LOCALIZATION OF THE CATSPER1 PROTEIN AND INDUCTION OF HYPERACTIVATED-LIKE MOTILITY IN EQUINE SPERMATOZOA

A Thesis

by

KRISTIN ROSE ROLKE

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

December 2008

Major Subject: Veterinary Physiology

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Approved by:

Chair of Committee,	Katrin Hinrichs
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ABSTRACT

Localization of the CatSper1 Protein and Induction of Hyperactivated-Like Motility in Equine Spermatozoa. (December 2008)

Kristin Rose Rolke, B.S., Texas A&M University

Chair of Advisory Committee: Dr. Katrin Hinrichs

In vitro fertilization is not efficient in the horse, and this may be due to a failure of induction of hyperactivated motility in stallion sperm in vitro. Hyperactivated motility is characterized by high curvilinear velocity and amplitude of lateral head movement, and is required for the sperm to penetrate the zona pellucida of the oocyte in order to achieve fertilization. In mice, hyperactivated motility is induced by calcium influx into the flagellum of the sperm through the CatSper channel, a tetrameric cation channel made up of CatSper proteins 1 to 4. The CatSper channel is located specifically in the principal piece of the sperm tail, and opens in response to an increase in intracellular pH.

Factors associated with the induction of hyperactivated motility of sperm have been reported in most domestic and laboratory species, with the notable exception of the horse. In addition, presence of CatSper proteins has not been demonstrated in the horse. Our objective was to determine the presence and location of the CatSper1 protein on stallion sperm, and to determine whether alkalinization of the intracellular pH induces hyperactivated motility in stallion sperm. Presence of the CatSper1 protein on the

principal piece of the flagellum of stallion sperm was confirmed by immunocytochemistry with an anti-human CatSper1 C-terminus antibody. Incubation of stallion sperm with the cell-permeant weak base NH_4Cl increased curvilinear velocity and amplitude of lateral head movement values in stallion sperm as measured by computer-assisted sperm analysis. These measures are indicative of hyperactivated motility in other species. Hyperactivated-like motility in response to NH_4Cl was dependent upon the CO_2 atmosphere in which the sperm were incubated and was enhanced by presence of glucose in the medium. Maximum motility values were reached with 25 mM NH_4Cl at 60 minutes in a 5% CO_2 atmosphere.

These results indicate that the CatSper1 protein, and thus presumably the CatSper channel, is present on the principal piece of stallion sperm and that treatments inducing a rise in intracellular pH increase hyperactivated-like motility. These findings represent a basis for establishment of in vitro fertilization protocols that include induced hyperactivated motility to ensure zona penetration.

DEDICATION

In Loving Memory of my Mother!

Kathleen Ann Rolke

December 12, 1957 – January 03, 2008

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In honor of my mother, Kathleen Ann Rolke, who was called home to our heavenly father too soon but will never be forgotten. You were indeed my hero, best friend and most of all cherished mother! I dedicate this work to you as you have been my greatest inspiration!

Last, but certainly not least, I would like to thank my father, Randolph J. Rolke, and my loving husband, Christopher A. Crawford, for their unwavering support, love and encouragement. Without them, I would have never reached for my dreams. Thank you for always being my light at the end of the tunnel.

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INTRODUCTION

Knowledge of the events surrounding fertilization is essential to the application of assisted reproductive technologies. In vitro fertilization (IVF) has been a successful tool both for research on the fertilization process and for clinical production of offspring in many species. However, there is limited knowledge of the events leading to fertilization of the equine oocyte, and this has posed significant problems in the development of a repeatable IVF protocol in this species.

The failure of equine IVF seems to be related to the inability of the sperm to penetrate the zona pellucida (Choi et al., 1994), and this has been attributed to incomplete capacitation of the stallion spermatozoa in vitro; there is no data to support an alternative hypothesis at this time. The term capacitation refers to complex changes in the plasma membrane of the sperm enabling the sperm to undergo the acrosome reaction when given the proper stimulus (in vivo, binding to the zona pellucida). The acrosome reaction initiates release of hydrolytic enzymes from the sperm acrosome. These enzymes dissolve a hole in the zona pellucida, allowing the sperm to penetrate the oocyte. Hyperactivated motility (strong, whip-like beating of the sperm flagellum) is considered to be a necessary stage of sperm capacitation (Ren et al., 2001).

While the inability of equine sperm to penetrate the zona pellucida during IVF has been attributed to inadequate capacitation, many studies have shown that equine

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sperm treated under “capacitating conditions” show changes similar to those seen in capacitated sperm in other species. For instance, although cholesterol content of equine sperm is notably high compared to that of other quadrupeds (Gadella et al., 2001), during capacitation equine sperm undergo cholesterol efflux as is seen in other species, increasing fluidity and permeability of membranes and enabling membrane fusion and the acrosome reaction (Landim-Alvarenga et al., 2001; Langlais et al., 1985). Reorganization of the plasma membrane (advancement of lipid components to a higher order), indicating capacitation, has been reported in incubated equine spermatozoa stained with merocyanine 540 (Rathi et al., 2001). Phosphorylation of tyrosine residues on intracellular proteins, another feature of capacitated sperm in other species, has also been reported in equine spermatozoa under “capacitating” culture conditions (Pommer et al., 2003; McPartlin et al., 2006).

