Spring 2019

Oxyrase and Pyruvate Inclusion in Extenders for Cold Stored Stallion Spermatozoa

Jordan T. Shore
Missouri State University, Shore549@live.missouristate.edu

OXYRASE® AND PYRUVATE INCLUSION IN EXTENDERS FOR COLD STORED STALLION SPERMATOZOA

A Master's Thesis

Presented to

The Graduate College of

Missouri State University

In Partial Fulfillment

Of the Requirements for the Degree

Master of Science, Agriculture

By

Jordan Todd Shore

May 2019

OXYRASE® AND PYRUVATE INCLUSION IN EXTENDERS FOR COLD STORED STALLION SPERMATOZOA

Agriculture

Missouri State University, May 2019

Master of Science

Jordan Todd Shore

ABSTRACT

A current method in addressing subfertility in stallions include manipulating the semen extender media by supplementing antioxidants and energy sources. Two experiments (EXP 1/EXP 2) were conducted to assess the effects of pyruvate and Oxyrase® suspended in commercial diluents for preservation of stallion spermatozoa. Assessment of total (TM) and progressive motility (PM), velocity and direction of movement (VAP, VSL, VCL, and elongation) were recorded by CASA. In EXP 1, 3 different ejaculates were collected from each of 4 stallions. Aliquots of each ejaculate were suspended into 4 treatments of INRA96 with or without Oxyrase® supplemented at 2.4 U/ml and centrifuged or not centrifuged. Aliquots of each treatment were split for analysis by CASA after 2 hours, 24 hours, and 48 hours of storage. In EXP 2, 3 different ejaculates were collected from each of 3 stallions. Two of five aliquots were suspended in skim-milk glucose (SKMG) or SKMG modified by the addition of sucrose (SKMG+S). The remaining diluents were formulated with 2.4 instead of 4.9 g of glucose per 100ml and with increasing amounts of pyruvate. Method of storage and analysis was consistent through EXP 2. In EXP 1 the addition of Oxyrase® did not improve ($P<.05$) post storage motility of stallion spermatozoa. At all times in EXP 2 the diluents SKMG+S and SKMG demonstrated numerically higher TM and PM than extenders with substituted pyruvate. In conclusion, addition of Oxyrase® in INRA96 and substitution of pyruvate in diluents with formulas commonly used demonstrated no beneficial effects to stallion spermatozoa survival.

KEYWORDS: stallion, spermatozoa, oxyrase, pyruvate, semen extenders

OXYRASE® AND PYRUVATE INCLUSION IN EXTENDERS FOR COLD STORED STALLION SPERMATOZOA

By

Jordan Todd Shore

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

May 2019

Approved:

Gary Webb, Ph.D., Thesis Committee Chair

William McClain, Ph.D., Committee Member

Lacy Sukovaty, DVM, Committee Member

Elizabeth Walker, Ph.D., Committee Member

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

ACKNOWLEDGEMENTS

During the course of my graduate studies I have learned more than just information from the classroom. The experiences and skills I have developed from numerous people and teaching opportunities will leave an everlasting impact on my life. I would like to thank the following people for their support during my graduate studies:

Dr. Gary Webb and Sue Webb gave me the opportunity to gain invaluable skills as a teacher, coach, and student during my journey at Missouri State. The Webb's have left a perpetual influence on my life and have helped mold me into the teacher and coach I am today. I will forever be grateful for that. I would also like to thank William Boyer for his help during the project. Will is a "behind the scenes" worker and deserves recognition for all of the hard work he has done. Without their help and support this would not have been possible.

I would also like to recognize the rest of my committee; Dr. William McClain, Dr. Lacy Sukovaty, and Dr. Elizabeth Walker for their guidance in the process of completing this thesis. Thank you to my fellow graduate students, especially Taylor Godwin, for your assistance during the project and in the process of writing. This would have been much more difficult without your help. Finally, I would like to thank my family and friends for helping me in any way they could and believing in me to finish this goal.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

Justification

Cold storage of spermatozoa acts on the metabolic process by slowing and decreasing depletion of the energy stores. The effect cold storage has on each stallion is variable. When researching the optimal extender for stallions, one must acknowledge that one extender may work better than another for an individual stallion. Therefore, no single protocol has been identified as the most efficient among the population of stallions. Developing an extender to prolong and improve the ability of stallion spermatozoa to handle cold storage stress improves several factors [1]:

1. Increase breeder access to stallion's superior genetics.
2. Increase overall market area for stallion semen.
3. Increase longevity of spermatozoa allowing for an increase in flexibility, which will allow a way to circumvent problems with delivery schedules of shipped inseminations.
4. Decrease the number of shipments required per pregnancy.
5. Allow for more insemination doses per ejaculate.

A current method in addressing subfertility in stallions include, manipulating the semen extender media by supplementing antioxidants and energy sources. The goal of supplementing these components is to increase post-storage survivability for cooled and cryopreserved spermatozoa [1 - 11]. Energy sources including skim milk, glucose, and egg yolk compose the majority of popular extenders. However, semen extender media are not limited to those specific substrates. Other potential energy source include pyruvate, the product of glycolysis.

Pyruvate acts as an intermediate compound in the metabolism of simple sugars (mainly glucose), fatty acids, and amino acids. Compared to glycolytic substrates (glucose), pyruvate is a more direct substrate in the mitochondrial pathway of oxidative phosphorylation [2]. Stallions prefer the oxidative phosphorylation pathway to glycolysis as the main energy provider for spermatozoa function [12]. An inadequacy of an energy substrate is detrimental and leads to mitochondrial dysfunction/decreased motility of spermatozoa [2]. For this reason, mitochondrial energy inputs, like pyruvate, are crucial to energy production.

The aforementioned inadequacy of an energy substrate causing mitochondrial dysfunction not only decreases motility but also increases reactive oxygen species (ROS) production [2]. Oxidative stress, brought on by the upregulation of ROS, and the byproducts it produces are thought to be a major factor in spermatozoa cell death [3]. To counter this effect, antioxidants, which reduce free radicals produce from oxidative stress, can be supplemented into the media of cooled or cryopreserved spermatozoa. Several compounds have been studied as possible antioxidant additives to semen extenders, including, glutathione peroxidase, catalase, tocopherol, pyruvate, and superoxide dismutase [4 - 6]. Oxyrase®, has been recently used in cryopreservation protocols as a supplemental antioxidant to aid in protection from freeze-thaw damaging effects in stallions, monkeys, and mice [3,13,14]. In recent studies, contradicting evidence regarding Oxyrase® has been demonstrated to improve or not effect motility of stallion spermatozoa [3,7]. Variability within stallions and ejaculates from each study could be a major contributing factor for the inconsistent results. Cryopreservation effects were found with Oxyrase®. However, there is little research on effects of cooled stallion spermatozoa supplemented with Oxyrase®. The addition of Oxyrase® in extended treatments for cold storage should be further researched.

Determination of the effectiveness of pyruvate and Oxyrase® capability to prolong and improve the ability of the spermatozoa when under cold storage was analyzed at increasing time increments (2, 24 and 48 h). Motility and velocity were recorded in order to analyze the effect on the spermatozoa.

Problem Statement

Deleterious effects from cold storage on stallion spermatozoa can decrease the motility and velocity, thus, incapacitating the paternal DNA transporters and decreasing the chance of fertilization. Suspension of spermatozoa in an extender with adequate supply of energy and/or protective compounds will allow for prolonged survival in cold storage.

Objective

Objectives of the two experiments reported in this thesis were to determine the effects of supplemented Oxyrase® and pyruvate in commercially available extenders on motility and velocity of cold stored stallion spermatozoa.

Null Hypotheses

1. EC-Oxyrase® will not increase post storage motility and velocity of stallion spermatozoa
2. Substituted pyruvate in a skim milk glucose extender will not improve post storage motility and velocity of stallion spermatozoa when compared to commercially available extenders.

LITERATURE REVIEW

Spermatozoa Structure

Anatomically, the structure of the spermatozoa consists of four major parts: the head, middle piece (midpiece), principle piece, and end piece (Figure 1). The head, midpiece, and entire sperm cell is enveloped in a phospholipid bilayer membrane. The previous components of the spermatozoa structure will be the focus of this literature review.

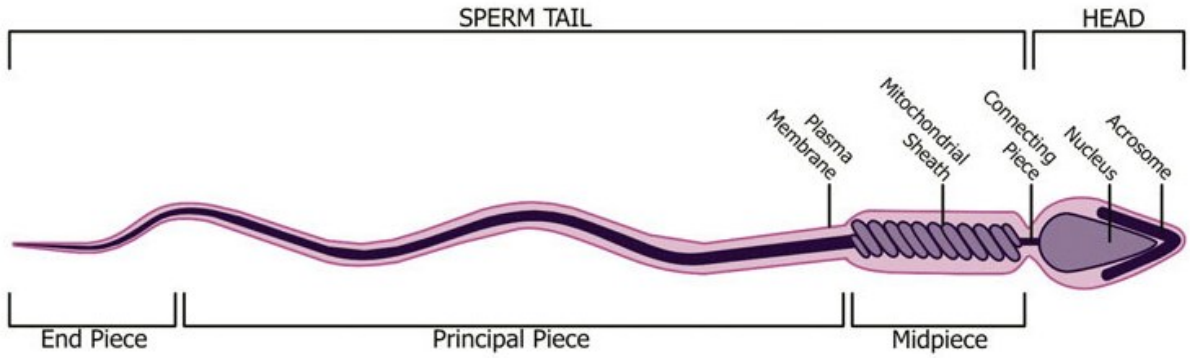

Figure 1. Anatomical structure of a spermatozoon [15].

Phospholipids are amphipathic and have a polar head that contain a phosphate and glycerol. Nonpolar, hydrophobic fatty acids make up the tail of the phospholipid [16]. Alignment of the phospholipids from both sides of the bilayer serve as a barrier to deter water and other polar or charged substances from crossing the membrane. Embedded within the membrane are proteins, carbohydrates, and cholesterol [16].

Proteins make up approximately 50% of the total membrane molecular weight. Placement of the protein influences the role that it serves to the spermatozoa. Integral proteins act as ion channels, pores, receptors, and signal transduction components. Proteins receptors for various

hormones are located on the outer layer and glycocalyx (densely packed oligosaccharides attached to cell-surface proteins and lipids) of spermatozoa [17]. Proteins associated on the outside of the spermatozoa include lipid-linked proteins, which contain covalently attached lipids that anchor itself to the lipid membrane. In addition, peripheral membrane proteins lie on the exterior of the membrane and are not bound to any lipids but most likely bound to integral proteins [17]. Carbohydrates are strictly present on the outer portion of the plasma membrane, attaching to proteins or lipids forming glycoproteins or glycolipids [16].

Cholesterol, a four ringed carbon lipid, is located within the cell membrane core and aids in minimizing the change in fluidity from temperature changes. In low temperatures, cholesterol helps maintain a high fluidity by preventing phospholipids from packing tightly together. Stallion spermatozoa have a lower cholesterol to phospholipid molar ratio, 0.36, when compared to bulls, 0.45. This likely contributes to the higher susceptibility of cold shock in stallion spermatozoa [18 – 20]. Species with a higher cholesterol to phospholipid ratio, similar to bulls, exhibit a greater tolerance to temperature changes due to the stability of the membrane at low temperatures.

