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L-Carnitine and Pyruvate Inclusion in Diluents for Cold-Stored Stallion Spermatozoa

Daniel Scott Potter

L-CARNITINE AND PYRUVATE INCLUSION IN DILUENTS FOR COLD-STORED STALLION SPERMATOZOA

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By

Daniel Scott Potter

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ABSTRACT

Spermatozoa survivability is of utmost importance in delivering equine semen for artificial insemination. Semen extender supplementation may allow for increased survivability. L-carnitine (CARN) has been studied for its role in the mobilization of fatty acids. Two experiments (EX1/EX2) assessed the effects of CARN and pyruvate (P) on spermatozoa survival. Total (TM) and progressive motility (PM), velocity and direction of movement (VAP, VSL, VCL, and elongation) were assessed by CASA, membrane status (SYBR 14/PI stains) and acrosome status (FITC-PSA stain) by flow cytometer. In EX1, 4 ejaculates were collected from 2 stallions. Aliquots were diluted in skim-milk glucose extender (SKMG) or INRA 96 (INRA) with and without CARN and P added at levels of 0.00806 g/mL and 0.0011004 g/mL. Split aliquots were placed in separate Equine Express II™ containers for CASA after 2 hours, 24 hours, and 48 hours. Flow cytometery was conducted at 24 hours. In EX 2, 4 ejaculates were collected from 3 stallions. Aliquots were diluted in SKMG, INRA, and Revolution (REV) with or without CARN+P. In EX1/EX2 dilution in INRA resulted in higher motility compared to dilution in other extenders ($p<0.05$). In EX1 CARN+P ($p<0.01$) improved TM and PM. Intact membrane percentages were higher ($p<0.05$) for with CARN + P (74.3% with vs. 71.7% without). In EX2 CARN+P ($p<0.05$) improved TM and PM and also improved ($p<0.05$) percentage of intact membranes (78.0% with vs. 75.0% without) and intact acrosomes (78.9% with vs. 66.2% without). In conclusion, addition of L-carnitine + pyruvate may improve multiple factors of spermatozoa survival.

KEYWORDS: stallion, spermatozoa, carnitine, flow cytometer, semen extenders

This abstract is approved as to form and content

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Chairperson, Advisory Committee
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INTRODUCTION

Justification

In the field of equine fertility and reproduction, overall survival of spermatozoa after suspension in extender is of utmost importance and concern in the effectiveness of providing viable and motile semen for artificial insemination. A majority of infertility issues within the male sex of a species are related to spermatozoa malfunction (Sikka, 1996.).

Processing techniques have profound impact on semen characteristics. Some of these processes include cooling, freezing, dilution, and the process of centrifugation to remove the seminal plasma.

Different compounds have been tested as supplemental components for semen extenders and assessed for their ability to increase post-storage survival for both cooled and frozen equine spermatozoa. Since the science of artificial insemination was first developed items such as skim milk, glucose, glycerol and egg yolk have been used to aid in preservation of equine, bovine, caprine, ovine, and human spermatozoa. Carnitine (L-carnitine specifically) and pyruvate are two pivotal compounds for overall health and bodily function. This study was conducted to further assess the roles they play in survival of spermatozoa and to determine if they are an asset in equine semen handling, preservation, shipping and effectiveness once delivered in utero.

Spermatozoa differ greatly from other cells in the stallion. Although spermatozoa function and survival is often assessed post-ejaculation, modifications and damages to its ability to perform can happen throughout generation and maturation within the stallion.

According to Amann and Graham (Equine Reproduction, 1993), the ability of spermatozoa to successfully fertilize the egg depends on five factors:

1. Spermatozoa must have the ability to produce energy through metabolism.
2. Spermatozoa must be progressively motile.
3. The acrosome and the enzymes within must have the ability to break down and penetrate the wall of the ovum.
4. There must be adequate lipid availability within the membranes for pre-fertilization stability and capacity for membrane fusion when the spermatozoa and oocyte connect.
5. Proteins that are necessary for survival in the mare tract and for spermatozoa to egg attachment must be present.

Although the concept of fertilization is simple in theory, the constituent pieces that allow for a successful pregnancy can be complex. When studying the male of any species, there are a significant number of factors that contribute to fertility. The difficulty in semen research is the lack of a singular technique that can be applied across all the males of one species. This issue is derived from the variation not only across breeds but also within breeds. In the equine, different breeds have been developed for their abilities in certain disciplines and events. The characteristics that are selected are usually related to these abilities rather than to reproductive efficiency.

This study evaluated motility, velocity, membrane status and acrosome status of the spermatozoa at increasing time increments post collection (2 hours, 24 hours, 48 hours). The practical application of this study was to ascertain the effectiveness of L-carnitine and pyruvate in their ability to help with the longevity of the sample as is necessary for the 2-3 day time frame required for shipping frozen or cooled equine semen.

Problem Statement and Objective

Delivery of viable and motile equine spermatozoa for artificial insemination can be difficult due to the varying shipping schedules of mail carriers. In order for spermatozoa to stay viable over the two to three day shipping time, spermatozoa must be suspended in extenders that provide adequate energy and protection. The objective of this study was to determine the effects of L-carnitine and pyruvate on motility, velocity, membrane status and acrosome status of cold stored stallion spermatozoa when added to diluents in commercially available equine semen extenders.

Null Hypotheses

1. L-carnitine and pyruvate supplementation of semen extenders will have no effect on motility, velocity, membrane status and acrosome status for stallion spermatozoa.
2. There will be no difference in effects among extenders.

LITERATURE REVIEW

Spermatozoa Structure

The general structures of spermatozoa are the plasma membrane, head, neck, midpiece, principal piece and end piece. The structures of focus within this literature review are the plasma membrane, head and midpiece.

The lipid bilayer, the phospholipid-water interface, and the glycocalyx comprise the three zones of the plasma membrane. Within the lipid bilayer are inward facing fatty acyl groups, which are the hydrophobic polar phospholipids, as well as outward facing charged polar head-groups, which are hydrophilic in nature. The most common lipids in the plasma membrane are phospholipids and cholesterol.

Proteins are likewise a significant part of the plasma membrane and make up approximately 50% of its matter (Dupuy et al., 2007). Integral proteins provide necessary structure while peripheral proteins can be easily removed or replaced. Integral proteins may also serve as pores, channels and receptors for media based substrates. Often times these proteins have carbohydrate chains, which have the ability to bind with medium-based proteins due to the net negative charge of the carbohydrate chain.

Properties and composition of the glycocalyx have the potential for change depending on the effects caused by the addition of new material attaching to the carbohydrate chains of the integral proteins. Within the plasma membrane in stallions, the ratio of cholesterol to phospholipid is 0.36 (Parks and Lynch, 1992). The major classes of phospholipids in the spermatozoa of most domestic species are: choline, ethanolamine, and sphingomyelin (Amann and Graham, 1993). The cholesterol to

phospholipid (specifically those phospholipids that contain polyunsaturated acyl side chains) ratio generally characterizes the fluidity of the membrane. Higher levels of cholesterol will make the membrane more rigid. Proper cholesterol levels maintain the integrity of the bilayer. Most of the plasma membrane attachment is around the tail of the spermatozoa. There are not a significant amount of attachment points that rigidly join the plasma membrane to the head of the spermatozoa (Aman and Graham, 1993).