While capacitation-like changes have been shown to occur in the head of treated equine sperm, there are no reports demonstrating the induction of hyperactivated motility in stallion spermatozoa. Hyperactivation is characterized by a less progressive nature of movement, asymmetrical motility, increased lateral head displacement and increased amplitude of motion of the principal piece in the flagellum in other species. Hyperactivation in vivo is thought to enable the sperm to navigate the oviduct, separate from the oviductal epithelium and have the vigor required for penetration of the zona pellucida (Suarez and Ho, 2003).

Marquez et al. (2007) used computer-assisted sperm analysis (CASA) to assess hyperactivated motility in bull spermatozoa. Sperm classified as hyperactivated showed

an increase in both curvilinear velocity (VCL; measuring the rate of travel of the sperm head) and amplitude of lateral head movement (ALH; measuring mean width of head oscillations). Similarly, in vitro hyperactivated hamster spermatozoa became less progressive but more vigorous, and could be distinguished from non-hyperactivated spermatozoa because the hyperactivated sperm swam in circles and had low progressive velocity, decreased linearity (LIN), and increased amplitude of lateral head displacement (Shivaji et al., 1995). Rathi and coworkers (2001) evaluated equine spermatozoa by CASA; these workers classified hyperactivated motility in equine sperm somewhat arbitrarily as having a VCL greater than 180 µm/sec and ALH greater than 12 µm. However, they were not able to demonstrate a treatment in sperm incubated in a tyrodes bicarbonate medium, with or without calcium ionophore, which increased the proportion of sperm showing these parameters. To our knowledge, the study of Rathi et al. (2001) is the only study assessing equine spermatozoa for hyperactivated motility.

Given the above data, i.e. that capacitation-like changes have been observed in equine spermatozoa under "capacitating conditions," but that hyperactivated motility has not been documented in vitro, we hypothesize that the inability of the equine sperm to penetrate the zona pellucida during IVF is due to the lack of induction of hyperactivated motility in equine sperm in vitro.

Hyperactivated motility in mouse spermatozoa is associated with activation of voltage-gated Ca^{2+} channels in the sperm principal piece. These channels are termed the cation channels of sperm or CatSper channels. These channels are specific to the sperm tail, and are distinct from the ion channels in the sperm head associated with capacitation

and induction of the acrosome reaction. CatSpers1 to 4 assemble as a tetramer to form an alkalinization-activated calcium channel, in association with a less-related protein, CatSper β (Liu et al., 2007). In mice, CatSper proteins are localized on the sperm membrane in the principal piece of the sperm (Ren et al., 2001). Proper functioning of the CatSper channel is vital for the flagellar motility required for penetration of the zona pellucida. Ren and coworkers (2001) found that in the mouse, CatSper is essential for the sperm to penetrate the zona pellucida, as sperm from CatSper-null mice were able to adhere to the zona in vitro but were unable to penetrate. Sperm from CatSper-null mice did not show hyperactivated motility under physiological conditions and could fertilize only eggs in which the zona pellucida had been enzymatically removed (Ren et al., 2001; Liu et al., 2007). CatSper-null murine spermatozoa showed impairment of the motility parameters of path velocity, progressive velocity and track speed when exposed to capacitating conditions and assessed with time-lapse photomicrography, and were seen to lack vigorous beating and deviation of the flagellum (Ren et al., 2001). Thus, sperm lacking the CatSper protein lose the ability to hyperactivate along with the capacity to fertilize the oocyte. It remains unknown whether these events are associated or completely separate functions.

Patch-clamp experiments indicate that in the mouse, alkalinization of intracellular pH promotes a Ca^{2+} influx through CatSper 1-associated channels (Ren et al., 2001). Intracellular pH can be increased by addition of NH_4Cl to media. The NH_4Cl dissociates into ammonia gas (NH_3) and hydrogen and chloride ions. Ammonia gas dissolves through the plasma membrane into the cell cytoplasm, where it goes into

equilibrium to form NH_4^+ and Cl^-, thus reducing H^+ concentration in the cytoplasm, causing intracellular alkalinization to occur. Addition of NH_4Cl to media induced Ca^{2+} influx in CatSper-intact mouse spermatozoa, but not in CatSper-null spermatozoa (Kirichok et al., 2006). Hyperactivated motility in bull sperm, as assessed by CASA, was induced by treatment with NH_4Cl (Marquez et al., 2007), presumably via activation of the CatSper ion channel.