Located between the plasma membrane and the nucleus, lies the acrosomal matrix. Within the matrix, the inner acrosome is in close contact with the nuclear envelope surrounding the nucleus, while the outer acrosome lies beneath the plasma membrane. Enzymes within the acrosome include proacrosin, acrosin, hyaluronidase, and lipases that are important for penetration of the oocyte after the acrosome reaction [21]. During the acrosome reaction, hybrid vesicles and pores are created when the outer portion of the acrosome attaches with the overlying plasma membrane, therefore, allowing the release of the acrosomal contents and paternal genetic material from the fertilizing sperm [22]. Chromatin, containing the paternal genetic material

DNA, is densely packed within the nucleus and is surrounded by the nuclear envelope. Posterior to the chromatin is the implantation fossa, the point of attachment for the flagellum [23].

Attaching to the implantation fossa, the capitulum, is a specialized structure within the connecting piece, forms the cranial border of the flagellum. Proteins rich in disulfide bonds make up the cranial border. The connecting piece is responsible for attaching the midpiece to the head of the spermatozoon. Within the midpiece is the mitochondrial matrix. In spermiogenesis, the final stage of spermatogenesis, mitochondria assume a final spiral alignment in the matrix. Alignment of mitochondria in this position are known as gyri (Figure 2) [24].

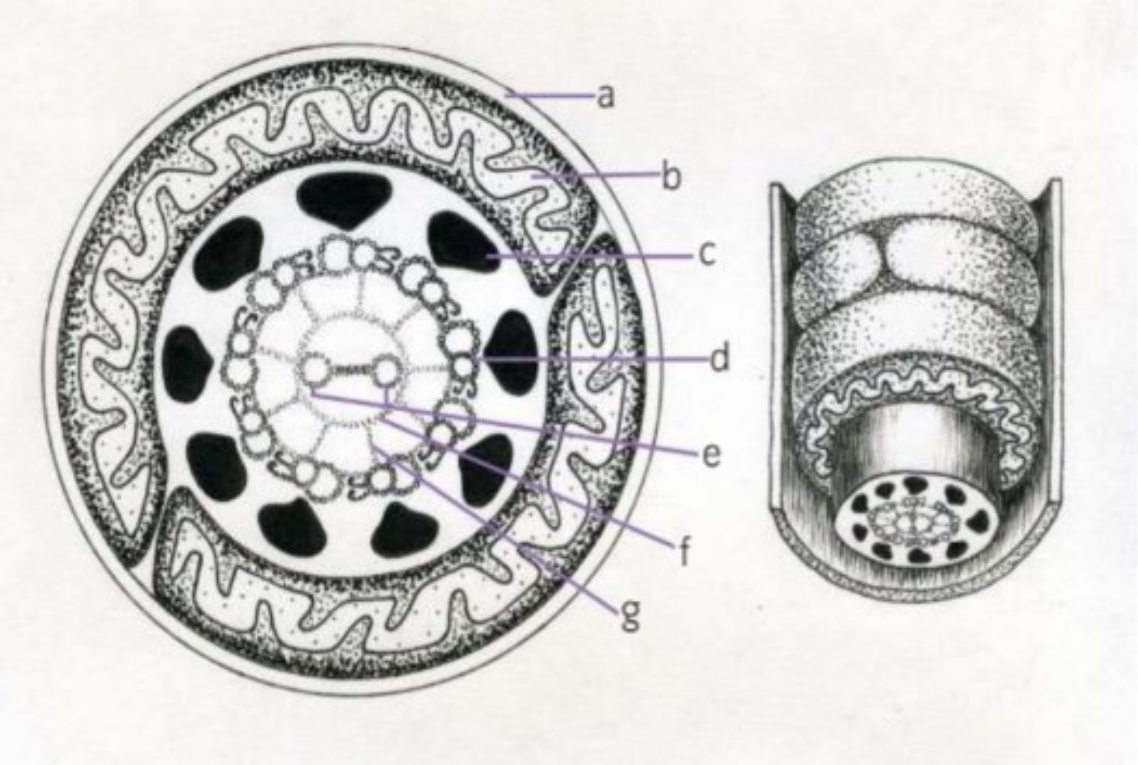

Figure 2. Cross sectional view of the middle piece (midpiece) in stallion spermatozoa [23].

Each gyri contains approximately 40-50 mitochondria. Order and symmetry of gyri play a significant role in mitochondria as they may express a functional effect. Disruptions of the

order and symmetry of the gyri have been linked reducing fertility rate in stallions [24]. Fertilization relies heavily on the function of mitochondria to produce and store ATP for spermatozoal motility and survival.

Spermatozoa Metabolism

Spermatozoa function as carriers of paternal DNA, and triggers/activates the female oocyte upon fertilization, a process that is highly energy dependent. Spermatozoa are not solely responsible for movement to the oviduct, uterine contractions also aid in the transport to site of fertilization. For successful fertilization to occur, spermatozoa require the production of ATP for capacitation, hyperactivation, and the acrosomal reaction. When undergoing capacitation, structural and functional changes occur which include membrane modifications, modulation of enzymes, and protein phosphorylation. Hyperactivation accompanies capacitation and is indicated by an increase in flagellar bend amplitude. Hyperactivation is critical for movement through the ovidcutal mucus and to penetrate the zona pellucida of the oocyte after the acrosomal reaction.

The site of sperm deposition, the uterus, is distant from the site of fertilization, the oviduct. Therefore, sufficient production of ATP is essential for motility in order for spermatozoa to reach the oviduct for fertilization [21]. Two major metabolic pathways that provide ATP for spermatozoa are glycolysis and oxidative phosphorylation (OXPHOS).

Glycolysis. Glucose is broken down in a two-phase process known as glycolysis. Phase one of glycolysis consumes two molecules of ATP in order to initiate glucose catabolism. During phase one, the glucose is phosphorylated and split to yield two molecules of glyceraldehyde-3-phosphate. During phase two, the two molecules of glyceraldehyde-3-phosphate are converted

into pyruvate, subsequently, generating four ATP from the process. Thus, glycolysis has a consumption of two ATP and a production of four ATP for a net total of two ATP produced per glucose [17].

Glycolytic enzymes and glucose transporters (GLUTs) allow passage through the lipid bilayer and determine location of glycolysis. Glycolytic activity, transporters of glucose and enzymes are entirely localized in the fibrous sheath of the flagellum and the acrosome [25]. Distribution of GLUTs suggest that glycolytic processes mainly produce energy for the changes induced on the cell membrane during capacitation and the acrosomal reaction. Bucci et al., [26] described the dispersal of GLUTs as evidence of the function status of the sperm in dogs; this has yet to be seen in equine [26].

Production of ATP between the two metabolic pathways differs. Oxidative phosphorylation produces an approximate ATP total of 26 – 28 and is more ATP productive than glycolysis. Glycolysis produces an approximate net total of two ATP and two NADH. Some species (e.g. humans and mice) rely almost entirely on glycolysis for ATP production [25], but the preferred metabolic pathway is highly species specific [27]. Both pathways run in concurrence with one another. When supplementing energy is considered, then the knowledge of the pathway desired by a specific species is required to optimize a more direct energy production.

Oxidative Phosphorylation. Recent studies indicated that stallion spermatozoa rely almost entirely on OXPHOS for energy supply [12,28]. In order to ascertain which pathway is primarily relied on, a comparison between stallions and a known glycolysis utilizer (humans) was conducted. Diphenyleneiodonium (DPI; a flavoprotein inhibitor of the mitochondrial electron transport chain) was selected as a mitochondrial (OXPHOS) inhibitor on motility and

velocity. Within human spermatozoa, no effect was observed when DPI was added as human spermatozoa does not rely on OXPHOS. Therefore, these results were expected. Stallions, however, were detrimentally effected by the DPI indicating a heavy reliance on OXPHOS for energy [12].

Stallions preferred metabolic pathway, OXPHOS, takes place within the mitochondrial matrix in the intermembrane spaces. At this location, energy is produced through protein complexes. Oxidative phosphorylation utilizes more diverse substrates when compared to glycolysis. Unlike glycolysis, there is not a single main substrate (e.g. glucose) metabolized to further the process of ATP production. Substrates utilized, generally stem from derivatives from the catabolism of carbohydrates, lipids, and proteins.

Utilization of OXPHOS over glycolysis for the main production of ATP is appropriate due to stallion spermatozoa velocity parameters being 60% faster than human parameters; requiring a longer lasting energy producing system for motility. Subsequently, high-energy demands of the stallion spermatozoa lead to a lower concentration of ATP when compared to human spermatozoa [12]. Gibb et al. [12] hypothesized, higher levels of OXPHOS in more fertile stallions, increased mitochondrial activity, leading to an increase in the byproduct of oxidative stress.

Reactive Oxygen Species

Reactive oxygen species (ROS), byproducts of oxidative stress, effect fertilization and embryonic development. Therefore, ROS can cause developmental abnormalities in offspring and pregnancy loss. The latter likely result from oxidative damage done to DNA within the spermatozoa cells [29]. Vulnerability of spermatozoa to oxidative attack is intensified by the fact

that these cells actively generate reactive oxygen species. But, production of ROS is necessary for capacitation of spermatozoa prior to fertilization. However, when spermatozoa are stressed, the role of ROS turns to an intrinsic apoptotic pathway [30].

Reactive oxygen species can be detrimental to spermatozoa [31]. Spermatozoa membranes consist of polyunsaturated fatty acids (PUFAs), particularly docosahexaenoic acid, which are highly vulnerable to free radical attack [30]. Free radicals attack the PUFAs generating a variety of lipid metabolites, including lipid radicals (Figure 3a.) and aldehydes like 4-hydroxynonenal (4HNE) [32,33]. Lipid radicals then combine with oxygen, an electron acceptor, producing a peroxyl radical (Figure 3b.). Destabilization of the lipid membrane occurs by the extraction of hydrogen atoms to stabilize the radicals own structure. To achieve stability, the peroxyl radical eradicates a hydrogen from a nearby PUFA to form a lipid hydroperoxide. The eradication process perpetuates the production of lipid peroxyl radicals continuing the progress of lipid peroxidation. (Figure 3c.) [30].

Lipid aldehydes (4HNE) produced from peroxidation attach to mitochondrial proteins. Aldehydes can bind to mitochondrial proteins, like succinic acid dehydrogenase, triggering the electron leakage forming additional ROS, continuing peroxidation [30]. As the cycle continues, spermatozoa enter an apoptotic pathway losing membrane potential and eventually resulting in damage to DNA strands [34]. Spermatozoa DNA damage is mostly due to hydroperoxide, which is the only apoptotic product that can pass from the midpiece to the head. Continuation of oxidative stress will increase the lipid peroxidation cycle and lead to cell death [35].