The main regions of the head of the spermatozoa cell are the plasma membrane, the post-acrosomal lamina, the acrosome, the nuclear envelope and the nucleus (this is from the outer-most region to the innermost region). In stallions, the head is wide and mostly flat. The head generally tapers down from a thicker region near the midpiece to a thinner region approaching the rostral tip of the cell. The enzymes housed in the acrosome, which are responsible for the penetration of the oocyte, are hyaluronidase, proacrosin, acrosin, and varying lipases. A feature known as the perfatorium can sometimes be observed in the rostral tip of the acrosome and is believed to help provide structure. The structures in the plasma membrane allow the spermatozoon to attach to the zona pellucida of the ovum, which catalyzes the beginning of the acrosome reaction (Flesch and Gadella, 2007).

The region from the base of the neck to the annulus is known as the midpiece. The matrix of mitochondria is held within this area of the spermatozoa cell. Approximately 50 helical turns (known as gyri) of mitochondria exist in the typical stallion spermatozoon. A series of nine fibrous structures extend through the midpiece and provide the mechanical ability for movement. The axoneme is in the central most region of the tail and is responsible for giving spermatozoa the potential for propulsion.

The annulus conjoins is a significant apparatus in joining the head and the tail of a spermatozoon (Gadella et al., 2008).

Spermatozoa Metabolism

The study of spermatozoa metabolism helps discover the function of spermatozoa and the specific characteristics that need to be addressed to aid in preservation and survivability. Spermatozoa, like other cells, must receive energy in the form of adenosine tri-phosphate, or ATP. Spermatozoa have the ability to utilize aerobic and anaerobic pathways for energy production. The breakdown of glucose, lactate, and pyruvate as well the oxidation of amino acids and lipids are the subsidiary components of spermatozoa metabolism. Diffusion may provide substantial energy for flagella in species that produce spermatozoa of a smaller size; however, diffusion does not provide enough energy (ATP) through the mitochondria for continued function of the flagellum in species with larger spermatozoa (Ford, 2005). This energy conundrum can be solved in part through the quick delivery of ATP through the use of the adenylate-kinase shuttle (Ford, 2005). The presence of lactate within a population of spermatozoa is a player in spermatozoa metabolism. Lactate has historically been condemned as metabolic waste, but is now being classified as an essential component of cell metabolism as well as a player in intercellular and intracellular redox reactions (Gladden, 2004). There is some correlation in the production of L-lactate and overall spermatozoa survival. In swine, the production of L-lactate and the ratio of insemination to farrowing show a "relatively high correlation." (Rigau et al.,1996).

Cholesterol

The focus of this study was on the effects of L-carnitine and pyruvate; however, knowledge of the role of cholesterol in the overall production of ATP due to its influence over the process of fatty acid oxidation is essential. Unsaturated fatty acids are present in large quantities in the membranes of spermatozoa cells in mammals (Sikka, 1996). As an integral component of structural integrity and survivability of spermatozoa, fatty acids must be maintained at adequate levels and ratios. Intracellular fatty acids act as an energy source for spermatozoa as they can be oxidized to generate ATP for energy for the spermatozoa cells. Subsequently, a lack of fatty acids will result in lack of substantial ATP production. Lack of ATP means lack of motility for spermatozoa, lack of motility leads to lack of ability to fertilize an egg and thus an overall reduction in conception rates of females can occur.

One important cholesterol-binding molecule is known as HBCD (2-hydroxypropyl-2-cyclodextrin). This molecule has the capacity to serve as a sink or a shuttle for cholesterol, depending on its current environment, which can subsequently raise or lower the concentration of cholesterol in the plasma membrane of the spermatozoa (Galantino-Homer et al., 2006). A higher ratio of cholesterol to phospholipids lowers the risk of cold shock to the spermatozoa.

A major issue with adding fatty acid compounds to semen extender is the hydrophobic nature of lipids that results in the inability of most fatty acid compounds to bind and integrate with water based media. The addition of cyclodextrins to a hydrophobic compound can allow for the compound to change its nature and become hydrophilic. This process is conceptually simple. A cyclodextrin is a cyclic sugar

molecule that can attach itself by surrounding another compound. When the cyclodextrin surrounds a lipid molecule, such as cholesterol, it allows the cholesterol to integrate with the water. The ability to add these fatty acids to semen extenders increases available substrates for spermatozoa metabolism. A study on cholesterol (Christian et al., 1999) found that adding cyclodextrins at low levels to serum serves as a catalyst for a cholesterol shuttle that can transport cholesterol from cells to the lipoproteins within the serum. As a result, cyclodextrins possess the ability to manipulate the metabolism of cholesterol. The highest efficiency cholesterol shuttle in the tests conducted was the "double-decker" cyclophane.

The ratio of cholesterol to phospholipids in an ejaculate is highest directly after ejaculation and decreases over time (Cross, 1998). When spermatozoa are being capacitated, protein acceptor cells obtain the cholesterol that is released from the membrane of the spermatozoa while phospholipids are being brought in. The most important and abundant sterol in an ejaculate is cholesterol. Density of cholesterol in the membranes of spermatozoa differs significantly across species. The recovery of motile spermatozoa post-thaw is improved when exogenous cholesterol is administered to the membranes of the spermatozoa prior to freezing (Purdy et al., 2004).

Carnitine

Carnitine was named from the Latin word *carnus*, which means meat, because meat was the first location in which the compound was discovered. Different groups of Russian and German scientists discovered carnitine (the Russian fraction being credited with the initial isolation) in 1905. In 1948, scientists realized the necessity of this

compound in the survival of the larvae of mealworms. They did not know that the compound they were dealing had been previously discovered and so it was dually named as vitamin B_T until they later identified that this "vitamin B_t" was not fully functional as a vitamin and had already been classified as carnitine (Harmeyer, 2002). The preliminary findings on the function of carnitine as a fatty acid transporter were conducted in the late 1950's. For many years, scientists questioned which isomer of the compound was biologically active. L-carnitine was accepted as the biologically significant isomer in 1962 and the early studies of the effects of carnitine deficiency were conducted in the early 1970's.

Carnitine is a quaternary ammonium compound. The proper chemical name of L-carnitine is *3-Carboxy-2-hydroxy-N,N,N-trimethyl-1-propanaminium*. L-carnitine has seven carbon atoms, fifteen hydrogen, one nitrogen, and three oxygen; lending the chemical formula of $C_7H_{15}NO_3$. In comparison to many organic molecules that exist within the body of an animal, carnitine is not a large compound.

L-carnitine is mainly produced in the kidneys and the liver. Carnitine does not have to be directly consumed in the diet of most mammals in order to maintain adequate levels for bodily function. The chief derivatives of carnitine are lysine and methionine (Higdon, 2002).

At most, there is only a variance of two carbon atoms, four hydrogen atoms, one nitrogen atom, one oxygen atom, and one sulfur atom when comparing these three molecules on any level. Most species create their own carnitine by a process that adds a methyl group to the amino acid lysine known as methylation (Bremer, 1983).

Carnitine plays a pivotal role as a transporter in the process of long-chain fatty acid oxidation (Hoppel, 2003). Carnitine transports acyl groups to the mitochondrial stations within a cell once B-oxidation of these long-chain fatty acids has occurred. Once these acyl groups have arrived at the mitochondria, they are used for the production of acetyl-CoA, which can be used in the citric acid cycle for the production of ATP (Figure 1). Acetyl groups can also be transported within the cell. Carnitine acetyl-transferase converts acetyl-CoA into acetyl-carnitine in a process known as the acetyl-carnitine shuttle (Hynes et al., 2011). To condense, carnitine aids in taking fatty acid substrates and mobilizing them for energy production by the cell (Childress et al., 1967). Carnitine is not only helpful in fatty acid metabolism, but is completely necessary for the process of oxidation of these molecules. In order for oxidation of fatty acids (specifically within skeletal muscle) to occur; there must be adequate levels of carnitine present. Within the mitochondrial matrix of a cell, species that use fatty acids for energy supply during flight, such as the common fly, must have high levels of carnitine to support and sustain this function. However, species like the bee that utilize carbohydrates instead of fatty acids for this same function, do not need the same supply of carnitine (Childress et al., 1967).

