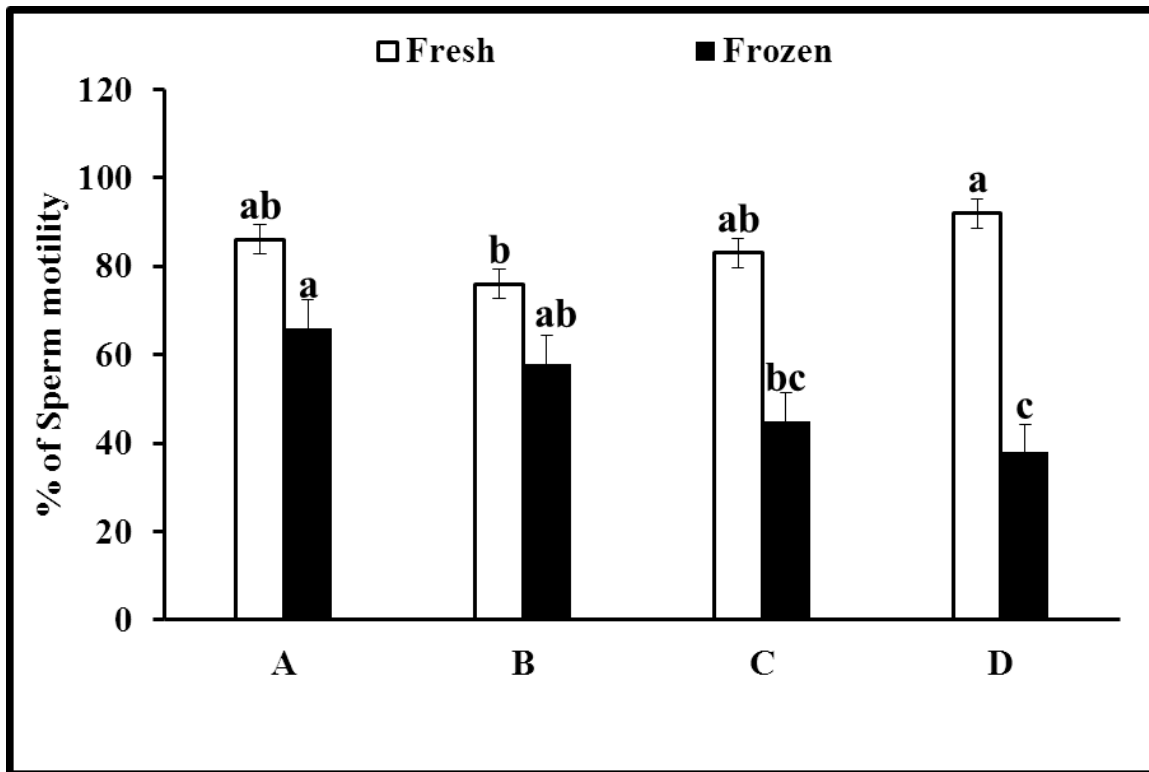


Figure A.1 Effect of cryopreservation on total motility of stallion spermatozoa.

Data are shown as mean percentage  $\pm$  SEM of 4-7 ejaculations per stallion. Letters (<sup>a, b, c</sup>) indicate significant differences within the same column across individuals ( $P < 0.05$ ).

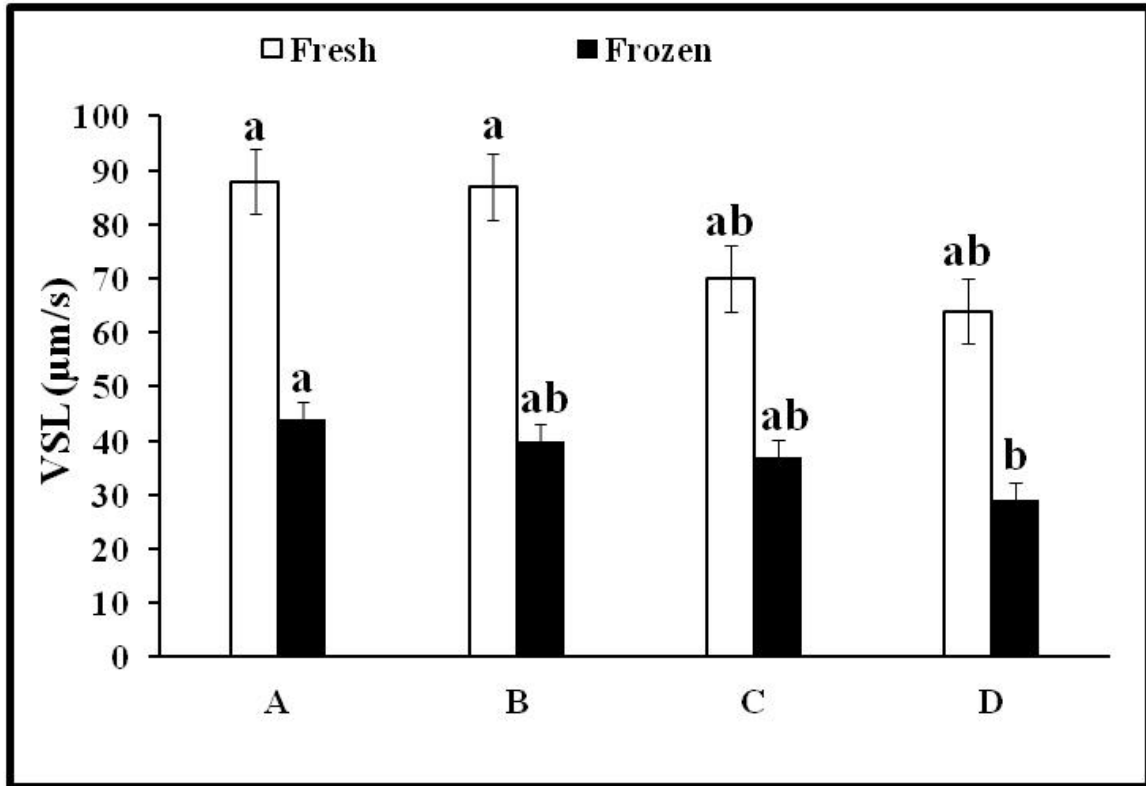


Figure A.2 Effect of cryopreservation on straight line velocity ( $\mu\text{m/s}$ ) of stallion spermatozoa.

Data are shown as mean percentage  $\pm$  SEM of 4-7 ejaculations per stallion. Letters (<sup>a, b, c</sup>) indicate significant differences within the same column across individuals ( $P < 0.05$ ).