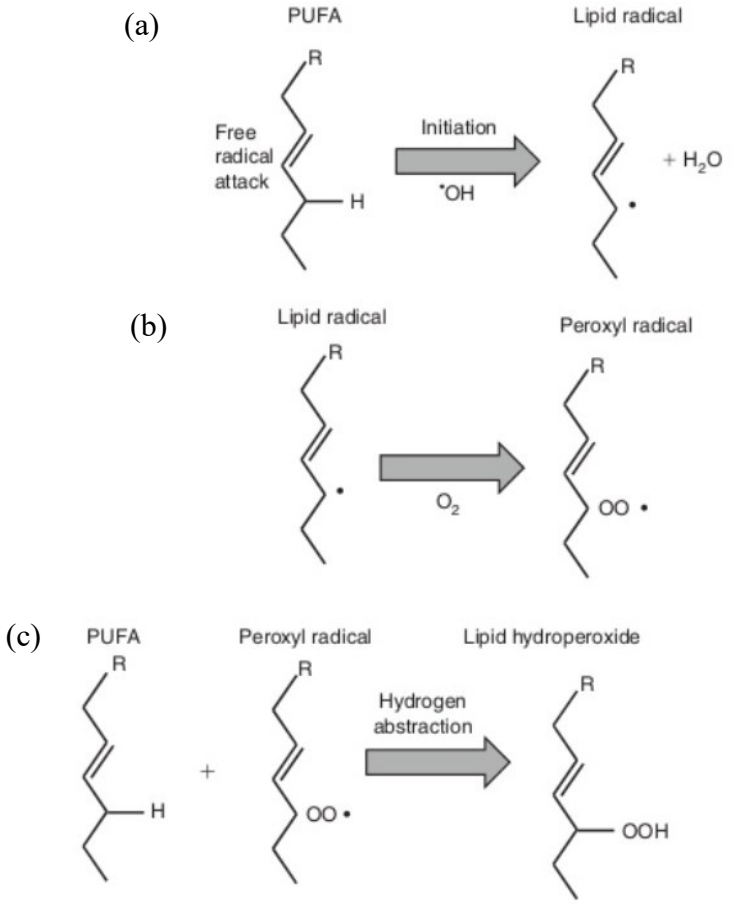

Figure 3. Lipid peroxidation in the mammalian cell [30].

Spermatozoa also lack intracellular antioxidant protection as provided by catalase and glutathione peroxidase, common metabolizing enzymes of ROS. In stallions, catalase is abundant in the seminal plasma (SP) but limited intracellularly due to restricted volume of space within the spermatozoa membrane [30,36]. Catalase, along with glutathione peroxidase, is used for the removal of hydroperoxide within the spermatozoa [31]. If catalase and/or the glutathione reductase system is inefficient, hydroperoxide will generate more lipid radicals via the Fenton reaction (e.g., iron or copper reaction with hyrdoperoxide). However, this can be eliminated by nonenzymatic antioxidants like ascorbic acid and α-tocopherol within the cell wall [37].

Consequently, removal of the SP, also removes several enzymatic and nonenzymatic antioxidants, which subsequently, removes the beneficial actions of antioxidants. In contrast, the removal of SP can be beneficial to sperm motility. Dawson et al. [38], suggested that decreasing the amount of time spermatozoa remained in an undiluted state without SP, increased the post storage recovery of progressive motility and acrosome-intact stallion spermatozoa [38]. Removal of SP is utilized to concentrate the number of spermatozoa and remove detrimental components [39]. Cryopreservation and cooling protocols of semen, generally, consist of centrifugation, a method used to remove SP. Consequently, natural antioxidants in the SP are removed when semen is centrifuged, thus allowing for an increased amount of ROS produced [11].

Hydroxyl radicals have been shown to be detrimental to spermatozoa DNA [40]. Even with the presence of SP (which includes catalase), hydroxyl radicals were deleterious to DNA integrity. Fragmentation of DNA without SP occurred at 92.7 ± 3.2%, compared to fragmentation with SP at 13.0 ± 6.1% [40]. Therefore, demonstrating presence of antioxidants aid in reducing the damage from radicals. Antioxidant protection is crucial to breaking up hydroperoxide via catalase and glutathione peroxidase [41], subsequently inhibiting formation of hydroxyl radicals [40]. Addition of antioxidants has been suggested when SP is not present as a method to prevent negative reactions from occurring [41].

Oxyrase®

Oxygen has a primary role in formation of ROS; consequently, this may lead to an effect on amount of O_2 radicals within the spermatozoa. The continuing revolution of lipid peroxidation in spermatozoa is a linear function of partial pressure of oxygen (O_2) in the medium [42]. In order to attain low O_2 tension, integration of commercially available antioxidants, like Oxyrase®,

may be beneficial [14]. Pyruvate acts as an antioxidant and aid in deterring induced hydroperoxide damage by peroxidation of lipids in ram and bull spermatozoa [5,10]. However, stallion spermatozoa (frozen-thawed and nonfrozen) are less susceptible to peroxidation than bull spermatozoa [43] and contain a higher amount of catalase [8]. Therefore, pyruvate in stallion spermatozoa act as a readily energy substrate and not as an antioxidant to stallion spermatozoa [1]. Based on the prior research, the antioxidant Oxyrase® could be beneficial when supplemented to stallion spermatozoa.

Oxyrase® is obtained from the cytoplasmic membrane of the microorganism *Escherichia coli* (EC), and contains an excerpt of bacterial electron transport systems [44]. The product EC-Oxyrase® (product number: EC-0005, Oxyrase® Inc.), contains 100mM DL of lactate. With the presence of lactate, a suitable hydrogen donor, Oxyrase® can reduce oxygen in solutions [44]. Oxyrase® is suggested to be "nature's antioxidant," due to its ability to reduce free radicals in a solution creating an anaerobic environment (Oxyrase® Inc). Stallion spermatozoa survivability has been hypothesized to improve when cooling or freezing procedures include Oxyrase®, creating a more anaerobic environment [2,7]. Oxyrase® has not only been used in cryopreservation of stallion spermatozoa but also supplemented in monkey and mouse cryopreservation protocols with a significant ($P<0.05$) beneficial effect [13,14].

Darr et al. [2] analyzed the effects of reducing oxygen concentration using Oxyrase®, the oxygen scavenger, on motility of frozen-thawed stallion spermatozoa. Six stallions with low progressive motility measurements (<50% PM) were selected for treatments to increase sensitivity of Oxyrase®. Results are listed in Figure 4 and demonstrate the effectiveness of Oxyrase® compared to a control group without Oxyrase®. Treatment of 2.4 U/ml Oxyrase® supplemented prior to cryopreservation significantly increased motility over 24 h of

measurements ($P < 0.05$) when compared to control. Other treatments (0.6, 1.2 and 5 U/ml) tended to have a higher motility but were not significant. However, results from the previous study indicated a post thaw (T0) progressive motility at approximately 30%, which is considered fair semen quality and results in an approximate pregnancy rate of 33% [45]. Oxyrase® demonstrated improvement in stallions with <50% progressive motility but still demonstrated a lower fertility.

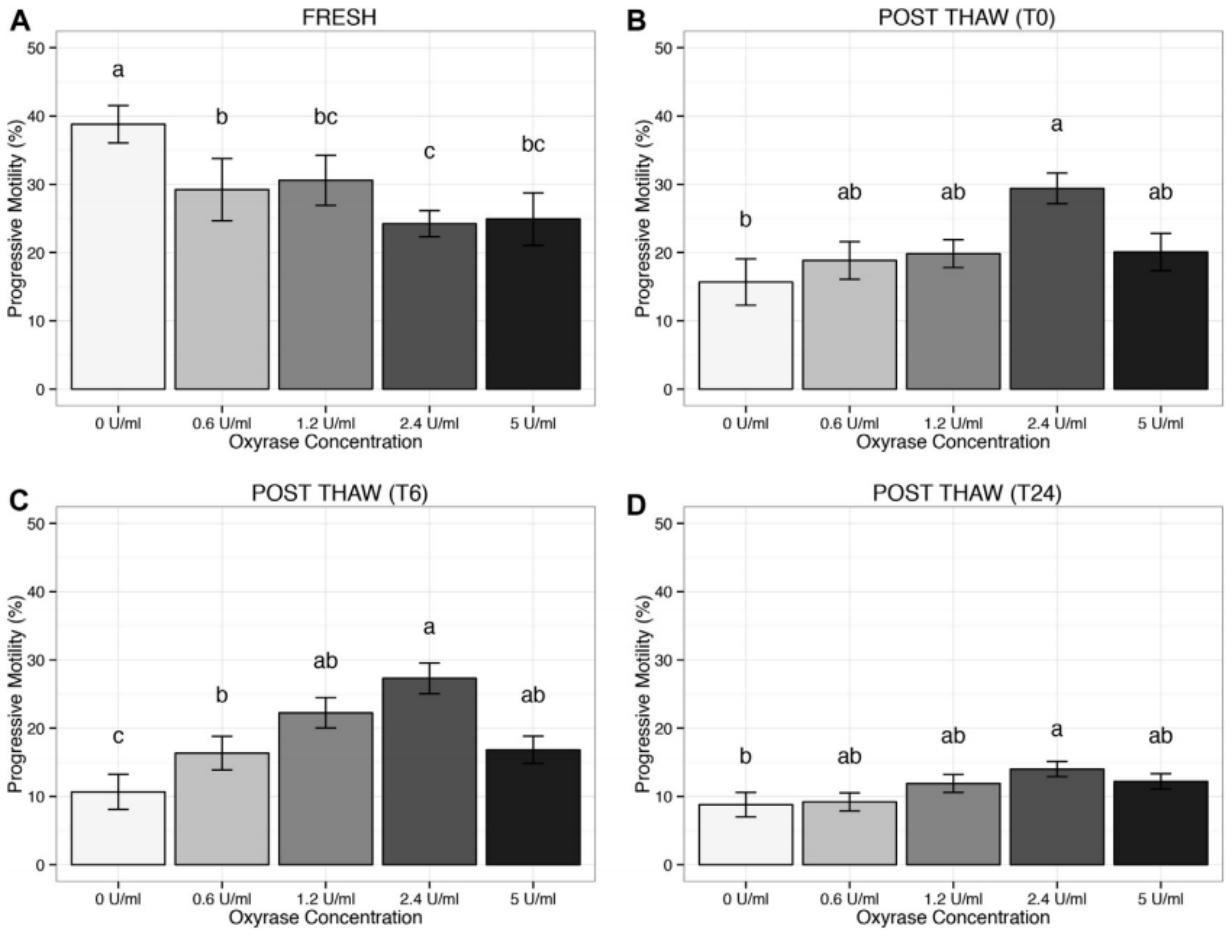

Figure 4. Oxyrase® concentration effect on progressive motility over a 24 hour period post thaw [2].

Contrary to the previously study, London et al. [7] demonstrated Oxyrase® did not have any significant effect improving motility parameters or display any protective effects on the cell membrane viability ($P > 0.05$) [7]. Stallions selected were not of lower progressive motility as used in the Darr et al. [2] study. Stallions displaying a higher rate of fertility are hypothesized to have higher levels of OXPHOS, with oxidative stress as a by-product of this increased level of metabolism [12]. Level of fertility, extender used and variability within stallions selected could explain the contradicting data from both London et al. [7] and Darr et al. [2].