A study on prematurely weaned swine conducted by showed that overall metabolism of lipids was increased when swine were given exogenous L-carnitine. The summary of data and research on L-carnitine suggests that the oxidation/metabolism of fatty acids is greatly improved due to the ability of L-carnitine to serve as an efficient transporter of metabolic substrates (Coffey et al., 1991). However, it should be noted that in a study of the effects of oral L-carnitine on semen quality in boars, no significant

($p<0.05$) treatment effects were observed in the control diet versus a 500 mg per day supplemental diet (Kozink et al., 2004).

Carnitine deficiencies lead to less efficient mobilization of fatty acids, decreased ATP production due to the lack of substrates available for ATP production, and the inability to oxidize lipids at a significant rate. Carnitine has great influence on heart health (Lango et al., 2001). Although carnitine deficiency did not have an effect on cardiac function when observed on a short time scale in humans, an extended period of time with insufficient levels of carnitine could cause weakened performance of the heart and irregular patterns of function (Paulson, 1998). The ability of the heart to contract with adequate strength and timing is especially reduced while performing under strenuous circumstances. In relation to the function of carnitine in fatty acid metabolism, Paulson predicts that these low levels of carnitine lead to an inability of the cells within the heart to produce enough ATP to sustain proper energy output for contraction (Paulson, 1998). A study on ischemic swine hearts showed that high levels of carnitine increase the ability of the cells to oxidize free fatty acids which may aid in removing these extra fatty acids from the cardiac cycle thus increasing the performance and health of the animal (Liedtke et al., 1981). Carnitine deficiencies not only result in insufficient ATP production, as was observed in the Paulson study, but also in the inability of the system to rid itself of excessive amounts of free fatty acids that may be deleterious to overall function and performance of the heart.

Human supplementation has become the most popular and widely applied area of carnitine use. The role that carnitine plays in fatty acid metabolism makes it highly attractive to people desiring to be efficient in the burning of lipids during exercise and the

use of these lipids as a source of energy (Brass, 2000). This same research suggests that carnitine may positively affect the balance of glucose within the system, biosynthesis of acyl-carnitine, and the ability of muscle to have longer endurance against fatigue (Roberts et al., 2004). These exercise physiology researchers admit that more in depth and controlled trials are necessary to determine the absolute results of supplemental carnitine on overall bodily performance during exercise.

Oral L-carnitine supplements have been shown to increase spermatozoa motility in humans when administered in a 3 to 4 month clinical trial (Higdon, 2002). Added carnitine is hypothesized to aid in the prolonged survival of spermatozoa cells by increasing the ability of the individual spermatozoa to mobilize its fatty acids for sustained energy production. This sustained energy production results in spermatozoa that stay viable and motile, meaning they have the ability to successfully fertilize an egg for a longer period of time. Few studies have been conducted on the effects of carnitine with the stallion but there has been evidence that seminal carnitine may serve as a potential semen quality marker and that carnitine may improve spermatozoa survival during storage (Stradaioli et al., 2000).

Research interest in carnitine has increased significantly from its earliest discovery and isolation to its most recent applications as a supplement for metabolic, cardiac and reproductive health and efficiency. From the time that it is biosynthesized until it completes its role in transporting substrates for fatty acid oxidation, carnitine is an essential player in the bodily function of most mammals. Although there are visible effects of deficiencies and observed improvements with supplementation, much research still needs to be conducted on the molecule that we know as carnitine.

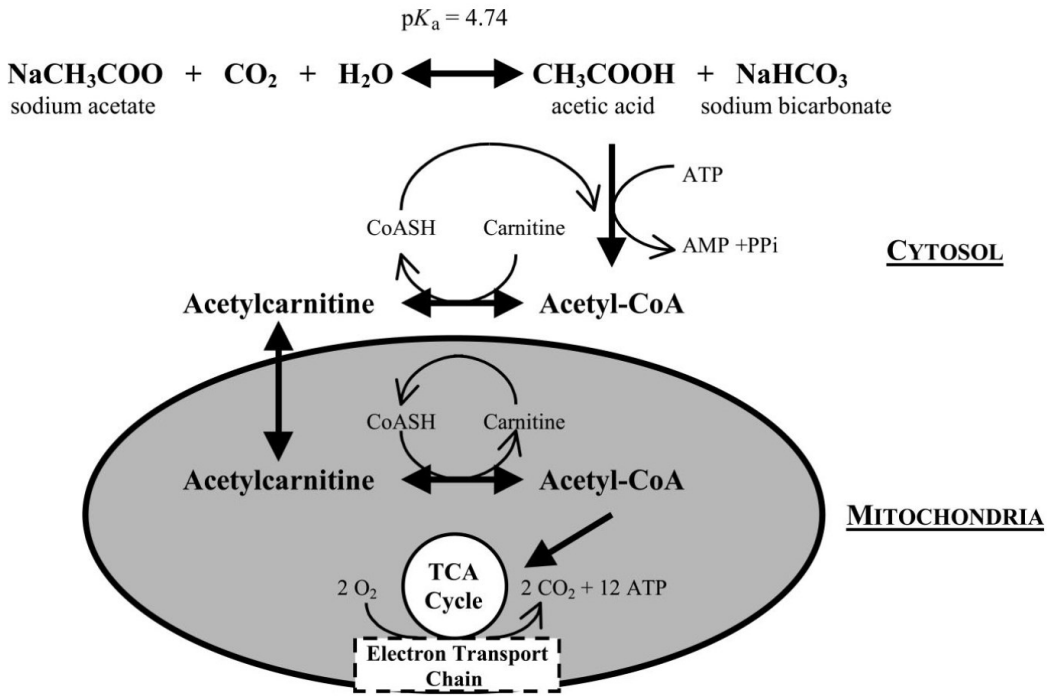

Figure 1. The role of L-carnitine in the production of ATP and substrate transfer between the cytosol and mitochondrial matrix. (Roberts et al., 2004).

Semen Processing

When handling and processing semen for packaging, transport and use for breeding mares there is a significant effect on the survivability of the spermatozoa based upon the procedures and processing techniques used.

Using a centrifuge to remove the seminal plasma from an ejaculate is one of the most common techniques and procedures used in semen processing. The amount of seminal plasma in an ejaculate is reduced to less than 5% of the original volume after centrifugation. Removal of seminal plasma for cold storage is not as much of a routine procedure in Europe as it is in the United States, leading to decreased spermatozoa survival and fertility when shipping cold stored semen (Johannisson et al., 2009).

Centrifugation of semen in the equine is used to increase the concentration of spermatozoa within the ejaculate (Edmond et al., 2012). Greater motility, viability, spermatozoa chromatin integrity in treated compared to untreated samples (Johannisson et al., 2009). In contrast, the body of centrifugation research shows individual animal variation in their response to centrifugation. Two common centrifugation techniques are known as centrifugal fractionation and cushioned centrifugation. The technique of centrifugal fractionation is observed to enhance overall spermatozoa survival as it relates to motility, acrosome status and morphology (Edwards et al., 2012). The process of cushioned-centrifugation has the ability to collect large volumes of spermatozoa per ejaculate (Waite et al., 2008).