It is not known whether the CatSper protein is present in equine spermatozoa, nor, if it is present, whether it responds to intracellular alkalinization in a similar way to that seen in other species, (i.e., by allowing influx of calcium into the sperm and thus inducing hyperactivated motility). Furthermore, a clear description of hyperactivated motility in equine spermatozoa is not available. It is notable that the only repeatable method for IVF in horses (achieving about 10% fertilization) is treatment of equine spermatozoa with calcium ionophore, which causes direct influx of calcium into the sperm, and thus would bypass the need for activated CatSper channels.

REVIEW OF THE LITERATURE

Background

Equine in vitro fertilization has many potential applications in the equine industry, but unfortunately equine IVF has become known for its meager efficiency and poor repeatability. The inability for the sperm to become fully and properly capacitated and penetrate the equine oocyte is believed to be the reason behind the slow progress in equine IVF. The zona pellucida appears to be the main barrier to sperm at the time of fertilization, as bypassing the zona via zona drilling, partial zona removal, use of zona free oocytes, or intracytoplasmic sperm injection (ICSI) results in higher rates of fertilization (Choi et al., 1994; Li et al., 1995; Landim-Alvarenga et al., 2001; Dell'Aquila et al., 1997).

Capacitation

Capacitation is a necessary transition in which sperm gain the level of maturity that allows them to penetrate and fertilize the oocyte. Capacitation -associated events include, but may not be limited to: bicarbonate-induced increase in adenylate cyclase activity, increase in cyclic AMP (cAMP) which activates protein kinase A, lipid synthesis and breakdown, phosphorylation of tyrosine residues of sperm proteins, and increased cellular calcium influx (Gadella et al., 2001; Pommer et al., 2003).

Spermatozoa are exposed to progressively increasing levels of bicarbonate while undergoing maturation and capacitation, starting with male accessory gland fluid and

ending with the female reproductive tract secretions (Okamura et al., 1985). These conditions can be replicated in vitro in a bicarbonate-buffered medium. The presence of bicarbonate has been shown to stimulate sperm adenylate cyclase, which directly increases cAMP. Intracellular rises in cAMP support capacitation-like changes by increasing protein kinase A while in the presence of bicarbonate (Visconti et al., 1997). Increasing protein kinase A levels stimulate mechanisms for phosphorylation of tyrosine residues within the flagellum of the sperm tail (Pommer et al., 2003). These tail-associated tyrosine residues, when phosphorylated, have been directly linked to motility and hyperactivation (Nassar et al., 1999).

Protein kinase A also aids in lipid re-organization, causing collapse of normal phospholipid asymmetry within the sperm cell plasma membrane. The resulting phospholipid scrambling initiates cholesterol efflux from the sperm plasma membrane; which is associated with bicarbonate influx into the plasma membrane resulting in a subsequent rise in intracellular pH, associated with fusion of the sperm plasma membrane lipid bilayer (Cross, 1998). Cholesterol efflux from the plasma membrane is directly correlated to capacitation in that the higher the levels of cholesterol, the longer the period of capacitation, which could explain the variation in capacitation time frames among species (Gadella et al., 2001).

Heparin-induced capacitation in bovine sperm involves an increase in cAMP and protein phosphorylation, and can be inhibited by glucose, possibly via acidification of intracellular pH during glycolysis (Parrish et al., 1988). The inhibitory effects of glucose

may be specific to bovine sperm, as a stimulatory effect of glucose on capacitation has been noted in mice, rats, hamsters and humans (Galantino-Homer et al., 2004).

Capacitation also initiates the rapid flux of calcium into the sperm cell in response to the appropriate stimulus (i.e. the zona pellucida), which is essential for the acrosome reaction. Only capacitated spermatozoa are capable of undergoing the acrosome reaction in response to calcium. In capacitated sperm, membrane fusion of the plasma and outer acrosomal membranes occurs due to the rapid swelling of the membrane layers resulting from increased calcium permeability and decreased ATPase activity (Yanagimachi and Usui, 1974). Observations of two net inward movements of calcium were noted by Singh et al. (1978). These workers designated the secondary calcium uptake of importance as its time course parallels that of the acrosome reaction; thus indicating a calcium requirement for the induction of the acrosome reaction (Singh et al., 1978).