Utilization of Oxyrase® as a supplement for increasing motility and membrane protector should be further studied due to the high variability in current research. Treatment of Oxyrase® has demonstrated effects with cryopreservation. However, there is little research on effects of cooled stallion spermatozoa supplemented with Oxyrase®. Manipulating spermatozoa metabolism with the extender is a technique to increase preservation and function of spermatozoa prior to insemination [3]. Oxidative stress and production of ROS are detrimental to spermatozoa health, thus an extender should be used. Furthermore, the origin of the issue may stem from an inadequate amount of metabolizable substrate, which can lead to an increase in production of ROS and more oxidative stress on the spermatozoa [3].

Pyruvate

Pyruvate is a 2-oxo monocarboxylic acid anion that stems from deprotonation of the carboxylic group of pyruvic acid. Deriving from a propionate, pyruvate's IUPAC ID is 2-oxopropanoic acid with the chemical formula of $C_3H_3O_3$. This compound is a cornerstone molecule crucial to a variety of anabolic and catabolic pathways including: oxidative

metabolism, gluconeogenesis (re-synthesis of glucose), lipogenesis (synthesis of new lipids), cholesterol synthesis, and maintenance/support of citric acid cycle [46].

Major sources of pyruvate are phosphoenolpyruvate via pyruvate kinase (PK), lactate via lactate dehydrogenase (LDH) and alanine via alanine aminotransferase (ALT) [47]. The predominant production of pyruvate comes from the anaerobic breakdown of glucose via glycolysis into two pyruvate molecules. Pyruvate kinase, is the enzyme that takes phosphoenolpyruvate, the final molecule in glycolysis, and breaks it down into the two subsequent molecules of pyruvate (Figure 5a) [46]. Oxidation of lactate is another significant source of pyruvate. Lactate dehydrogenase, the enzymes responsible for the oxidation of pyruvate, produces a significant portion of pyruvate for metabolism (Figure 5b) [46]. One last important source of pyruvate is catabolism of the three-carbon amino acid, alanine, via ALT (Figure 5c) [47]. Within the mitochondrial matrix, ALT is also found to convert alanine, which was transported into the matrix, into pyruvate (Figure 5d) [48]. Other amino acid sources of pyruvate within the cytosol include serine, threonine, glycine, cysteine, and tryptophan [46]. Overall, these enzymes and other molecules contribute to the production of pyruvate within most cells, including spermatozoa.

Pyruvate present in the cytosol is transported into the matrix via mitochondria pyruvate carrier [49]. The mitochondria pyruvate carrier assists as a highly important linkage between the cytosol and mitochondrial pyruvate metabolism [47]. Once pyruvate reaches the matrix, one of two enzymes, pyruvate dehydrogenase complex (PDH) or pyruvate carboxylase (PC) [46], can metabolize it. The majority of the pyruvate is oxidized via PDH to form acetyl-CoA and NADH from NAD^+. Acetyl-CoA is primarily converted to $2CO_2$ and energy created during the process produces NADH and $FADH_2$, which are crucial to the proton gradient required for OXPHOS

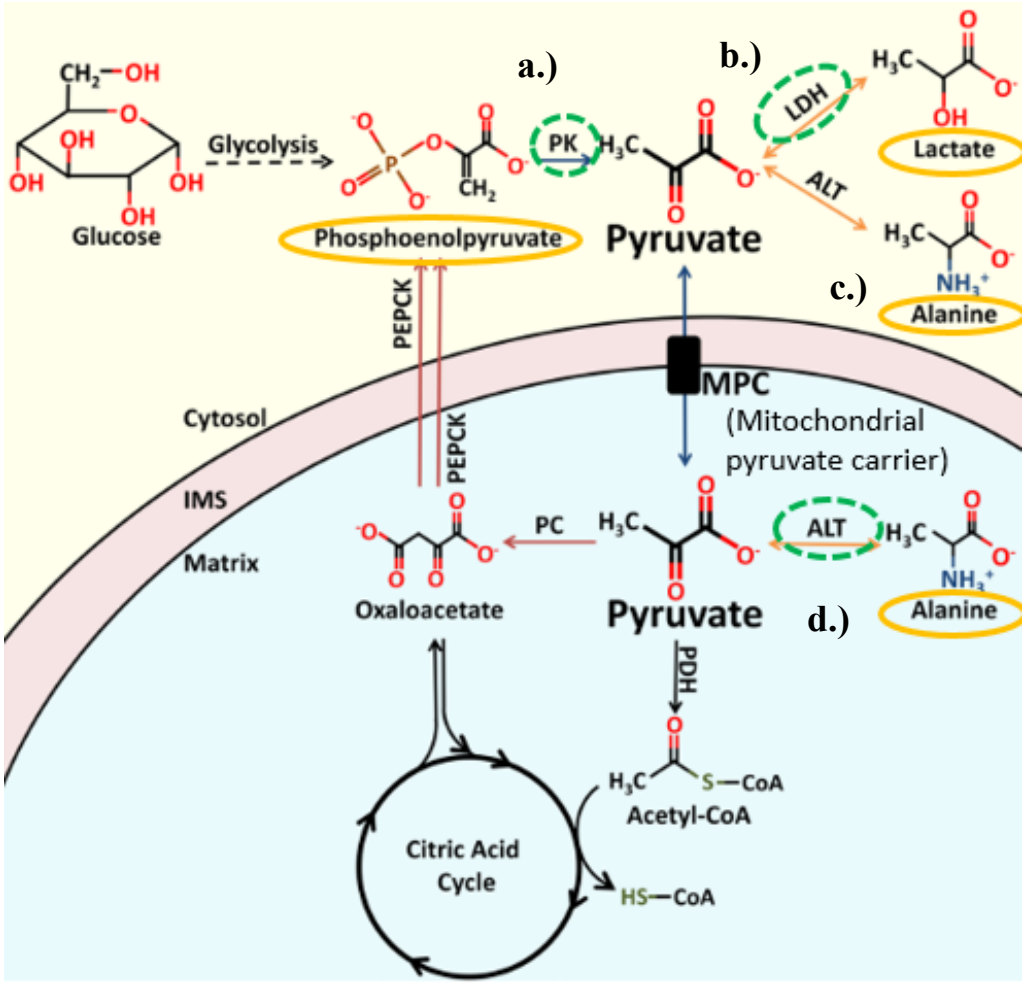

Figure 5. Sources and enzymes involved with the production of pyruvate [47].

and ATP production [46]. Pyruvate carboxylase, the second enzymes acting on pyruvate, generates oxaloacetate and serves as an acceptor for acetyl-CoA. The PC reaction also acts to replenish citric acid cycle intermediates [50].

Research has suggested pyruvate can have possible antioxidant effects on rams, bulls, and stallions [5,6,10,51]. In order to ascertain antioxidant actions of pyruvate in spermatozoa, hydrogen peroxide (H_2O_2) has been used to induce lipid peroxide damage that could potentially be offset by different antioxidants, including pyruvate. In a study on frozen- thaw bull spermatozoa challenged with H_2O_2, the addition of pyruvate or other antioxidants was able to

offset the effects of induced lipid peroxidation from H_2O_2 [5]. However, studies within stallions have demonstrated contradicting results on pyruvates action as an antioxidant. Bruemmer et al. [51] indicated pyruvate to have antioxidant like effects when supplemented into a skim milk diluent that was subjected to cold storage for 48 h. These results indicated a higher motility and velocity from the addition of pyruvate when compared to a treatment without pyruvate [51].

In agreement with the previous research, Cavinder et al. [6] found inclusion of pyruvate to be effective in improving motility in a skim milk extender, as well as, protecting against low levels of 0.1, 0.3, and 0.6 mmol H_2O_2 for up to 72 hours of storage [6]. Furthermore, the previous study had indicated that a level of at least 0.8 mmol of H_2O_2 was needed in order to cause detrimental effects to spermatozoa. A study from Webb and Arns [1] found levels of 0.8 and 1.6 mmol H_2O_2 to a skim milk extender with the addition of pyruvate and lactate did not result in consistent improvement in total motility. Results concluded pyruvate was unable to prevent the damaging effects from H_2O_2, therefore indicating pyruvate serves predominantly as an energy source and not an antioxidant within stallion spermatozoa [1]. Furthermore, an explanation for an increase in the resistance from peroxidation in stallions, when compared to bulls, could be due to a higher concentration of catalase within the seminal plasma as well as the spermatozoa [8].

Potter et al. [9] have recently researched inclusion of pyruvate in popular extenders. Within that study, the extenders SKMG, INRA 96 (IMV Technologies, Maple Grove, MN), and Revolution™ (Santa Cruz Biotechnology, Dallas, TX) extenders were utilized as subjects for pyruvate supplementation. The extender SKMG and Revolution™ extenders tended to benefit from the supplementation of pyruvate. However, a strong statistical significance was not demonstrated within the two. The extender INRA 96 did not show a benefit ($P>0.05$) from the supplemented pyruvate. Within the trial, INRA 96 proved to be the superior extender when

compared to SKMG and Revolution extenders. Although INRA 96 has shown to not benefit from pyruvate supplementation, other popular extenders have benefitted from the addition of the energy substrate [9]. As stated by Potter et al. [9], increasing the sample size could have added to a more significant statistical difference within the extenders [9].

Research by Darr et al. [2] has indicated that pyruvate is a precursor/primary substrate utilized in OXPHOS within spermatozoa mitochondria and could be a more efficient source of energy when compared to glucose. Popular extenders, like SKMG, contain exclusively glucose as the energy source. Levels of mitochondrial oxygen consumption (MITOX) and motility from media containing solely glucose were incapable of maintaining the high levels produced from pyruvate and lactate, which directly contribute to the citric acid cycle [3]. Mitochondrial health has been linked to oxygen consumption and is positively correlated with sperm motility in humans and stallions, indicating pyruvate can support a higher mitochondrial health [2,3,52,53]. Darr et al. [2] suggested, without OXPHOS substrates in the media, glucose could be the main energy source due to breakdown into pyruvate via glycolysis. However, this could likely cause a decrease in mitochondrial function due to lower concentration of pyruvate transport into mitochondria [2]. Results from Darr et al. [2], indicate pyruvate as a more effect supplemental substrate than the popular substrate, glucose. Manipulating the extender to increase more effective energy substrates will allow for better preservation techniques and increased spermatozoa health prior to insemination.

METHODS

Research took place between mid-May and late-June at Missouri State University Darr Agricultural Center in Springfield, MO. Housing, collection of stallions, and mares used within the study were conducted at Pinegar arena. All procedures involving the care, management, and use of equine in this project was approved by the Institutional Animal Care and Use Committee of Missouri State University (IACUC #18-020.0-A). Funding of this project was by Missouri State University Graduate College and Department of Animal Science funds.