Factors as seemingly unimportant as the shape of the tube that the sample is centrifuged in can also play a significant role in overall spermatozoa survival. For example, recovery rate of the spermatozoa may be higher in tubes with a conical base, but overall spermatozoa survival values are greater when the tubes with the nipple base are used while being centrifuged (Waite et al., 2008). Tube size can also have significant effects on spermatozoa survival. Spermatozoa handled in 50-ml tubes recover slower and have lower velocities than do spermatozoa handled in 15-ml tubes (Webb and Dean, 2009). Volumes and concentrations of spermatozoa in extender likewise effect spermatozoa characteristics. When the percent seminal plasma in the sample is high, overall ratios of progressively motile spermatozoa are reduced (Dawson et al., 2000). Similarly, excessive amounts of semen per tube centrifuged can be damaging to spermatozoa. When volumes of semen are centrifuged at levels less than or equal to 20 ml, overall loss of spermatozoa is reduced (Ferrer et al., 2012). In addition, raising the

amount of spermatozoa in the sample to15-ml per tube may be deleterious to the recovery of spermatozoa (Edmond et al., 2012).

Temperature at which spermatozoa are stored as well as sequence of cooling and thawing contributes to spermatozoa survival. When semen is stored close to the 0°C level, DNA integrity and overall spermatozoa motility is reduced, compared to the result that spermatozoa remained quite viable over a 22-hour period when kept in conditions that ranged from 4°C to 15°C (Vidament et al., 2012). Progressively motile spermatozoa are shown to decrease after semen is thawed when samples are cooled prior to centrifugation (Squires et al., 2001). The process of centrifugation might briefly disrupt the spermatozoa membrane; however, further tests showed that they returned to normal after 48 hours (Johannisson et al., 2009).

Several different types of semen extenders have been used as the general medium, pre-supplementation of other compounds, for the suspension of equine spermatozoa. Overall, INRA 82 (INRA and IMV-Technologies. L'Aigle, France) was shown to be a more adequate semen extender versus alternative treatments (Ijaz et al., 1995). When using INRA 82 and Kenney's extenders (Reproduction Resources, Walworth, WI) overall spermatozoa survivability was greater to the 96-hour (INRA 82) and 48-hour (Kenney's) markers when the compound taurine was added to the extender. Survivability of spermatozoa (cleaned and housed in Sp-Talp) that are incubated beforehand in extenders with taurine added increases ($p<0.05$) an extra 12 hours (INRA 82) or 24 hours (Kenney's). The most current INRA extender is INRA 96 and is an improvement upon the original INRA 82.

The flow cytometer is a machine used for the analysis of spermatozoa at a rate of thousands of cells in a matter of seconds. When an ejaculate is collected and suspended in extender it can be inserted into the flow cytometer for analysis. A flow cytometer functions by stimulating individual cells to align one after another and pass through the reading apparatus. The cells are exposed to a variety of dyes, depending on what factors the researcher desires to observe, in order for the machine to read specific characteristics. A high-energy beam of light or laser stimulates the cells to assort them in a single-file stream. Some of the traits that can be assessed by a flow cytometer are membrane status, acrosome status, sex, volume, size, mitochondrial status, apoptosis indicators, presence of ROS (reactive oxygen species), damaged DNA and spermatozoa capacitation (Marchetti et al., 2001). This technology is often used in the medical field to look for disorders within the blood, cancer markers and other medical indicators but has been widely adapted for use in reproductive and fertility research and practice.

Most recently, flow cytometers are being calibrated to sort semen by sex of individual spermatozoa. This practice is becoming more widely used in the cattle industry. The equine industry is beginning preliminary work on the use of the flow cytometer for sex-sorting stallion semen; however, there has been some difficulty (Gibb et al., 2010) This issue arose from the inability of the flow cytometer to recognize differences due to the opaque nature of the extenders (skim milk, egg yolk, etc.). When a more transparent semen extender was used instead of skim milk, there was a significant increase in the efficiency of sex sorting by the flow cytometer (Gibb et al., 2010).

Flow cytometers have been used to look for spermatozoa that have suffered damage to their chromatin. An assay known as the DNA Fragmentation Index (DFI) is

positively correlated with lower fertility, decreased conception rates, and increased "spontaneous miscarriage" in humans (Virro et al., 2004). A myriad of problems still exist with flow cytometers that necessitate further study or investigation.

Cost issues can exist with technologies for each machine. For instance, specific lasers can only excite certain dyes. Purchase of multiple dye-specific lasers is costly. Cost of the machine itself is also quite heavy, many machines starting around the $50,000 range. Operator efficiency and accuracy is of paramount importance with flow cytometers. Using the wrong dyes or lasers, calibrating the machine incorrectly or improperly setting parameters through the machine's software can completely ruin an analysis or give inaccurate results. Electronic interference from other machines near the flow cytometer can likewise cause issues. When using dyes, it is important to know how different dyes affect one another. For instance, there can be fluorescent cross-contamination of dyes or complete blocking of the effectiveness of one dye by another dye.

The machine can also produce a series of false results depending on the scenario. It is important to analyze spermatozoa by human quantification through a microscope (subjective analysis) along with the use of the machine (flow cytometers, CASA machines, etc.) to verify machine settings and accuracy (Johannisson et al., 2009). In CASA analysis a correlation of $r=0.65$ was observed between "subjective" analyzed spermatozoa and CASA analyzed spermatozoa, showing good calibration of the CASA machine (Johannisson et al., 2009). Some dyes are designed to stain the acrosome to check for acrosome status; however, over-damaged acrosomes lose their receptors that act as binding sites for the dye. A completely damaged acrosome cannot absorb the dye,

just as a completely intact acrosome would. Misreading debris within the sample can cause false-positive or false-negative results. Bacteria that have membranes similar to spermatozoa are often counted as spermatozoa by the flow cytometer leading to an over estimation of viable cells. Similar misreading can occur in the machine from blood, epithelial cells, gel and extender particles that are present in the sample.

METHODS

Experiment 1

Collection and Processing. Four ejaculates from each of two stallions (1 American Quarter Horse, 1 Miniature horse) were collected using an artificial vagina (AV) and the semen passed through a filter into the collection bottle. Directly after the collection, the gel portion of the ejaculate was once again filtered off of the semen if necessary. Semen was then analyzed for volume (mL) and concentration (million spermatozoa per mL) using a 590B Densimeter (Animal Reproduction Systems, Chino, CA). Semen was then divided into two sets of four test tubes, half of which were centrifuged at 350 g-force for two to four minutes depending on visual estimation of proper effects of centrifugation.

Extenders were prepared and frozen for use throughout the experiment. Two different extenders were used for Experiment 1, Kenney's SKMG (K) and INRA 96 (I). Base tubes were labeled as A1-A8 for stallion 1 (QH) and C1-C8 for stallion 2 (MH) and were comprised of one of the two extenders (K or I), centrifuged or non-centrifuged (S for centrifuged), and supplemented with L-carnitine and pyruvate or not supplemented (C for carnitine and pyruvate). Labels were as follows: 1: K, 2: KS, 3: KC, 4: KCS, 5: I, 6: IS, 7: IC, 8: ICS. Treatment numbers were also preceded with A or C for stallions 1 and 2 respectively. Carnitine was added to the extender at the rate of 1.612 grams per 200 mL of extender. Pyruvate was added at the rate of 0.22008 grams per 200 mL. INRA 96 is delivered in 200 mL bottles, hence the 200 mL basis. The sterile water portion of Kenney's is delivered in 92 mL bottles; therefore, the amount of Kenney's extender was

doubled to make 184 mL and then 16 mL of distilled water was added to create an equal volume to add carnitine and pyruvate to both extenders.