Hyperactivated Motility

Yanagimachi (1969) was the first to detect an abrupt change from linear to whiplash-like motility in hamster spermatozoa in vitro, and hypothesized that the vigorous motility assisted in penetration of the zona pellucida of the oocyte. Hyperactivated motility is seen as an integral part of capacitation, however, these two phenomena may be induced separately (Marquez and Suarez, 2004). Hyperactivated motility is most often characterized by vigorous motility accompanied by large amplitude whiplash-like flagellar beats and erratic sperm head trajectories (Katz and

Yanagimachi, 1980). Spermatozoal hyperactivation is thought to aid sperm in maneuvering oviductal mucosa and the oocyte cumulus complex, and in penetration of the oocyte zona pellucida. A common method to evaluate sperm for hyperactivated motility is to use a computer-assisted sperm motility analyzer (CASA) to evaluate semen samples that have been incubated under capacitating conditions, and to analyze movement patterns that increase over time in comparison to a control sample (Suarez and Ho, 2003). Shivaji et al. (1995) classified sperm as hyperactivated when they possessed either circular or helical motility patterns, and as non-hyperactivated when they possessed planar-type motility patterns.

Rathi and coworkers (2001), examined in vitro capacitation of stallion spermatozoa, and classified hyperactivation by cell location on a scatter graph of CASA values. Spermatozoa were suspended in bicarbonate- and calcium- containing medium, believed to lead to sperm cell capacitation. Cells were then evaluated for sperm motility pattern analysis with specific regard to hyperactivated-like motility, and these data were graphed into a four-quadrant scatter plot. Based upon the quadrant, cells were designated as possessing hyperactive motility (VCL $\geq$180 µm/sec and ALH $\geq$12 µm). However, throughout their study only 5.3% $\pm$ 2.4% of sperm were considered hyperactive and this percentage did not change significantly with time in any treatment. The lack of change in motility parameter values brings into question the basis for the authors designating these parameters as defining hyperactive motility. In other species, hyperactive motility is associated with the presence of high VCL and ALH values, and low or regressing

linearity (LIN), progressive motility, and straightness (STR) values (Shivaji et al., 1995; Marquez et al., 2007).

The factors regulating induction of hyperactivated motility are similar to those regulating capacitation, including bicarbonate, cAMP and calcium. A study by White and Aitken (1989) showed that calcium-dependent elevation of cAMP levels preceded and led to hyperactivated motility. Calcium is required to induce and maintain hyperactivated motility in hamster spermatozoa (Yanagimachi, 1994). Inositol 1,4,5-triphospahte (IP_3), plasma membrane calcium channels, voltage sensitive calcium channels and channel proteins have been localized to the base of the flagellum and show evidence of intracellular calcium stores and major calcium transport across the membrane, all of which may be related to regulation of induction and maintenance of hyperactivated motility. Intracellular calcium levels are higher in hyperactivated sperm cells (Suarez and Ho, 2003). Hyperactivated motility is further regulated by the presence of cAMP, thought to help maintain calcium levels, and bicarbonate, which modulates adenylate cyclase activity of the flagellum, all promoting flagellar asymmetry (Suarez and Ho, 2003).

It was shown as early as 1984 that intracellular pH regulated spermatozoal motility (Acott and Carr, 1984). Studies by Parrish et al. (1989) suggested that pH elevation may contribute to activation of calcium-permeable channels, hence capacitation was suppressed when intracellular alkalinization was inhibited.

Studies conducted by Marquez and coworkers (2007) analyzed sperm motility parameters including curvilinear velocity, lateral head movement and linearity upon the

addition of NH_4Cl to bovine sperm suspended in a balanced salt solution medium. Data points were analyzed within two min of NH_4Cl addition, and values for curvilinear velocity and lateral head movement peaked at 25 mM NH_4Cl; concomitantly, linearity was lowest at this concentration. There was a dose-dependent response to NH_4Cl concentrations up to 25 mM, however motility declined after addition of more than 25 mM NH_4Cl. Marquez and Suarez (2007) showed that NH_4Cl treatment of sperm rapidly evoked elevations of intracellular pH and Ca^{2+} concentrations that persisted as long as NH_4Cl was present. The intracellular pH rise preceded that of Ca^{2+}, and Ca^{2+} influx was identified in both the head and flagellum. Upon the addition of 25 mM NH_4Cl, VCL and ALH values both increased >170% from baseline, whereas LIN decreased by >40% from baseline; characteristic traits of hyperactivated motility. Further evidence for the involvement of pH-modulated Ca^{2+} entry promoting hyperactive motility was demonstrated by an increase in asymmetrical flagellar beating in the presence of an alkaline medium (Marquez and Suarez, 2007).