Experiment 1

Collection and Processing. In order to stabilize extragonadal sperm reserves, stallions were collected 4 times during the week prior to the beginning of the experiment. During the experiment, three different ejaculates were collected each day on alternating days from four different stallions. Stallions consisted of three American Quarter Horses (1 = Playboy; 3 = Blue; 4 = Dundee) and one miniature horse (2 = Roanie). Ejaculates were attained via a Missouri artificial vagina (AV) lubricated with Priority Care® (First Priority, Inc., Elgin, IL), a non-spermicidal sterile lubricating jelly. Collection bottles were attached to the end of the AV and warmed to 35°C prior to ejaculation into the bottle. Filters were placed in the collection bottle to separate out gel portion of ejaculate. Stallions were teased by mares in estrus, if needed, and their sheath cleaned prior to mounting of the phantom and collection. Once collected, 180 ul of the ejaculate was pipetted into a 4 ml plastic cuvette containing formalin 10 solution. Cuvette was then inverted and placed in a 590a Densimeter (Animal Reproduction Systems, Chino, CA) for analysis of volume (ml) and concentration (million spermatozoa per ml).

Treatments. Aliquots of semen were then divided into four 15 ml Falcon™ (Fisher Scientific, Germany) test tubes. Two of the four tubes were centrifuged at 350 g-force for two to four minutes or until consistency of a sperm pellet was attained. The base extender utilized during experiment one consisted of the commercially available INRA 96 (IMV Technologies, Maple Grove, MN). A factorial design was implemented in which two aliquots were centrifuged and two had the INRA 96 supplemented with Oxyrase® at a level of 2.4 U/ml such that there were four combinations which served as treatments for experiment 1 (Table 1.). The supplemental amount of Oxyrase® was based off of the results from the study by Darr et al. [2].

Table 1. Centrifugation and Oxyrase® treatments for experiment one.

	INRA96			
	$OxySP^-$	$OxySP^+$	SP^-	SP^+
Centrifugation	+	-	+	-
Oxyrase®	2.4 U/ml	2.4 U/ml	0	0

Extenders were premade, froze prior to collection, and thawed reaching a temperature between 30°C to 35°C before suspension to ensure spermatozoa did not undergo cold shock. Once thawed, treatment $OxySP^-$ and SP^- were suspended into centrifuged semen, while the remaining treatments ($OxySP^+$ and SP^+) were suspended into non-centrifuged semen. Aliquots for each treatment were diluted at ratios (extender: semen) of 3:1 to 5:1 to insure concentrations between 20 and 40 X 10^6 sperm per ml of diluted semen after centrifugation. Aliquots of each treatment were then divide into three Fisherbrand® (Fisher Scientific, Germany) microcentrifuge 1.5 ml tubes. The three microcentrifuge tubes from each treatment were allocated into three different Equine Express II™ (Exodus Breeders, York, PA) shipping containers for analysis after

2, 24 and 48 h of storage. Once shipping containers had the allocated microcentrifuged tubes, a Thermosafe® Polarpack® (Sonoco, Hartsville, SC) was placed inside the container for cooling and storage in the lab at approximately 22°C. Cooling packs were replaced daily to maintain a temperature of approximately 5°C. Each container was analyzed via computer assisted sperm analysis (CASA; Hamilton Thorne Biociences, Beverly, MA).

CASA. Inversion of each treatment was performed by hand prior to analysis to insure proper mixing. Samples were placed on a 4 chamber, 20 micron Leja® (IMV Technologies, Netherlands) slide via a micropipette. Slide were placed on a slide warmer at 35°C for five minutes to ensure cooling effects were not present and spermatozoa had full motility. Chambers were analyzed individually on a microscope (Olympus, CX41, Japan). Within each chamber, 300 – 400 sperm cells were analyzed for parameters measured by CASA. The parameters measured were total motility (TM), progressive motility (PM), average path velocity (VAP), straight-line velocity (VSL), curvilinear velocity (VCL), and elongation (ELONG). Total motility is defined as the population of cells that are moving at or above a predetermined minimum speed. Progressive motility are cells moving with a predetermined speed and straightness. Average path velocity is recorded as the average velocity of the smoothed cell path in microns/second. Straight-line velocity is the average velocity measured in a straight line from the beginning to the end of track. Curvilinear velocity is the average velocity measured over the actual point to point track followed by the cell. Elongations is determined by the average value of the ratio of minor and major axis of all sperm heads. The following instrument settings were used for CASA analysis: frame rate, 60 Hz; number of frames acquired, 45; minimum contrast, 70; minimum cell size, 5; motile VAP cutoff, 15.0 μ/s; motile VSL cutoff, 30.0 μ/s; progressive VAP, 30.0 μ/s; progressive STR, 50.0%.

Statistics. The general linear model procedure of ANOVA was used in Minitab 18 (Minitab Inc., State College, PA) for treatment analysis. Within ANOVA, the model consisted of stallion and treatment split by time. Tukey's pairwise comparisons analyzed stallion and treatment effects.

Experiment 2

Collection and Processing. For experiment 2, three ejaculates were collected from each of three stallions. Two stallions were American Quarter Horses (1 = Playboy; 3 = Blue) and one stallion was a miniature horse (2 = Roanie). Ejaculates were collected and processed using the same procedures as experiment 1, excluding the centrifugation procedure. All ejaculates collected for experiment 2 were centrifuged at 350 g-force for two to four minutes or until desired effect from centrifuge was attained. Once processed, aliquots of each ejaculate were divided into five treatments allocated in 15 ml Falcon™ test tubes.

Treatments. Formulas for each treatment are listed in Table 2. Control treatments consisted of the formulas similar to commercially used extenders, (SKMG=S) the Modified Kenney Extender (Exodus Breeders, York, PA), containing 2.4 g of glucose and 4.0 g of sucrose, and (SKMG) Next Generation® Kenney Extender, which contains 4.9 g of glucose. In order to determine if some of the glucose could be replaced by pyruvate as an energy source, remaining treatments were supplemented in the Kenney Extender (SKMG) and had a reduced glucose amount of 50% from the original 4.9 g to 2.4 g within the extender. Treatments PYR2, PYR4, and PYR8 were supplemented with increasing amounts of sodium pyruvate, 0.2 g, 0.4 g, and 0.8 g, respectively. Each extender was diluted into 92 ml/dl of BioWhittaker™ deionized water (Cambrex Bio Science, Walkersville, MD). Antibiotics supplemented into each dilution included

amikacin sulfate 1 g/dl and potassium penicillin 1×10^6 units/dl (KPenn; purchased from reproductive resources). The optimal range for extender osmolality is between 300 and 400 mOsm/L [54]. Osmolality was adjusted by supplemented sodium chloride (NaCl) in treatments PYR2 and PYR4 to equal that of the SKMG, PYR 8, and SKMG+S treatments (350 mOsm/L). Each ejaculate made five separate treatments that followed the processing protocol, spermatozoa parameter analysis, and statistical analysis listed in experiment. Treatment formulas with differing amounts of pyruvate substituted in the SKMG extender were based off of results from a study by Darr et al. [2].

Table 2. Formulas for semen extenders tested in experiment two.

	Treatments				
	SKMG+S	SKMG	PYR2	PYR4	PYR8
Skim Milk Powder	5.0 g	5.0 g	5.0 g	5.0 g	5.0 g
Glucose	2.4 g	4.9 g	2.4 g	2.4 g	2.4 g
Sucrose	4.0 g	0	0	0	0
Na Pyruvate	0	0	0.2 g	0.4 g	0.8 g
Deionized Water	92 ml	92 ml	92 ml	92 ml	92 ml
*Antibiotics	+	+	+	+	+
**NaCl	-	-	+	+	-

SKMG+S = Kenney Extender (addition of sucrose); SKMG = Skim Milk Glucose; PYR2 = 0.2 g pyruvate; PYR4 = 0.4 g pyruvate; PYR8 = 0.8 g pyruvate
*Antibiotics = Amikacin sulfate 1 g/dl and potassium penicillin 1×10^6 units/dl (KPenn)
**Added sufficient amount of salt to PYR2 and PYR4 in order to achieve osmality equal to all other extenders

RESULTS

Experiment 1

Mean values ± standard error were recorded for Oxyrase® treated aliquots compared to untreated aliquots assessed by CASA after 2 hours, 24 hours, and 48 hours of storage post collection. Results for all treatments for TM and PM are listed in Table 3, while the effects on VAP, VSL, VCL, and ELONG are displayed in Table 4. Pairwise comparisons were made via a Tukey test and results from each were recorded in Tables 5 – 7. Comparisons of stallions 1, 2, 3, and 4 were analyzed for all sperm parameters at 2 hours, 24 hours, and 48 hours and recorded on Tables 5, 6, and 7, respectively.

When all treatments were compared, a difference ($P<0.05$) was found in parameters VAP and VCL with Oxyrase® supplemented treatments at 2 hours. No difference ($P>0.05$) was found at 24 and 48 hours. However, contrary to results reported by Darr et al [2], throughout the experiment Oxyrase® treatments tended to displayed lower motility and velocity percentages when compared to treatments without Oxyrase®. In addition, numerical differences were found among centrifuged and not centrifuged treatments, indicating centrifugation optimized spermatozoa survivability, which is in agreement with previous research [38,54].

In comparison, stallions displayed several significant effects ($P<0.05$) on sperm motility parameters. Total motility analyzed at 2 hours, 24 hours, and 48 hours, stallion 2 had a lower motility ($P<0.05$) than that of stallions 1 and 3, while being numerically lower from stallion 4. Progressive motility within stallion 2 also showed numerically lower results at all times when compared to stallion 1, 3, and 4. However, a lower PM ($P<0.05$) was recorded at 48 h compared to stallion 1.

Table 3. Effects of Oxyrase® supplementation of extenders on motility of stallion spermatozoa centrifuged and non-centrifuged and stored at 5 ℃ (experiment 1).

	Treatments			
	Centrifuged		Not Centrifuged	
	Oxyrase® (+)	Oxyrase® (-)	Oxyrase® (+)	Oxyrase® (-)
TM				
2h	45.9±5.0[a]	54.2±4.6[a]	45.9±6.1[a]	48.8±4.7[a]
24h	44.4±6.7[a]	50.8±5.2[a]	37.7±6.4[a]	45.3±4.8[a]
48h	44.4±6.3[a]	50.5±4.5[a]	40.8±6.8[a]	44.6±5.3[a]
PM				
2h	30.1±3.7[a]	30.5±4.1[a]	25.9±3.8[a]	25.5±3.6[a]
24h	27.3±4.3[a]	26.8±3.1[a]	21.1±3.4[a]	21.6±3.3[a]
48h	28.2±3.8[a]	22.3±2.6[a]	18.3±3.7[a]	19.0±3.7[a]

[a, b] Row means (± Standard Error) with different superscripts are significantly different ($P<0.05$, Tukey's pairwise comparisons). Row and column means (± Standard Error) demonstrate percent Total Motility (TM) and Progressive Motility (PM) of each treatment analyzed via Computer Assisted Sperm Analysis (CASA) at 2 hours, 24 hours, and 48 hours.

Table 4. Effects of Oxyrase® supplementation of extenders on velocity and elongation of stallion spermatozoa centrifuged and non-centrifuged and stored at 5 °C (experiment 1).