Extenders were thawed prior to collection of the stallions and were quality controlled to a temperature of 30°C to 35°C before semen was added to ensure that the spermatozoa did not undergo cold shock. Each sample was extended from 3:1 (extender: semen) to 5:1 depending on the concentration of the sample to ensure that there were at least 300 million spermatozoa per test tube. Each test tube (A1 through A8 and C1 through C8) was then divided into four 1-½ mL aliquots to be used as the four sub-samples for analysis. The four sub-samples of each treatment were placed in Equine Express II™ containers for cooling and were labeled for computer assisted sperm analysis (CASA) at 2 hours, 24 hours, and 48 hours post-collection and for flow cytometer analysis at 24 hours post-collection.

CASA. Each sample was inverted by hand to ensure proper mixing when removed from the cooling containers. Droplets were taken from each sample with a micropipette and placed on 20 micron 4 chamber slides. Slides were placed on a slide warmer for five minutes to ensure that spermatozoa thawed to full motility and were no longer experiencing the effects of cooling. Each chamber was then assessed by CASA and results were recorded manually. The parameters assessed by CASA were total motility (TM), progressive motility (PM), average path velocity (VAP), straight-line velocity (VSL), curvilinear velocity (VCL) and elongation (ELONG).

Flow Cytometer. Each sample was vortexed on a Fisher Vortex Genie 2 (Scientific Industries Inc. Bohemia, NY) for one to two seconds to ensure the sample was mixed homogenously. Samples of 50 µL were taken from each treatment's base tube

using a micropipette and placed into 1 mL tubes for staining and preparations for the flow cytometer. Three different stains were used. Intact membranes and disrupted membranes were assessed using SYBR14 and Propidium Iodide (PI) stains. FITC-PSA was used to stain for reacted acrosomes (unstained cells signified acrosomes that were not yet reacted). 16 tubes were used for each ejaculate (8 for SYBR14/PI and 8 for FITC-PSA). A quantity of 5 μL of SYBR14 and 5 μL of PI was added to each of 8 samples of 50 μL of semen. Treatments were vortexed for one to two seconds and then placed in a cabinet to incubate in the dark for 20 minutes. 10 μL of FITC-PSA were added to each of 8 samples of 50 μL of semen. Treatments were vortexed for one to two seconds again and then placed in a cabinet to incubate in the dark for 10 minutes. FITC-PSA is extremely time sensitive and caution must be taken to not over-stain the samples. After the incubation period, 100 μL of Ham's 10 HEPES diluent was added to each sample and then samples were once again vortexed for one to two seconds prior to flow cytometer analysis. Samples were then run through the flow cytometer and results were recorded manually and within the memory of the computer.

Experiment 2

Collection and processing. Four ejaculates from each of three stallions (2 Arabians (A), 1 Miniature horse) were collected in the same manner as described in Experiment 1. Volume and concentration were recorded in the same manner as Experiment 1. Three different extenders were prepared and frozen for use throughout the experiment. Extenders used were EZ-Mixin (Animal Reproduction Systems) SKMG (K), INRA 96 (I), and Revolution (Santa Cruz Biotechnology, Dallas, TX) SKMG (R).

Centrifugation was eliminated from Experiment 2 due to the deleterious effects of centrifugation observed in some of the stallions during Experiment 1. Treatment tubes were labeled as C1-C6 for stallion 1 (MH), T1-T6 for stallions 2 (A), and X1-X6 for stallion 3 (A). Treatments consisted of one of the three extenders and were either supplemented with carnitine/pyruvate combination as in Experiment 1 or were not supplemented. Carnitine/pyruvate supplemented extenders were annotated with the label of (+). Treatment labels were as follows 1: K, 2: K+, 3: I, 4: I+, 5: R, 6: R+. Treatment labels were also preceded with C, T, or X for stallions 1, 2, and 3. In this experiment, distilled water was not added to the extenders to equal the 200mL that we standardized from the INRA 96 volume. Concentration per unit volume; however, was standardized from Experiment 1 to Experiment 2 and was equivocated to the ratio of 1.612 grams of carnitine and 0.22008 grams of pyruvate per 200mL volume of extender. All further processing of semen was done according to the protocol described in Experiment 1.

CASA. CASA analysis was conducted according to the same procedures and protocols discussed in Experiment 1.

Flow Cytometer. Flow cytometer analysis was likewise conducted according to the procedures and protocols discussed in Experiment 1.

Statistics

The GLM procedure of ANOVA was used in Minitab for treatment analysis. Tukey's pairwise comparisons were used for analysis of L-carnitine and pyruvate effects as well as extender effects. For ANOVA, the model consisted of stallion, ejaculate (nested within stallion) and time (nested within ejaculate).

IACUC Compliance

These experiments were conducted in compliance with the International Animal Care and Use Committee (IACUC) and can be found under the protocol name "Techniques to Improve Stallion Semen Preservation." The IACUC identification number is 13-027.0-A.

RESULTS

Experiment 1

Experiment 1 mean values ± standard deviations for L-carnitine and pyruvate treated aliquots compared to untreated aliquots assessed by CASA at 2 hours, 24 hours and 48 hours post collection and by flow cytometer at 24 hours post collection were recorded. Total motility and progressive motility are recorded in Table 1. The effects of VAP, VSL, VCL and elongation are recorded in Table 2. The percentage of intact membranes (% SYBR), disrupted membranes (% PI), intact acrosomes (% unstained), and reacted acrosome (% FITC-PSA) are recorded in Table 3. Main effects of stallion, ejaculate (nested within stallion), centrifugation, extender, L-carnitine and pyruvate treatment, and time are listed with p-values in Table 4. Tukey's comparisons of L-carnitine and pyruvate treatment effects are listed in Table 5 and Table 6. Tukey's comparisons of extender effects are listed in Table 7 and Table 8.

When observing L-carnitine and pyruvate treatment effect, the only statistically different period ($p<0.05$) of observation was 48 hours post-collection progressive motility. The visual means show trends; the reasons for the lack of within time difference will be addressed within the "Discussion" section of this thesis.

Multiple significant ($p<0.05$) main effects existed when observed across all stallions, ejaculates, times and treatments. Stallion effect was significant for VCL. Ejaculate effect was significant for TM, PM, VAP, ELONG, % SYBR, % PI, % unstained and % FITC-PSA. Centrifuge effect was significant for VAP. INRA was superior to SKMG ($p<0.05$) for TM and PM. L-carnitine and pyruvate treatment effect

was superior to control for TM and PM. Time effect was significant for TM, PM, VAP, VSL, VCL and ELONG (note: Time was not a factor for % SYBR, % PI, % unstained and % FITC-PSA).

Experiment 2

Experiment 2 mean values ± standard deviations for L-carnitine and pyruvate treated aliquots compared to untreated aliquots assessed by CASA at 2 hours, 24 hours and 48 hours post collection and by flow cytometer at 24 hours post collection were recorded. Total motility and progressive motility are recorded in Table 9. VAP, VSL, VCL and Elongation are recorded in Table 10. The percentage of intact membranes (% SYBR), disrupted membranes (% PI), intact acrosomes (% unstained), and reacted acrosome (% FITC-PSA) are recorded in Table 11. Main effects of stallion, ejaculate (nested within stallion), centrifugation, extender, L-carnitine and pyruvate treatment, and time are listed with p-values in Table 12. Tukey's comparisons of L-carnitine and pyruvate treatment effects are listed in Table 13 and Table 14. Tukey's comparisons of extender effects are listed in Table 15 and Table 16.

There were no significant treatment effects ($p<0.05$) when comparing means across individual times. As in Experiment 1, the reasons for the lack of difference will be addressed within the "Discussion" section of this thesis.