CatSper

CatSper is a unique cation channel that is localized to the principal piece of the flagellum of the sperm, and is believed to open when exposed to an alkaline intracellular environment, allowing for an influx of calcium into the sperm and initiating hyperactive motility. The cation channel protein CatSper1 has been identified as a six-transmembrane-spanning protein and has been localized by immunocytochemistry to the principal piece of the flagellum of murine sperm (Ren et al., 2001). After the CatSper1

protein was isolated, a related protein, CatSper2 was identified (Quill et al., 2001). In total, a family of four related sperm-specific cation channels, CatSper1 through CatSper4, have been identified. It was initially unknown if the four CatSper proteins acted as a single heterotetrameric channel or whether they acted alone (Quill et al., 2001); however, subsequently it was found that the four CatSper proteins, along with a less-related protein, CatSper β, together form the functional calcium ion channel in the principal piece of the flagellum of sperm (Liu et al., 2007). CatSper exhibits pore features of calcium-sensitive ion channels, a putative S4 domain and single repeat structure resembling that of cyclic-nucleotide gated channels, high voltage-gated calcium channels and transient receptor potential channels (Ren et al., 2001). An abundance of histadine residues within the amino terminus of the CatSper gene are believed to be sensitive to intracellular pH, indicating the mechanism for the opening of CatSper upon alkalinization, allowing subsequent calcium influx (Ren et al., 2001).

Ren et al. (2001) determined that CatSper1 protein was essential for induction of hyperactivated motility of murine sperm by comparing CatSper1-knockout mice to wild-type mice. They found that the mice lacking this protein were sterile, and their sperm failed to penetrate eggs in vitro. Furthermore, ablation of CatSper1 abolished cAMP-induced calcium influx in these sperm.

It has been shown that each CatSper protein individually appears to be required for male fertility (Ren et al., 2001; Quill et al., 2003; Qi et al., 2007). The pore is made of CatSpers1 to 4 along with CatSper β, as deletion of any one of these proteins causes loss of hyperactivated motility. Thus, findings for any one CatSper protein probably

reflect function of the calcium channel (CatSper tetramer) as a whole. Carlson et al. (2003) concluded that CatSper1 is required for depolarization-evoked calcium entry into the membrane of the flagellum, thereby forming functional voltage-gated calcium channels. These results were based upon the administration of depolarizing medium K8.6, known to open voltage-gated calcium channels, resulting in a rise in intracellular calcium, to both wild-type and CatSper1-null sperm. Whereas treatment of sperm of wild-type mice induced a large intracellular calcium increase, treated CatSper1-null murine sperm displayed minimal to zero depolarization-evoked calcium increase (Carlson et al., 2003). Similarly, CatSper2 null mice fail to display the transition from active motility to calcium-dependent hyperactivated motility, indicating that activation of CatSper2 also drives hyperactivated motility required for fertilization (Ren et al., 2001; Quill et al., 2003).

Patch-Clamp Detection of Alkaline Activated Ca^{2+} Channels

Patch-clamp is an electrophysiological technique that allows for the examination of excitable cells. In this technique, a fine pipette is applied to the plasma membrane, and membrane is broken to create a continuous environment between the cytoplasm and the contents of the pipette. Kirichok and co-workers (2006), applied patch-clamps to whole-cell spermatozoa to study flagellar calcium channel activation following intracellular alkalinization. A secure seal between the plasma membrane and the glass pipette could not be accomplished using mature sperm, so the patch pipette was attached to the cytoplasmic droplet of corpus epididymis mouse sperm cells. The patch-clamp

studies showed that calcium influx was induced in wild-type corpus epididymal sperm by intracellular alkalinization. Intracellular alkalinization occurred when 10 mM of NH_4Cl was added to the bath solution, resulting in calcium current stimulation attributed to CatSper ($I_{CatSper}$). Kirichok et al. (2006) concluded that under capacitating conditions, inducing an alkaline intracellular pH, $I_{CatSper}$ is strongly stimulated. Notably, the patch-clamp studies (Kirichok et al., 2006), showed no calcium influx in CatSper null sperm in response to intracellular alkalinization.

Ionic currents were also recorded from sperm cell fragments consisting of either the mid- and principal pieces together, lacking the head of the sperm, or from the head and attached mid piece, lacking the principal piece. The calcium current attributed to CatSper ($I_{CatSper}$) was detected in measurements taken only from the segments having the principal piece, indicating CatSper channel localization to the principal piece only. These findings confirmed the previous immunocytochemical findings of Ren et al. (2001), in which the protein was localized to the principal piece.

Goals and Objectives

The long-term goal of this research was to define factors affecting equine fertilization in vitro, to enable development of a repeatable, effective procedure for IVF in this species.

The specific goals of this research were:

1. Determine the presence and location of the CatSper1 protein in stallion sperm.

2. Determine whether exposure to NH_4Cl induces hyperactivated-like sperm motility in stallion sperm.

3. Determine whether glucose affects hyperactivated-like sperm motility in stallion sperm.

MATERIALS AND METHODS

General Procedures

Collection of Equine Semen (for all experiments)

Ejaculates were collected from a Texas A&M University-owned stallion by use of a Missouri-model artificial vagina (NASCO, Ft. Atkinson, WI). The ejaculate volume was recorded, concentration was determined by a Nucleo-Counter SP-100 (Chemometec, Allerød, Denmark), and the total and progressive motility of each ejaculate was determined by CASA (IVOS Version 12.2 L, Hamilton Thorne Biosciences, Beverly, MA) after dilution of the ejaculate in INRA 96 extender, a milk based extender containing purified fractions of milk micellar proteins (IMV Technologies USA, Maple Grove, MN). Previously frozen equine semen was also used in some experiments.