	Treatments			
	Centrifuged		Not Centrifuged	
	Oxyrase® (+)	Oxyrase® (-)	Oxyrase® (+)	Oxyrase® (-)
VAP				
2h	96.2±6.2[b]	113.5±5.2[a]	99.4±5.0[ab]	112.1±4.2[a]
24h	104.3±4.4[a]	113.0±5.2[a]	103.0±4.6[a]	114.9±4.6[a]
48h	100.2±3.5[a]	103.4±4.0[a]	107.9±5.2[a]	114.4±5.6[a]
VSL				
2h	76.3±4.7[a]	85.6±4.1[a]	78.0±3.8[a]	85.7±4.2[a]
24h	80.8±3.1[a]	84.9±4.3[a]	79.7±3.0[a]	83.6±3.6[a]
48h	78.5±2.0[a]	73.6±2.0[a]	80.7±3.7[a]	80.8±4.7[a]
VCL				
2h	182.3±11.8[b]	208.7±12.7[a]	177.1±9.0[b]	194.5±9.5[ab]
24h	203.5±7.8[a]	210.5±9.3[a]	190.5±9.7[a]	210.4±12.1[a]
48h	185.7±16.7[a]	203.9±7.7[a]	210.5±10.4[a]	218.6±12.5[a]
ELONG				
2h	64.3±1.2[a]	65.4±1.4[a]	65.8±1.2[a]	64.3±1.0[a]
24h	64.8±1.0[a]	64.9±1.2[a]	66.1±1.2[a]	64.2±1.3[a]
48h	64.3±1.0[a]	64.9±1.0[a]	63.7±1.4[a]	64.5±1.3[a]

[a, b] Row means (± Standard Error) with different superscripts are significantly different ($P<0.05$, Tukey's pairwise comparisons). Row and column means (± Standard Error) demonstrate percent average path velocity (VAP), straight-line velocity (VSL), curvilinear velocity (VCL), and elongation (ELONG) of each treatment analyzed via Computer Assisted Sperm Analysis (CASA) at 2 hours, 24 hours, and 48 hours.

Table 5. Tukey's comparisons of stallion effect on sperm motility, velocity, and elongation at 2 hours post collection (experiment 1).

	Stallion			
	1	2	3	4
TM	59.6±3.0[a]	33.5±3.0[b]	51.4±6.5[a]	50.3±4.2[ab]
PM	32.1±2.4[a]	20.0±1.5[a]	31.3±5.8[a]	28.6±3.2[a]
VAP	98.3±3.5[b]	94.5±2.6[b]	102.8±6.6[b]	125.6±3.5[a]
VSL	74.2±1.5[b]	75.1±1.3[b]	80.4±5.1[b]	95.9±4.5[a]
VCL	168.2±4.1[b]	167.2±3.7[b]	187.7±11.5[b]	239.5±7.0[a]
ELONG	67.6±1.0[a]	65.9±1.0[a]	65.8±1.0[a]	60.1±1.0[b]

[a, b] Row means (± Standard Error) with different superscripts are significantly different ($P<0.05$). Sperm parameters total motility (TM), progressive motility (PM), average path velocity (VAP), straight-line velocity (VSL), curvilinear velocity (VCL), and elongation (ELONG) were measured via Computer Assisted Sperm Analysis System (CASA).

Table 6. Tukey's comparisons of stallion effect on sperm motility, velocity, and elongation after 24 hours of storage at 5 °C (experiment 1).

	Stallion			
	1	2	3	4
TM	58.4±3.6[a]	28.6±3.8[c]	52.1±6.9[ab]	39.0±4.5[bc]
PM	27.7±2.2[a]	17.8±2.0[a]	26.7±5.0[a]	24.7±3.7[a]
VAP	105.0±3.1[b]	96.3±2.6[b]	110.0±4.6[ab]	124.0±5.0[a]
VSL	77.4±1.2[b]	75.8±1.3[b]	82.3±3.3[ab]	93.5±4.5[a]
VCL	182.4±2.9[b]	176.7±5.2[b]	220.1±8.5[a]	235.8±8.9[a]
ELONG	67.4±0.3[a]	66.9±0.5[a]	65.7±1.0[a]	60.0±1.1[b]

[a, b, c] Row means (± Standard Error) with different superscripts are significantly different ($P<0.05$). Sperm parameters total motility (TM), progressive motility (PM), average path velocity (VAP), straight-line velocity (VSL), curvilinear velocity (VCL), and elongation (ELONG) were measured via Computer Assisted Sperm Analysis System (CASA).

Table 7. Tukey's comparisons of stallion effect on sperm motility, velocity, and elongation after 48 hours of storage at 5 ℃ (experiment 1).

	Stallion			
	1	2	3	4
TM	60.4±2.5[a]	27.4±4.7[b]	48.8±5.9[a]	43.7±5.0[ab]
PM	25.7±2.3[a]	13.4±2.6[b]	23.7±4.6[ab]	25.1±3.5[ab]
VAP	106.2±2.9[a]	99.2±2.9[a]	107.5±3.3[a]	113.0±7.8[a]
VSL	76.5±1.0[a]	74.3±1.1[a]	77.1±1.1[a]	85.7±6.0[a]
VCL	197.5±4.2[a]	190.0±6.2[a]	214.3±18.2[a]	216.9±15.0[a]
ELONG	66.1±1.0[a]	65.1±1.0[a]	65.2±1.0[a]	61.1±1.3[b]

[a, b] Row means (± Standard Error) with different superscripts are significantly different ($P<0.05$). Sperm parameters total motility (TM), progressive motility (PM), average path velocity (VAP), straight-line velocity (VSL), curvilinear velocity (VCL), and elongation (ELONG) were measured via Computer Assisted Sperm Analysis System (CASA).

Experiment 2

Mean values ± standard error were recorded for pyruvate treated aliquots compared to untreated aliquots assessed by CASA at 2 hours, 24 hours, and 48 hours post collection. Results for all treatments for TM and PM are listed in Table 8, while the effects on VAP, VSL, VCL, and ELONG are displayed in Table 9. Pairwise comparisons were made via a Tukey test and results from each were recorded in Tables 10 – 12. Comparisons of stallions 1, 2, and 3 were analyzed for all sperm parameters at 2 hours, 24 hours, and 48 hours and recorded on Tables 10, 11, and 12, respectively.

Differences ($P<0.05$) were observed throughout experiment 2 at 2 hours, 24 hours, and 48 hours. The SKMG+S extender, while not always significant, numerically was generally higher than all other extenders within the experiment. Along with the SKMG+S extender, SKMG, when compared to all pyruvate treatments, numerically always resulted in higher spermatozoa parameter measurements. Within the pyruvate treatments, few statistical differences were found and showed little variation between substituted amounts of pyruvate, especially at 24 hours and 48 hours. Results gathered in this experiment displayed the commercially available extenders were superior to the SKMG extender with substituted pyruvate.

As in experiment 1, a stallion effect was present. Stallion 1 spermatozoa showed higher ($P<0.05$) TM at 24 hours and PM at 24 and 48 hours than stallion 3. Numerically, stallion 1 spermatozoa displayed a higher TM and PM at 2 hours. Both stallion 1 and 3 were more motile ($P<0.05$) in TM and PM than stallion 2 at all times recorded. In addition, stallion 3, at 2 hours, exhibited a lower ($P<0.05$) VCL when compared to stallion 1.

Table 8. Effect of pyruvate substitution for glucose in skim milk extenders on motility of stallion spermatozoa stored at 5 ℃ (experiment 2).

	Treatments				
	SKMG+S	SKMG	PYR2	PYR4	PYR8
TM					
2h	87.6±4.2[a]	86.7±4.6[a]	85.8±4.4[a]	65.6±9.5[b]	77.0±5.7[ab]
24h	83.33±4.1[a]	82.4±4.8[a]	63.2±9.0[ab]	58.3±8.7[b]	58.0±9.1[b]
48h	78.4±6.0[a]	73.6±7.3[a]	52.3±12.6[ab]	55.2±9.2[ab]	43.9±12.4[b]
PM					
2h	58.9±4.3[a]	61.2±4.5[a]	52.3±5.1[ab]	38.7±7.9[b]	48.6±6.1[ab]
24h	54.8±4.8[a]	47.4±5.7[a]	25.4±5.2[b]	27.4±6.1[b]	23.7±5.8[b]
48h	45.8±6.1[a]	37.4±6.2[ab]	19.6±5.5[c]	21.4±4.7[c]	16.4±6.4[c]

[a, b] Row means (± Standard Error) with different superscripts are significantly different ($P<0.05$, Tukey's pairwise comparisons). Row and column means (± Standard Error) demonstrate percent Total Motility (TM) and Progressive Motility (PM) of each treatment analyzed via Computer Assisted Sperm Analysis (CASA) at 2 hours, 24 hours, and 48 hours. SKMG+S = Kenney Extender (addition of sucrose); SKMG = Skim Milk Glucose Extender; PYR2, PYR4, and PYR8 = 0.2 g, 0.4 g, and 0.8 g of pyruvate, respectively.

Table 9. Effect of pyruvate substitution for glucose in skim milk extenders on velocity and elongation of stallion spermatozoa stored at 5 ℃ (experiment 2).

	Treatments				
	SKMG+S	SKMG	PYR2	PYR4	PYR8
VAP					
2h	140.4±4.4[a]	132.5±5.8[ab]	117.2±4.2[bc]	71.4±7.3[d]	96.3±4.8[c]
24h	130.3±7.0[ab]	133.1±7.0[a]	108.5±5.7[bc]	75.9±4.9[d]	94.3±5.6[cd]
48h	142.0±6.1[a]	141.2±4.1[a]	98.5±19.1[b]	78.0±7.3[b]	89.5±8.6[b]
VSL					
2h	106.1±3.1[a]	107.7±4.4[a]	94.3±5.3[ab]	63.0±6.2[c]	77.1±4.3[bc]
24h	92.5±3.9[a]	94.1±5.8[a]	73.5±4.8[b]	60.2±3.8[b]	65.4±3.5[b]
48h	96.3±4.3[a]	96.6±3.5[a]	66.3±13.1[b]	60.0±4.7[b]	65.9±4.6[b]
VCL					
2h	244.2±9.0[a]	209.7±9.9[ab]	194.8±7.5[bc]	119.7±11.3[d]	171.4±9.6[c]
24h	236.5±15.1[a]	228.5±12.5[ab]	213.6±10.5[ab]	130.4±9.1[c]	185.3±9.5[b]
48h	258.4±8.9[a]	247.3±7.0[ab]	183.1±35.3[bc]	139.7±11.8[c]	164.0±19.3[c]
ELONG					
2h	61.0±1.0[b]	61.3±1.0[b]	61.9±1.0[b]	65.9±1.0[a]	61.9±1.0[b]
24h	60.8±1.0[ab]	61.6±1.0[ab]	61.1±1.0[ab]	64.9±1.0[a]	60.4±1.3[b]
48h	61.3±1.2[a]	61.6±1.0[a]	46.6±8.3[b]	64.3±1.0[a]	55.0±3.4[ab]

[a, b] Row means (± Standard Error) with different superscripts are significantly different ($P<0.05$, Tukey's pairwise comparisons). Row and column means (± Standard Error) demonstrate percent Total Motility (TM) and Progressive Motility (PM) of each treatment analyzed via Computer Assisted Sperm Analysis (CASA) at 2 hours, 24 hours, and 48 hours. SKMG+S = Kenney Extender (addition of sucrose); SKMG = Skim Milk Glucose Extender; PYR2, PYR4, and PYR8 = 0.2 g, 0.4 g, and 0.8 g of pyruvate, respectively.