In parallel with Experiment 1, many main effects existed when observing means with all stallions, ejaculates, times and treatments included. Stallion effect was significant for TM, PM, VAP, VSL, VCL, ELONG, % unstained and % FITC-PSA. Ejaculate effect was significant for TM, VAP, % SYBR and % PI. The INRA extender

was superior ($p<0.05$) to SKMG and REV for TM and PM. These findings are in agreement with the literature. L-carnitine and pyruvate treatment effect was superior ($p<0.05$) to the control for TM and PM. Time effect was significant for TM, PM, VAP, VSL, VCL and ELONG (note: Time was not a factor for % SYBR, % PI, % unstained and % FITC-PSA.

Table 1. Experiment 1 CASA means ± SD for total motility (TM) and progressive motility (PM) across extender, centrifugation and L-carnitine/pyruvate treatment (CP) at 2 hours, 24 hours, and 48 hours post collection.

	SKMG				INRA 96			
	Not Centrifuged		Centrifuged		Not Centrifuged		Centrifuged	
	CP-	CP+	CP-	CP+	CP-	CP+	CP-	CP+
TM								
2h	65.9±19.3	79.3±11.7	70.7±16.0	75.0±11.7	76.5±13.0	78.1±13.8	73.8±10.7	78.8±11.8
24h	67.0±16.3	69.3±13.6	62.5±15.1	68.5±12.2	71.4±14.1	76.3±13.6	75.4±15.4	70.3±13.2
48h	58.6±18.8	70.0±10.0	67.9±14.9	62.4±16.9	76.6±14.9	76.3±9.7	74.5±15.2	69.5±10.4
PM								
2h	50.0±15.0	60.1±11.4	53.9±15.5	59.1±11.7	59.5±14.0	62.6±15.2	57.8±14.7	62.6±15.8
24h	47.0±14.2	56.4±13.1	48.5±16.4	59.1±11.8	53.6±16.5	59.6±13.0	59.6±18.6	55.9±13.4
48h	40.3±11.4^{b}	52.9±7.32^{ab}	54.8±14.2^{ab}	59.1±11.9^{ab}	58.5±12.9^{b}	56.3±7.6^{ab}	61.8±10.6^{a}	55.1±9.2^{ab}

Note: means and standard deviations within a row with different superscripts are different ($p<0.05$).

Table 2. Experiment 1 CASA means ± SD for active path velocity (VAP), straight-line velocity (VSL), curvilinear velocity (VCL), and elongation (ELONG) across extender, centrifugation and L-carnitine/pyruvate treatment (CP) at 2 hours, 24 hours and 48 hours post collection.

	SKMG				INRA 96			
	Not Centrifuged		Centrifuged		Not Centrifuged		Centrifuged	
	CP-	CP+	CP-	CP+	CP-	CP+	CP-	CP+
VAP								
2h	114.9±16.0	117.8±16.3	123.6±16.0	122.5±13.2	119.2±10.3	116.4±14.6	112.6±17.2	115.8±9.7
24h	122.2±5.8	119.0±9.7	119.7±9.9	114.2±7.1	125.0±10.5	118.9±8.0	117.7±11.9	111.7±7.4
48h	117.5±10.8	110.0±17.8	114.0±14.8	100.9±14.2	115.0±9.1	99.8±15.8	104.0±12.2	101.9±11.0
VSL								
2h	96.8±14.6	97.4±14.3	100.2±14.2	103.2±13.8	100.3±10.2	99.0±14.9	91.3±15.7	97.5±11.5
24h	98.2±11.3	103.3±12.2	97.5±9.6	100.0±6.4	101.8±11.5	98.5±8.5	94.0±10.7	95.1±7.4
48h	95.0±6.1	91.1±11.4	92.7±10.5	87.4±10.6	93.4±6.1	83.9±10.0	88.4±9.4	87.4±9.6
VCL								
2h	178.5±25.4	183.6±32.5	206.9±28.5	196.1±26.2	189.4±21.8	178.4±21.2	189.8±33.8	187.8±24.0
24h	204.5±11.1	196.1±39.7	204.2±27.4	182.1±22.4	205.3±29.6	193.5±31.1	202.5±28.1	181.5±24.9
48h	193.2±23.5	180.3±42.4	195.0±29.3	162.2±26.6	195.3±23.1	161.0±38.7	169.8±26.0	161.4±21.0
ELONG								
2h	65.5±4.3	65.1±4.7	65.3±6.1	64.8±5.1	65.1±6.1	65.8±4.5	65.9±6.1	66.0±5.4
24h	62.9±3.3	64.9±3.1	62.6±3.3	64.8±4.2	62.3±3.8	65.0±2.5	64.5±2.9	63.8±3.4
48h	63.5±4.7	64.8±4.7	64.3±4.9	65.4±4.0	64.4±4.3	64.6±5.5	65.0±5.4	64.5±3.9

Table 3. Experiment 1 flow cytometer means ± SD for intact membranes (% SYBR), disrupted membranes (% PI), unreacted acrosomes (% unstained) and reacted acrosomes (% FITC-PSA) across extender, centrifugation and L-carnitine/pyruvate treatment (CP) 24 hours post collection.

	SKMG				INRA 96			
	Not Centrifuged		Centrifuged		Not Centrifuged		Centrifuged	
	CP-	CP+	CP-	CP+	CP-	CP+	CP-	CP+
% SYBR	67.8±7.7	71.6±6.7	66.0±12.3	69.1±13.0	75.6±7.1	77.0±6.9	73.8±13.4	69.2±11.5
% PI	32.2±7.7	28.4±6.7	34.0±12.3	31.0±13.0	24.4±7.1	23.0±6.9	26.2±13.4	30.9±11.5
% unstained	65.6±13.0	64.6±12.7	63.5±16.1	64.4±15.1	61.5±12.5	67.5±15.8	65.0±19.0	64.3±15.9
% FITC	34.4±13.0	35.4±12.7	36.5±16.1	35.6±15.2	38.5±12.5	32.5±15.8	35.1±19.0	35.7±15.9

Table 4. Experiment 1 p-values for effect of stallion, ejaculate (nested within stallion), centrifugation, extender, L-carnitine/pyruvate (CP) and time on TM, PM, VAP, VSL, VCL, ELONG, % SBYR, % PI, % unstained and % FITC-PSA. (Note: % SYBR, %PI, % unstained and % FITC-PSA were not subject to multiple time analysis.

	Stallion	Ejaculate	Centrifuge	Extender	CP	Time
TM	0.121	0.000	0.610	0.000	0.001	0.021
PM	0.061	0.005	0.839	0.000	0.000	0.000
VAP	0.773	0.047	0.018	0.523	0.005	0.000
VSL	0.345	0.353	0.122	0.546	0.135	0.000
VCL	0.028	0.088	0.300	0.616	0.008	0.012
ELONG	0.988	0.001	0.852	0.815	0.003	0.000
% SYBR	0.080	0.000	0.176	0.000	0.015	N/A
% PI	0.080	0.000	0.176	0.000	0.015	N/A
% unstained	0.657	0.000	0.893	0.820	0.340	N/A
% FITC	0.657	0.000	0.893	0.820	0.340	N/A

Table 5. Experiment 1 Tukey's comparisons of CP effect on TM, PM, VAP, VSL, VCL and ELONG with all times and extenders included.

	CP-	CP+
TM	70.1±15.57[b]	72.8±12.8[a]
PM	53.8±15.1[b]	57.3±12.1[a]
VAP	117.1±12.9[a]	112.4±14.0[b]
VSL	95.8±11.2	95.3±12.2
VCL	194.5±27.0[a]	180.3±30.9[b]
ELONG	64.3±4.6[b]	64.9±4.1[a]

Note: means and standard deviations within a row with different superscripts are different ($p<0.05$).

Table 6. Experiment 1 Tukey's comparisons of CP treatment effects on TM, PM, VAP, VSL, VCL and ELONGATION by extender and centrifugation with all times included.