Collection of Mouse Sperm

Sexually mature male mice were received after euthanasia from the Comparative Medicine Program at Texas A&M University. Sperm were collected from the cauda epididymis by immersing the excised testicles in R-Tyrodes (Rathi et al., 2001) and puncturing the cauda repeatedly with an 18 gauge needle.

Collection of Bovine Semen

Fresh ejaculated bovine semen was obtained from Ultimate Genetics (Wheelock, TX). Previously frozen bovine semen was also used in some experiments.

Immunocytochemistry Procedures: Immunostaining for CatSper1 Protein

Media Preparation

A modified Tyrode medium (R-Tyrodes; Rathi et al., 2001) was used for sperm procedures. All chemicals were purchased from Sigma-Aldrich Co. (St. Louis, MO), with the exception of the gentamicin reagent solution, which was purchased from Invitrogen Corporation (Carlsbad, CA). Distilled water used in R-Tyrodes was purchased from Invitrogen. The modified Tyrode (R-Tyrodes) balanced salt solution was prepared according to Appendix A.

A stock medium lacking calcium chloride, pyruvate and bovine serum albumin (BSA), was prepared and stored for use in sterile containers at 4˚C for a period no longer than one week. Batches of working media were prepared by adding bovine serum albumin, calcium chloride and pyruvate 20 to 24 hours prior to semen collection. The batch of working medium was adjusted to pH 7.4 using NaOH (Sigma-Aldrich, S2770) or HCl (Sigma-Aldrich, H9892) as needed. The osmolarity of the R-Tyrodes working medium was recorded; if the osmolarity was not in a range of 290-300 mOsm/kg, this implied an error in production and the media was reconstructed. The medium was filtered through a 0.2 µm Acrodisc filter (Pall Corporation, Ann Arbor, MI) into 50 mL

conical tubes and placed in a humidified atmosphere of 5% CO_2 at 37˚C with loosened caps for equilibration.

Processing of Mouse Sperm for Immunostaining

Sperm acquired from the cauda epididymis was carefully applied to the bottom of a 5 mL tube containing 1 mL of R-Tyrodes medium. The tube was then placed in a 5% CO_2 incubator for 20 min, after which the top 600 µl of the medium was pipetted into an Eppendorf tube and centrifuged at ≈300 x g for 3 min. After centrifugation, the supernatant was removed and 200 µl of PBS (Invitrogen) was used to re-suspend the pellet, and centrifugation and re-suspension was repeated. Ten microliters of the re-suspended sperm sample was pipetted onto a superfrost plus slide (Fisher Scientific, Pittsburgh, PA). A coverslip was then used to gently spread the sample across the slide. The slide was allowed to dry for 30 min. A Pap smear pen was then used to circle the sample, creating a barrier around the sample.

Processing of Equine and Bovine Semen for Immunostaining

Both fresh and frozen/cryopreserved equine spermatozoa were used for immunostaining; while only cryopreserved bovine spermatozoa was used for this procedure. The fresh equine ejaculate was collected as described above and processed through swim-up procedures as outlined for mouse sperm. Equine and bovine frozen semen were thawed in a 37˚C water bath for 30 sec. The straws were then cut and the extended semen was collected into a 5 mL tube. Both fresh and frozen sperm were

prepared using the swim-up procedure, and placed on a slide for immunocytochemistry as described for mouse sperm.

Immunocytochemistry Protocol

Different procedures were investigated for processing of sperm for immunocytochemistry, including the method used by Okunade et al. (2004), for immunostaining analysis of plasma membrane calcium -ATPase (PMCA) in mouse sperm and the procedure used by Ren et al. (2001), for immunocytochemistry of CatSper1 in mouse sperm.

Okunade Immunostaining Procedure

The dried semen sample on the slide was fixed using methanol fixation and buffered formal saline (BFS) fixation. Methanol fixation was achieved by fixing the semen onto the slide for 5 min in -20˚C methanol and then air drying it for 30 min (as recommended by Santa Cruz Biotechnologies, Santa Cruz, CA). Buffered formal saline fixation was achieved by fixing the seeded slide overnight at 4˚C in 10% BFS.

Following the methanol and BFS fixation the slides were then washed within slide cases 4 times in PBS, at 2 min per wash. Following fixation, permeabilization was achieved by suspension in a 0.1% sodium dodecyl sulfate (SDS from Sigma Aldrich, L4390) in PBS solution for 15 min at room temperature in slide cases, followed by washing the sample 4 times in PBS at 2 min per wash.

After the spermatozoa were permeabilized, the methanol- and BFS-fixed samples were blocked by a 1 hour incubation in blocking medium). containing normal goat serum (Jackson Laboratory, West Grove, PA; Appendix C).