Table 10. Tukey's comparisons of stallion effect on sperm motility, velocity, and elongation at 2 hours post collection (experiment 2).

	Stallion		
	1	2	3
TM	92.2±1.7[a]	63.9±5.8[b]	85.4±3.1[a]
PM	62.4±2.6[a]	39.6±5.0[b]	53.8±4.5[a]
VAP	118.7±7.9[a]	106.9±8.2[a]	109.1±7.0[a]
VSL	93.8±6.0[a]	85.1±5.5[a]	90.0±5.8[a]
VCL	204.7±12.1[a]	182.2±515.4[ab]	177.0±10.6[b]
ELONG	61.2±0.7[b]	63.3±0.8[a]	62.7±0.7[ab]

[a, b] Row means (± Standard Error) with different superscripts are significantly different (P<0.05). Sperm parameters total motility (TM), progressive motility (PM), average path velocity (VAP), straight-line velocity (VSL), curvilinear velocity (VCL), and elongation (ELONG) were measured via Computer Assisted Sperm Analysis System (CASA).

Table 11. Tukey's comparisons of stallion effect on sperm motility, velocity, and elongation after 24 hours of storage at 5 °C (experiment 2).

	Stallion		
	1	2	3
TM	86.8±2.7[a]	48.5±5.0[c]	71.9±5.4[b]
PM	48.6±4.7[a]	23.1±4.6[c]	35.5±4.7[b]
VAP	114.4±6.8[a]	108.6±9.2[a]	102.3±5.3[a]
VSL	83.7±5.5[a]	74.2±5.2[a]	73.5±3.6[a]
VCL	202.9±11.3[a]	204.7±17.1[a]	188.8±10.6[a]
ELONG	62.1±0.9[a]	61.6±1.0[a]	61.5±0.8[a]

[a, b, c] Row means (± Standard Error) with different superscripts are significantly different (P<0.05). Sperm parameters total motility (TM), progressive motility (PM), average path velocity (VAP), straight-line velocity (VSL), curvilinear velocity (VCL), and elongation (ELONG) were measured via Computer Assisted Sperm Analysis System (CASA).

Table 12. Tukey's comparisons of stallion effect on sperm motility, velocity, and elongation after 48 hours of storage at 5 ℃ (experiment 2).

	Stallion		
	1	2	3
TM	81.3±6.2[a]	32.5±6.9[b]	68.3±5.1[a]
PM	42.0±5.2[a]	14.9±4.5[c]	27.5±3.5[b]
VAP	114.3±8.6[a]	96.3±13.3[a]	119.0±8.3[a]
VSL	80.4±5.8[a]	68.3±8.5[a]	82.4±5.0[a]
VCL	207.4±15.3[a]	171.1±23.9[a]	217.0±15.0[a]
ELONG	60.8±0.8[a]	50.9±5.8[a]	59.8±1.3[a]

[a, b] Row means (± Standard Error) with different superscripts are significantly different ($P<0.05$). Sperm parameters total motility (TM), progressive motility (PM), average path velocity (VAP), straight-line velocity (VSL), curvilinear velocity (VCL), and elongation (ELONG) were measured via Computer Assisted Sperm Analysis System (CASA).

DISCUSSION

Experiment 1

Previous studies have established contradicting effects from addition of Oxyrase® to diluents used for the preservation of stallion semen. Varying results are possibly due to the procedures used and/or differences in the stallions used in the studies. In a study that observed a positive effect from Oxyrase®, stallions with spermatozoa that had initial progressive motility lower than 50% were used. In contrast a second study found no advantage when Oxyrase® was added to extenders used to freeze semen from stallions that displayed an original motility >70%. The results of the latter study are similar to those from the current study, in which stallion spermatozoa were challenged by centrifugation and storage at 5ºC for 48 hours.

Variability between the semen of different stallions is a well-known issue and contributes as a major factor in results from treatments [55]. Dairy bulls, for example, have been continually selected based on spermatozoa ability to withstand the stress of a standard cryopreservation protocol. Subsequently, this has led to a positive response in the amount of bulls that can withstand a specific protocol [55]. Stallions, however, are not as uniform to a specific protocol as dairy bulls are. Selection criteria is much different for stallions and results in a wide variation in spermatozoa response to cooling or freezing protocols. Examining Tables 5, 6, and 7, stallion 2, which was used in both experiments, was lower ($P<0.05$) in TM than stallion 1 and 3, and numerically lower than stallion 4 at all experiment times recorded. The response from stallion 2 was different ($P<0.05$) than that of 1, 3, and 4, showing one extender may work better for one stallion than the other. A stallion effect was present ($P<0.01$) at all times for TM and ELG. Velocity measurements were different ($P<0.05$) at 2 and 24 h, but not at 48 h, while differences

($P<0.05$) in PM were only demonstrated at 48 h. Evidence of a stallion effect has demonstrated variability within the experiment and could be improved with a larger sample size to better represent results from each extender.

Tukey's comparisons within experiment 1 displayed a numerical difference between centrifuged treatments and not centrifuged treatments. Treatments that were centrifuged tended to have higher TM and PM when compared to treatments without centrifugation. This is in agreement with literature stating ejaculates that are centrifuged tend to have higher motility than those not centrifuged [1,38,54,56].

Experiment 2

In experiment 2, a stallion effect was present. Stallion 2 showed lower ($P<0.01$) TM and PM at all times measured when compared to stallion 1 and 3. Results were similar between both experiments, in which stallion 2 demonstrated lower motility than the other stallions within the experiments. As mentioned above, this lack of sample size increases variation within stallions and could affect the statistical differences within the experiment. However, it is important to test experimental procedures across stallions that are known to have lower post storage motility as these are common in the industry.

The diluents with formulas which are commonly used, SKMG+S and SKMG demonstrated numerically higher TM and PM than extenders in which pyruvate was substituted for glucose as an energy source. At 2 hours, a significantly ($P<0.05$) lower TM and PM was revealed in the extender PYR4. Analysis at 24 hours resulted in all pyruvate extenders but PYR2 to be significantly ($P<0.05$) lower than that of SKMG+S and SKMG. Finally, analysis at 48

hours demonstrated similar results suggesting replacement of glucose with pyruvate was not beneficial.

Contrary to results from Bruemmer et al. [51], Potter et al. [9], Cavinder et al. [6], and Darr et al. [2], the skim milk extenders with pyruvate did not tend to show any benefit from its inclusion compared to commonly used extender formulas. Furthermore, increasing the concentration of pyruvate in the diluent has also been suggested to result in an increase of motility, due to the more direct metabolism of pyruvate compared to glucose [2]. In agreement with results from Webb and Arns [1], inclusion and increasing concentrations of pyruvate did not improve motility of the supplemented aliquots. Conversely, the highest amount of pyruvate substituted was in PYR8 and at 48 hours, numerically, the extender had the lowest percent TM and PM. However it should be mentioned, that an increase in sample size may potentially lead to statistically different results across treatments.

Limitations

Spermatozoa are highly sensitive to the suspended environment and protocols implicated in the handling of each ejaculate. The reduction of error, such as operator error and environmental effects, can be attained by organized procedures and precise execution of such procedures. In agreement with previous research, stallion effects are a continuous difficult factor to adjust due to the nature of variability within different stallions. Diminishing variability could be achieved by executing precise procedures, increasing the sample size, and increasing the number of ejaculates collected, insuring no ejaculate effect could hinder results within different stallions. Increasing the stallion sample size could possibly result in an increase in the significance level within this current study.

CONCLUSIONS

Previous research has indicated that Oxyrase® may be beneficial when included in a cryopreservation protocol. However in this study, no significant differences were shown when EC-Oxyrase® was added to INRA 96 with or without centrifugation to remove the majority of the seminal plasma. Based on these results it was determined that supplementation of EC-Oxyrase® may not warrant the additional cost to include the antioxidant in diluents for cold storage of stallion spermatozoa. However, in agreement with previous research, results revealed a positive effect of seminal plasma removal by centrifugation. Centrifuged ejaculates, numerically, had a higher percent motility than ejaculates not centrifuged; thus, increasing data for beneficial effects of centrifugation to stallion semen before suspension in extender for some stallions.

Inclusion of pyruvate has been suggested by previous research to benefit stallion spermatozoa suspended in extenders for transportation with the intent of artificial insemination. However, potentially due to lack of sample size, handling/procedures and stallion effect, results from this study did not conclude substitution of pyruvate for glucose, in formulas similar to commercially available ones, was beneficial in increasing motility of spermatozoa. Due to the differences in stallions, an increase in the number of stallions collected would be beneficial. Certain stallions may respond better to certain extenders than others. Increasing the sample size will better represent the response from the general stallion population.

In the current equine market, sire selection is based on pedigree, performance record and conformation. Fertility should be considered along with athletic and performance characteristics of young stallions. Reproductive traits in stallions are heritable [57]. In addition, per cycle

conception within stallions, specifically thoroughbreds, is variable, ranging anywhere from 35% to 90% [58]. Variability has also been demonstrated in this study and can hinder results from an experiment with a smaller sample size.

More studies are necessary to determine the inclusion effects of the addition of energy substrates and antioxidants similar to and including pyruvate and Oxyrase®, respectively. The development of precise techniques and extenders will aid in alleviating error within spermatozoa preservation; this can prolong the capability of stallion spermatozoa to remain motile after cold storage. Thus, increasing the number of stallions available for breeding and access to superior genetics, while also increasing effectiveness of each ejaculate allowing for more inseminations and less shipments required to attain pregnancy.

REFERENCES

[1] Webb GW, Arns MJ. Effect of pyruvate and lactate on motility of cold stored stallion spermatozoa challenged by hydrogen peroxide. J Equine Vet Sci 2006;26:406–11.

[2] Darr CR, Varner DD, Teague S, Cortopassi GA, Datta S, Meyers SA. Lactate and pyruvate are major sources of energy for stallion sperm with dose effects on mitochondrial function, motility, and ROS production. Biol Reprod 2016;95:34–34.

[3] Darr CR, Cortopassi GA, Datta S, Varner DD, Meyers SA. Mitochondrial oxygen consumption is a unique indicator of stallion spermatozoal health and varies with cryopreservation media. Theriogenology 2016;86:1382–92.

[4] Aurich C. Recent advances in cooled-semen technology. Anim Reprod Sci 2008;107:268–75.

[5] Bilodeau JF, Blanchette S, Cormier N, Sirard MA. Reactive oxygen species-mediated loss of bovine sperm motility in egg yolk Tris extender: Protection by pyruvate, metal chelators and bovine liver or oviductal fluid catalase. Theriogenology 2002;57:1105–22.