	SKMG	SKMG	SKMG	SKMG	INRA	INRA	INRA	INRA
	Not-Cent	Cent	Not-Cent	Cent	Not-Cent	Cent	Not-Cent	Cent
	CP-	CP-	CP+	CP+	CP-	CP-	CP+	CP+
TM	63.8±17.8^{d}	67.0±15.1^{cd}	72.8±12.3^{abc}	68.6±14.2^{bcd}	74.8±13.6^{ab}	74.5±13.4^{ab}	76.9±12.0^{a}	72.8±12.1^{abc}
PM	45.8±13.7^{c}	52.4±15.0^{b}	56.5±10.8^{ab}	55.3±12.7^{ab}	57.2±14.1^{ab}	59.7±14.4^{a}	59.5±12.1^{a}	57.9±13.0^{ab}
VAP	118.2±11.5^{ab}	119.1±13.8^{ab}	115.6±14.9^{abc}	112.6±14.5^{abc}	119.7±10.4^{a}	111.4±14.5^{bc}	111.7±15.3^{abc}	109.8±10.8^{c}
VSL	96.7±10.8^{ab}	96.8±11.5^{ab}	97.3±13.1^{ab}	96.9±12.4^{ab}	98.5±9.9^{a}	91.2±11.9^{b}	93.8±13.1^{ab}	93.3±10.2^{ab}
VCL	192.1±22.8^{abc}	202.1±27.6^{a}	186.7±37.4^{abc}	180.1±27.9^{bc}	196.7±24.9^{ab}	187.36±31.3^{abc}	177.7±32.7^{c}	176.9±25.1^{c}
ELO	64.0±4.1	64.0±4.8	64.9±4.1	65.0±4.3	63.9±4.8	65.1±4.8	65.1±4.2	64.8±4.2

Note: means and standard deviations within a row with different superscripts are different ($p<0.05$).

Table 7. Experiment 1 Tukey's comparisons of extender effect on TM, PM, VAP, VSL, VCL and ELONG with all times and CP treatments included.

	SKMG	INRA
TM	68.1±15.1[b]	74.8±12.7[a]
PM	52.5±13.6[b]	58.6±13.3[a]
VAP	116.4±13.8[a]	113.2±13.3[b]
VSL	96.9±11.8[a]	94.2±11.5[b]
VCL	190.2±30.0	184.6±29.4
ELONG	64.5±4.3	64.7±4.5

Note: means and standard deviations within a row with different superscripts are different ($p<0.05$).

Table 8. Experiment 1 Tukey's comparisons of extender effects on TM, PM, VAP, VSL, VCL and ELONGATION with all times included.

	SKMG	INRA
TM	68.1±15.1[b]	74.8±12.7[a]
PM	52.5±13.6[b]	58.6±12.3[a]
VAP	116.4±13.8[a]	113.2±13.3[b]
VSL	96.9±11.8[a]	94.2±11.5[b]
VCL	190.2±30.0	184.6±29.4
ELONG	64.5±4.3	64.7±4.5

Table 9. Experiment 2 CASA means ± SD for total motility (TM) and progressive motility (PM) across extender and L-carnitine/pyruvate treatment (CP) at 2 hours, 24 hours, and 48 hours post collection.

	SKMG		INRA 96		REV	
	CP-	CP+	CP-	CP+	CP-	CP+
TM						
2h	78.0±13.9	82.8±13.3	85.9±11.8	87.8±9.7	78.0±14.6	82.8±11.9
24h	77.7±17.6	80.4±15.0	79.1±18.3	80.8±14.9	73.8±18.3	72.6±17.1
48h	68.8±21.5	74.3±18.4	75.4±18.9	78.7±13.5	58.3±23.0	66.4±17.8
PM						
2h	62.3±13.3	66.2±11.9	72.2±11.0	74.2±8.1	63.8±12.1	68.7±10.0
24h	57.3±16.6	57.8±17.4	59.4±17.9	62.1±15.4	53.8±17.5	56.4±16.7
48h	46.3±18.1	52.8±17.4	54.3±17.4	59.3±13.0	42.5±17.5	47.2±15.4

Table 10. Experiment 2 CASA means ± SD for active path velocity (VAP), straight-line velocity (VSL), curvilinear velocity (VCL), and elongation (ELONG) across extender and L-carnitine/pyruvate treatment (CP) at 2 hours, 24 hours and 48 hours post collection.

	SKMG		INRA 96		REV	
	CP-	CP+	CP-	CP+	CP-	CP+
VAP						
2h	114.9±13.9	123.1±15.7	120.4±10.1	117.6±13.8	121.0±14.1	119.6±14.7
24h	118.3±23.1	121.0±14.2	114.5±18.5	107.0±13.6	119.3±24.4	118.2±14.6
48h	113.6±22.5	111.8±18.7	117.7±21.4	105.9±10.4	115.1±18.3	105.0±11.7
VSL						
2h	98.7±12.1	104.6±14.6	103.5±6.4	101.8±10.5	106.4±11.7	105.7±12.5
24h	94.3±14.8	97.4±10.3	95.0±12.8	89.8±7.9	97.3±16.4	92.7±10.6
48h	88.8±15.3	88.3±11.6	95.7±12.3	85.5±7.3	92.0±11.8	84.4±6.6
VCL						
2h	181.7±19.5	192.9±26.1	186.9±22.1	178.8±28.1	182.4±23.6	117.2±25.9
24h	204.5±45.8	214.0±33.1	190.2±38.6	178.5±35.5	204.3±49.7	190.0±34.4
48h	200.8±43.5	202.2±43.7	203.6±46.3	184.5±33.9	208.1±154.6	190.5±35.1
ELONG						
2h	60.8±1.9	60.8±1.3	61.6±1.2	60.8±1.5	61.5±2.2	61.2±2.0
24h	60.7±1.8	60.4±2.4	60.8±2.1	61.3±1.7	60.3±2.1	60.5±2.6
48h	60.6±2.2	60.8±2.6	60.3±2.3	61.3±1.6	60.2±2.1	60.5±1.8

Table 11. Experiment 2 flow cytometer means ± SD for intact membranes (% SYBR), disrupted membranes (% PI), unreacted acrosomes (% unstained) and reacted acrosomes (% FITC-PSA) across extender and L-carnitine/pyruvate treatment (CP) 24 hours post collection.

	SKMG		INRA 96		REV	
	CP-	CP+	CP-	CP+	CP-	CP+
% SYBR	60.9±10.2	67.9±5.8	77.2±7.1	79.9±4.5	87.0±2.6	86.2±5.3
% PI	39.1±10.2	32.1±5.8	22.8±7.1	20.1±4.5	13.0±2.6	13.8±5.3
% unstained	63.0±21.2	77.9±4.6	65.8±17.6	80.7±6.4	69.8±13.7	78.0±7.3
% FITC-PSA	37.0±21.2	22.1±4.6	34.2±17.6	19.3±6.4	30.2±13.7	22.0±7.3

Table 12. Experiment 2 p-values for effect of stallion, ejaculate (nested within stallion), extender, L-carnitine/pyruvate (CP) and time on TM, PM, VAP, VSL, VCL, ELONG, % SBYR, % PI, % unstained and % FITC-PSA. (Note: % SYBR, %PI, % unstained and % FITC-PSA were not subject to multiple time analysis.