Following the blocking step, the samples were then incubated with their primary antibody, which was diluted in the blocking medium, for 1 to 24 hours at either 4˚C or at room temperature. After staining with the primary antibody, the slides were washed again in PBS, followed by incubation with the secondary Cy3-conjugated secondary antibody at room temperature. The samples were again washed with PBS then stained with 1.5 µg/mL of DAPI. Mounting medium and a coverslip was applied and the slides were examined 24 hours post procedure.

Ren Immunostaining Procedure

Only paraformaldehyde (PFA) fixation was used when following the protocol suggested by Ren (D. Ren, University of Pennsylvania, personal communication, 2007). Paraformaldehyde fixation was achieved after swim-up by fixing the processed semen in 4% PFA freshly prepared in PBS at room temperature for 20-30 min. After PFA fixation, the sample was washed by centrifugation three times in 1 mL PBS (700 x g for 3 min), and finally re-suspended in 500 µl of PBS. The sample was then seeded onto a superfrost slide and allowed to evaporate but not dry, then washed twice for two min each with PBS. The PFA samples were permeabilized in 0.2% triton in PBS for 15-20 min, and then washed 4 times in PBS at 2 min per wash.

Samples were blocked in a 10% goat serum for 15 min. The primary antibody was then prepared in a 10% goat serum- PBS blocking solution, and was incubated with the slide at room temperature for at least an hour. Following primary antibody application, the slide was washed 3 times for ten min with PBS. Secondary Cy3-conjugated antibody was again prepared in 10% goat serum in PBS and incubated as before at room temperature for at least an hour. The slide was washed three times for 5 min with 0.1% Triton in PBS. The samples were then stained with 1.5 µg/mL of DAPI. Antifade mounting medium and a coverslip were then applied.

Identification and Localization of CatSper1 on Equine Spermatozoa

Immunocytochemistry was used to determine the spermatozoal localization of equine Catsper1 and to compare it to that of the bull and mouse. Fresh and frozen stallion spermatozoa, fresh mouse spermatozoa, and frozen bull spermatozoa were processed following both the Okunade and Ren procedures, and utilizing a polyclonal antibody raised in the rabbit against the C-terminus of human Catsper1 (Santa Cruz Biotechnologies, Santa Cruz, CA) and a polyclonal antibody raised in the rabbit against a GST-fusion protein consisting the first 150 amino acids (N-terminus) of mouse CatSper1 provided by D. Ren. The sperm staining patterns were evaluated under three preset exposures using a Zeiss Axiovert inverted fluorescence microscope and Axiovision software to determine the intensity and pattern of staining. The location of the head of each sperm was determined by DAPI staining as viewed at 365 nm, and the location of the CatSper antibody was determined by detection of the secondary antibody,

a Cy-3 labeled goat anti-rabbit antibody (Jackson Laboratories, West Grove, PA). A minimum of 3 replicates were performed for each species.

Hyperactivated Semen Analysis Procedures: Marquez's Protocol

Media Preparation

The medium used for semen analysis procedures was that described by Marquez et al. (2007; M-Tyrodes). Following preliminary studies (see Results) this medium was used with the deletion of BSA and the addition of polyvinyl alcohol (PVA) in its place. All chemicals were purchased from Sigma-Aldrich Co. (St. Louis, MO), with the exception of the gentamicin reagent solution, which was purchased from Invitrogen. Distilled water used in M-Tyrodes was purchased from Gibco (Invitrogen). A stock medium lacking calcium chloride, sodium pyruvate and PVA was prepared and stored for use in a sterile container at 4˚C for a period no longer than one week. Batches of working media were prepared by adding BSA or PVA, calcium chloride, and sodium pyruvate 2 to 24 hours prior to semen motility trials. The M-Tyrodes was prepared according to Appendix B. The batch of working medium was adjusted to a pH of 7.6 using NaOH or HCl as needed. The osmolarity of the M-Tyrodes working batch medium was recorded; if the osmolarity was not in a range of 290-300 mOsm/kg this implied an error in production and the medium was reconstructed. Upon completion of the batch of working medium, the working batch was filtered through a 0.2 µm Acrodisc filter into 50-mL conical tubes and placed in a humidified atmosphere of 5% CO_2 at 37˚ with loosened caps for equilibration.

Semen Motility Analysis

Equine semen was immediately extended at a 1:5 dilution ratio of semen: medium with M-Tyrodes and processed immediately after dilution. Fresh ejaculated bovine semen was also diluted 1:5 and was transported in a warm water bath for 30 min back to the laboratory. Sperm concentration was determined by a Nucleo-Counter SP-100 from a raw sample of the equine ejaculate and from an extended sample of the bovine ejaculate. The extended samples were centrifuged in 15 mL conical tubes for 10 min at 170 x g.