[6] Cavinder CA, Harrison K, Webb GW. Antioxidant effects of pyru- vate as a component of extenders for stallion semen. Proc Equine Nutr Physiol Soc Symp; 2003:262–263.

[7] London KT, Christensen BW, Scott CJ, Klooster K, Kass PH, Dujovne GA, et al. The effects of an oxygen scavenger and coconut water on equine sperm cryopreservation. J Equine Vet Sci 2017;58:51–7.

[8] Ball B, Medina V, Gravance C, Baumber J. Effect of antioxidants on preservation of motility, viability and acrosomal integrity of equine spermatozoa during storage at 5°C. Theriogenology 2001;56:577–89.

[9] Potter DS, Webb GW, Onyango B, Northcutt T. 134 l-Carnitine and pyruvate inclusion in diluents for cold-stored stallion spermatozoa. J Equine Vet Sci 2015;35:440.

[10] Upreti GC, Jensen K, Munday R, Duganzich DM, Vishwanath R, Smith JF. Studies on aromatic amino acid oxidase activity in ram spermatozoa: Role of pyruvate as an antioxidant. Anim Reprod Sci 1998;51:275–87.

[11] Waheed MM, El-Bahr SM, Al-haider AK. Influence of seminal plasma antioxidants and osteopontin on fertility of the Arabian horse. J Equine Vet Sci 2013;33:705–9.

[12] Gibb Z, Lambourne SR, Aitken RJ. The paradoxical relationship between stallion fertility and oxidative stress. Biol Reprod 2014;91:1–10.

[13] Mazur P, Katkov II, Katkova N, Critser JK. The enhancement of the ability of mouse sperm to survive freezing and thawing by the use of high concentrations of glycerol and the presence of an Escherichia coli membrane preparation (Oxyrase) to lower the oxygen con- centration. Cryobiology 2000;40:187–209.

[14] Dong QX, Tollner TL, Rodenburg SE, Hill DL, VandeVoort CA. Anti- oxidants, Oxyrase, and mitochondrial uncoupler 2,4-dinitrophenol improved post thaw survival of rhesus monkey sperm from ejaculates with low cryosurvival. Fertil Steril 2010;94:2359–61.

[15] Borg CL, Wolski KM, Gibbs GM, O'Bryan MK. Phenotyping male infertility in the mouse: how to get the most out of a 'non-performer'. Human Reproduction Update 2010;16:205-224.

[16] Langlais J, Roberts KD. A Molecular Membrane Model of Sperm Capacitation and the Acrosome Reaction of Mammalian Spermatozoa. Gamete Research 1985;224:183–224.

[17] Voet D, Voet JG, Pratt CW. Fundamentals of Biochemistry. 4th ed. New York: Wiley; 1998.

[18] Parks JE, Lynch DV. Lipid composition and thermotropic phase behavior of boar, bull, stallion, and rooster sperm membranes preparation of sperm lipid extraction and fractionation. Cryobiology 1992;266:255–66.

[19] Moce´ E, Blanch E, Toma C. Use of cholesterol in sperm cryopreservation : present moment and perspectives to future treatment of sperm with cyclodextrins pre-loaded with cholesterol (CLC) 2010;45:57–66.

[20] Oldenhof H, Heutelbeck A, Blässe AK, Bollwein H, Martinsson G, Wolkers WF, et al. Tolerance of spermatozoa to hypotonic stress: role of membrane fluidity and correlation with cryosurvival. Reproduction, Fertility and Development 2015;27:285.

[21] Flesch FM, Gadella BM. Dynamics of the mammalian sperm plasma membrane in the process of fertilization. Biochimica et Biophysica Acta. 2000;1469:3:197-235.

[22] Buffone MG, Foster JA, Gerton GL. The role of the acrosomal matrix in fertilization. Int J Dev Biol 2008;52:511–22.

[23] Varner DD, Gibb Z, Aitken RJ. Stallion fertility: A focus on the spermatozoon 2015;47:16–24.

[24] Love CC, Varner DD, Thompson J.A. Intra- and inter-stallion variation in sperm morphology and their relationship with fertility. J. Reprod. Fertil. Suppl 2000. 56, 93-100.

[25] Storey BT. Mammalian sperm metabolism: Oxygen and sugar, friend and foe. Int J Dev Biol 2008.

[26] Bucci D, Rodriguez-Gil JE, Vallorani C, Spinaci M, Galeati G, Tamanini C. Gluts and mammalian sperm metabolism. J Androl 2011.

[27] Piomboni P, Focarelli R, Stendardi A, Ferramosca A, Zara V. The role of mitochondria in energy production for human sperm motility. International Journal of Andrology 2011;35:109–24.

[28] Davila MP, Muñoz PM, Tapia JA, Ferrusola CO, Da Silva C CB, Peña FJ. Inhibition of mitochondrial complex I leads to decreased motility and membrane integrity related to increased hydrogen peroxide and reduced ATP production, while the inhibition of glycolysis has less impact on sperm motility. PLoS One 2015.

[29] Aitken RJ, Baker MA. Oxidative stress and male reproductive biology. Reprod Fertil Dev 2004;16:581–8.

[30] Aitken JR, Gibb Z, Baker MA, Drevet J, Gharagozloo P. Causes and consequences of oxidative stress in spermatozoa. Reprod Fertil Dev 2016;28:1–10.

[31] Nordberg J, Arnér ES. Reactive oxygen species, antioxidants, and the mammalian thioredoxin system. Free Radic Biol Med 2001;31: 1287–312.0.

[32] Jones R, Mann T, Sherins RJ. Adverse effects of peroxidized lipid on human spermatozoa. Proc R Soc London - Biol Sci 1978;201:413–7.

[33] Moazamian R, Polhemus A, Connaughton H, Fraser B, Whiting S, Gharagozloo P, et al. Oxidative stress and human spermatozoa: Diagnostic and functional significance of aldehydes generated as a result of lipid peroxidation. Mol Hum Reprod 2015;21:502–15.

[34] Aitken RJ, Whiting S, De Iuliis GN, McClymont S, Mitchell LA, Baker MA. Electrophilic aldehydes generated by sperm metabolism activate mitochondrial reactive oxygen species generation and apoptosis by targeting succinate dehydrogenase. J Biol Chem 2012;287:33048–60.

[35] Aitken RJ, De Iuliis GN, Finnie JM, Hedges A, McLachlan RI. Analysis of the relationships between oxidative stress, DNA damage and sperm vitality in a patient population: Development of diagnostic criteria. Hum Reprod 2010;25:2415–26.

[36] Aitken RJ, Clarkson JS. Cellular basis of defective sperm function and its association with the genesis of reactive oxygen species by human spermatozoa. J Reprod Fert 1987;81:459–69.

[37] Baumber J, Ball BA, Gravance CG, Medina V, Davies-Morel MC. The effect of reactive oxygen species on equine sperm motility, viability, acrosomal integrity, mitochondrial membrane potential, and membrane lipid peroxidation. J Androl 2000;21:895–902.

[38] Dawson GR, Webb GW, Pruitt JA, Loughin TM, Arns MJ. Effect of different processing techniques on motility and acrosomal integrity of cold-stored stallion spermatozoa. J Equine Vet Sci 2000;20:191–4.

[39] Ramires Neto C, Monteiro GA, Soares RF, Pedrazzi C, Dell'aqua JA, Papa FO, et al. New seminal plasma removal method for freezing stallion semen. Theriogenology 2013;79:1120–3.

[40] Kawai GKV, Gurgel JRC, Losano JD de A, Dalmazzo A, Rocha CC, Tsunoda RH, et al. Susceptibility of stallion spermatozoa to different oxidative challenges: role of seminal plasma. J Equine Vet Sci 2017;55:76–83.

[41] Kankofer M, Kolm G, Aurich J, Aurich C. Activity of glutathione peroxidase, superoxide dismutase and catalase and lipid peroxidation intensity in stallion semen during storage at 5°C. Theriogenology 2005;63:1354–65.

[42] Alvarez J, Storey B. Spontaneous lipid peroxidation in rabbit and mouse epididymal spermatozoa: dependence of rate on temperature and oxygen concentration the considerably. Biol Reprod 1985:342–51.

[43] Neild DM, Gadella BM, Chaves MG, Miragaya MH, Colenbrander B, Agüero A. Membrane changes during different stages of a freeze-thaw protocol for equine semen cryopreservation. Theriogenology 2003;59:1693–705.

[44] Adler HI. The use of microbial membranes to achieve anaerobiosis. Crit Rev Biotechnol 1990;10:119–27.

[45] Metcalf ES. Pregnancy rates with cooled equine semen received in private practice. AAEP Proceedings 1998;44:16–8.

[46] Mccommis KS, Finck BN. Mitochondrial pyruvate transport: a historical perspective and future research directions. Biochem J 2015;466:443–54.

[47] Gray LR, Tompkins SC, Taylor EB. Regulation of pyruvate metabolism and human disease. Cell Mol Life Sci 2014;71:2577–604.

[48] Mendes-Mourão J, Halestrap A, Crisp DM, Pogson CI. The involvement of mitochondrial pyruvate transport in the pathways of gluconeogenesis from serine and alanine in isolated rat and mouse liver cells. FEBS Lett 1975;53:29–32.

[49] Herzig S, Raemy E, Montessuit S, Veuthey JL, Zamboni N, Westermann B, et al. Identification and functional expression of the mitochondrial pyruvate carrier. Science (80-) 2012;336:93–6.

[50] Owen OE, Kalhan SC, Hanson RW. The key role of anaplerosis and cataplerosis for citric acid cycle function. J Biol Chem 2002;277:30409–12.

[51] Bruemmer JE, Coy RC, Squires EL, Graham JK. Effect of pyruvate on the function of stallion spermatozoa stored for up to 48 hours. J Anim Sci 2002;80:12–8.

[52] Stendardi A, Focarelli R, Piomboni P, Palumberi D, Serafini F, Ferramosca A, et al. Evaluation of mitochondrial respiratory efficiency during in vitro capacitation of human spermatozoa. Int J Androl 2011;34:247–55.

[53] Ferramosca A, Pinto Provenzano S, Montagna, DD, Coppola L, Zara V. Oxidative Stress Negatively Affects Human Sperm Mitochondrial Respiration. Urology 2013;82:78-83.

[54] Brinsko SP, Crockett EC, Squires EL. Effect of centrifugation and partial removal of seminal plasma on equine spermatozoal motility after cooling and storage. Theriogenology 2000;54:129–136.

[55] Loomis PR, Graham JK. Commercial semen freezing: individual male variation in cryosurvival and the response of stallion sperm to customized freezing protocols. Anim Reprod Sci 2008;105:119–28.

[56] Webb GW, Dean MM. Effect of Centrifugation Technique on Post-storage Characteristics of Stallion Spermatozoa. J Equine Vet Sci 2009;29:675–80.

[57] Parlevliet J, Kemp B, Colenbrander B. Reproductive characteristics and semen Warmblood stallions quality in maiden Dutch Warmblood stallions. J Reprod Fertil 1994;101:183–7.

[58] Morris LHA, Allen WR. Reproductive efficiency of intensively managed Thoroughbred mares in Newmarket. Equine Veterinary Journal 2010;34:51–60.