	Stallion	Ejaculate	Extender	CP	Time
TM	0.005	0.050	0.000	0.003	0.000
PM	0.001	0.319	0.000	0.000	0.000
VAP	0.001	0.007	0.162	0.01	0.000
VSL	0.007	0.222	0.625	0.033	0.000
VCL	0.000	0.064	0.001	0.021	0.000
ELONG	0.000	0.290	0.354	0.618	0.001
% SYBR	0.224	0.012	0.000	0.028	N/A
% PI	0.224	0.012	0.000	0.028	N/A
% unstained	0.004	0.591	0.567	0.000	N/A
% FITC-PSA	0.004	0.591	0.567	0.000	N/A

Table 13. Experiment 2 Tukey's comparisons of CP effect on TM, PM, VAP, VSL, VCL and ELONG with all times and extenders included.

	CP-	CP+
TM	75.0±18.7[b]	78.5±15.5[a]
PM	56.9±17.5[b]	60.5±15.8[a]
VAP	117.2±18.5[a]	113.6±15.3[b]
VSL	96.8±13.5[a]	94.5±12.7[b]
VCL	195.8±38.5[a]	189.8±34.0[b]
ELONG	60.7±2.0	60.8±2.0

Note: means and standard deviations within a row with different superscripts are different ($p<0.05$).

Table 14. Experiment 2 Tukey's comparisons of CP treatment effects on TM, PM, VAP, VSL, VCL and ELONGATION by extender with all times included.

	SKMG	SKMG	INRA	INRA	REV	REV
	CP-	CP+	CP-	CP+	CP-	CP+
TM	74.8±17.9^{bcd}	79.2±15.7^{abc}	80.1±16.8^{ab}	82.4±13.1^{a}	70.1±20.3^{d}	73.9±16.8^{cd}
PM	55.3±17.1^{cd}	58.9±16.3^{bc}	62.0±17.1^{ab}	65.2±13.8^{a}	53.4±17.8^{d}	57.4±16.5^{bcd}
VAP	115.6±19.8^{ab}	118.6±16.6^{a}	117.5±17.0^{a}	110.1±13.4^{ab}	118.5±19.0^{a}	111.9±14.7^{ab}
VSL	93.9±14.3^{ab}	96.8±13.7^{ab}	98.1±11.3^{a}	92.4±10.9^{b}	98.6±14.4^{a}	94.26±13.3^{ab}
VCL	195.7±38.4^{ab}	203.0±35.2^{a}	193.6±36.7^{ab}	180.6±31.8^{c}	198.3±41.3^{ab}	185.9±31.8^{bc}
ELONG	60.7±1.9	60.7±2.1	60.9±2.0	61.1±1.6	60.7±2.2	60.7±2.1

Note: means and standard deviations within a row with different superscripts are different ($p<0.05$).

Table 15. Experiment 2 Tukey's comparisons of extender effect on TM, PM, VAP, VSL, VCL and ELONG with all times and CP treatments included.

	SKMG	INRA	REV
TM	77.0±16.9[b]	81.3±15.0[a]	72.0±18.6[c]
PM	57.1±16.7[b]	63.6±15.5[a]	55.4±17.2[b]
VAP	117.1±18.2	113.8±15.7	115.2±17.2
VSL	95.3±14.0	95.2±11.4	96.4±13.9
VCL	119.4±36.8[b]	187.1±34.7[ab]	192.1±37.1[a]
ELONG	60.7±2.0	61.0±1.8	60.7±2.2

Note: means and standard deviations within a row with different superscripts are different ($p<0.05$).

Table 16. Experiment 2 Tukey's comparisons of extender effects on TM, PM, VAP, VSL, VCL and ELONGATION with all times included.

	SKMG	INRA	REV
TM	77.0±16.9[b]	81.3±15.0[a]	72.0±18.6[c]
PM	57.1±16.7[b]	63.6±15.5[a]	55.4±17.2[b]
VAP	117.1±18.2	113.8±15.7	115.2±17.2
VSL	95.3±14.0	95.2±11.4	96.4±13.9
VCL	199.4±36.8[a]	187.1±34.7[b]	192.1±37.1[ab]
ELONG	60.7±2.0	61.0±1.8	60.7±2.2

DISCUSSION

Experiment 1

When data were analyzed within specific times (Tables 1-3) there was only one point of significant treatment difference observed (PM at 48 hours). However, when observing the means of each treatment, there are visible visual trends that show difference between these treatments. The lack of statistical significance is most likely due to inadequate degrees of freedom available when running the analysis of variance on treatment differences. The small sample size is causative of the limited degrees of freedom. Variation in the innate characteristics of our sample position may also be causative of this. One of the greatest difficulties in equine reproduction research is lack of uniformity in the different stallions used for a particular experiment. Similarly, there is a lack of uniformity in different ejaculates from the same stallion (this can be partially adjusted for by nesting the ejaculate effect within the particular stallion).

In this analysis the degrees of freedom are much higher; therefore the strength of extender as well as L-carnitine and pyruvate treatment are visible. An increase in sample size may result it significant differences in analysis of variance at each of the individual storage times.

Experiment 2

The results of the Experiment 2 are similar to the results of Experiment 1. Analysis at individual time points failed to show significant treatment effects. Limitations due to stallion effect and ejaculate effect were likewise an issue within this experiment.

Conversely, extender and L-carnitine and pyruvate treatment showed multiple points of significance when including all times in the analysis of variance. The assumption remains in this experiment that an increased sample size would have potentially led to statistically different results across different treatments for individual time analysis.

Limitations

Semen research can be highly sensitive to multiple extraneous factors. When dealing with such environmentally dependent material, it is imperative to maintain structured procedures and precise technical work to eliminate as much operator error and environmental influence as possible. As mentioned above, the issues of stallion effect and ejaculate effect are continuing areas of difficulty for adjustment in this research. Characteristic differences are highly variable across breeds but they are likewise variable within breeds.

Overall, in an increase in the number of stallions available, number of ejaculates from each stallion and continued improvement of technical precision would have led to an even stronger base of data to analyze the effects of extender and L-carnitine/pyruvate treatments.

CONCLUSIONS

The inclusion of L-carnitine and pyruvate to a variety of semen extenders has the potential to improve the media in which spermatozoa are housed during transportation for the purpose of artificial insemination. The response of spermatozoa to treatment is dependent on the type of extender used. SKMG and REV may benefit from the inclusion of L-carnitine and pyruvate while INRA seems to be optimized without the inclusion of these treatments.

Although a larger sample size would likely have led to an increased production of statistical significance across individual time points, the general trends in mean values show that the addition of L-carnitine and pyruvate may improve multiple viability and motility characteristics of equine spermatozoa.

With all times included, the significance of L-carnitine and pyruvate treatment is strongly apparent (see p-value tables in RESULTS section). The significance of extender is likewise strongly apparent. The addition of L-carnitine and pyruvate in diluents of extended equine semen may significantly improve multiple factors of spermatozoa survival analysis. The conclusion can be made that not all commercially available semen extenders are equal. INRA 96 consistently proved to be the best extender of the ones used in the trial (Tables 7 and 15) and often times only benefitted slightly from the addition of L-carnitine and pyruvate treatment whereas SKMG and Revolution extenders were inferior but improved drastically in the presence of L-carnitine/pyruvate.

Applying this knowledge may lead to an increased fertilizing capacity of a sample of equine semen. Increasing the level of fertility for the stallion will ultimately result in

an increased conception rate for the mares. Increased conception rates save the mare owner money by reducing the amount of attention due to missed cycles (ultrasounds, veterinarian checks, breeding farm fees, etc.).

All in all, stallions (specifically performance horse stallions) have been selected for athletic ability, conformation and trainability and by default their reproductive efficiency has gone by the wayside. More studies are necessary to say that L-carnitine and pyruvate inclusion are absolutely beneficial to equine semen; however, this research shows that there is strong evidence to support the use of these treatments in semen extenders to improve the reproductive capability of the stallion by enhancing the motility and viability characteristics of collected semen.

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