Upon completion of the first centrifugation, the supernatant was removed and the semen was then diluted to 50 x 10^6/mL (based on initial concentration) using equilibrated M-Tyrodes medium. The diluted samples were again centrifuged at 170 x g for 10 min, the supernatant was removed and the samples were diluted with M-Tyrodes to a concentration of approximately 30 x 10^6/mL (desired concentration for the CASA). The sample was divided into Eppendorf tubes in 1 mL aliquots for the individual treatments depending on the protocol. When NH_4Cl (Sigma-Aldrich, A0171) was used, an appropriate volume of 1 M NH_4Cl in distilled water was added to the aliquots to provide a desired final concentration of NH_4Cl. The Eppendorf tubes remained uncapped within the 5% CO_2 incubator at 37^{o}C unless the sample was being analyzed.

At pre-determined time intervals (see specific experiments) 6-µl aliquots were analyzed from each sample using a CASA. The system was programmed to the following settings for the stallion: frames acquired – 45; frame rate – 60 Hz; minimum contrast – 70; minimum cell size – 4 pixels; minimum static contrast – 30; straightness

threshold for progressive motility – 50%; average-path velocity (VAP) threshold for progressive motility—30; VAP threshold for static cells – 15; cell intensity – 106; static head size – 0.60 to 2.00; static head intensity – 0.20 to 2.01; static elongation – 31 to 85; LED illumination intensity – 2200. The system was programmed to the following settings for the bull: frames acquired – 45; frame rate – 60 Hz; minimum contrast – 80; minimum cell size – 7 pixels; minimum static contrast – 15; straightness threshold for progressive motility – 80 %; average-path velocity (VAP) threshold for progressive motility — 50; VAP threshold for static cells – 20; cell intensity – 80; static head size – 0.72 to 8.82; static head intensity – 0.14 to 1.84; static elongation – 0 to 47; LED illumination intensity – 2300.

CASA values for total motility, progressive motility, concentration, curvilinear velocity (VCL), linearity (LIN), and amplitude of lateral head displacement (ALH) were recorded. The changes in these parameters over time for each NH_4Cl concentration were compared to that for a control sample containing 0 mM NH_4Cl.

Experiment 1: Effect of NH_4Cl on Hyperactivated Motility of Bovine Spermatozoa

Preliminary findings in our laboratory indicated that equine sperm showed marked aggregation of sperm heads when centrifuged in M-Tyrodes, which contains BSA. Subsequently, 11 trials were performed to evaluate different media to identify a non-milk containing medium that supported multiple centrifugations of stallion spermatozoa without sperm agglutination. Different levels of glucose in M-Tyrodes (n = 2), different media (n = 6), and the use of PVA to replace BSA in the M-Tyrodes (n = 3)

were assessed. Results of these studies (data not shown) demonstrated that modifying the M-Tyrodes by replacement of BSA with PVA supported equine sperm motility without inducing aggregation. Therefore, a study was conducted with bovine sperm to 1) validate the procedure of assessment of NH_4Cl-induced hyperactivated motility by CASA, as described by Marquez et al. (2007) with bovine sperm in our laboratory and 2) to evaluate the effect of replacing BSA with PVA in M-Tyrodes on NH_4Cl-induced hyperactivation of bovine sperm.

Three replicates were performed. Freshly ejaculated bovine sperm were processed as described above using one of three media: M-Tyrodes containing BSA, M-Tyrodes containing 0.1% PVA, or M-Tyrodes containing 0.02% PVA. After washed sperm were aliquoted in 1-mL samples in Eppendorf tubes, NH_4Cl was added to final concentrations of 0, 5, 10, 25, 50 or 100 mM. Motility analysis was conducted at 1, 5 and 30 min after NH_4Cl addition. The order of NH_4Cl addition and initial reading was randomized among treatment groups.

Experiment 2: Effect of NH_4Cl on Hyperactivated Motility of Stallion Spermatozoa

M-Tyrodes with PVA was used in this experiment. Two medium PVA concentrations (0.1% and 0.02%) were evaluated. Equine semen was processed as above, aliquoted, and exposed to NH_4Cl as described in Experiment 1. Preliminary trials leading to the establishment of this technique suggested that response to NH_4Cl decreased when concentrations exceeded 10 mM, therefore the concentrations of NH_4Cl used in this study were 0, 5, 10 and 25 mM. Samples were read at 1, 5 and 30 min

intervals after NH_4Cl addition. The order of NH_4Cl addition and initial reading was randomized among treatments. Three replicates were performed.

Experiment 3: Further Determination of Optimal NH_4Cl Concentration to Induce Hyperactivated Stallion Sperm Motility

Findings of Experiment 2 (see Results) indicated that the effective concentration of NH_4Cl was approximately 5 mM. This experiment was conducted to determine the optimal level around this concentration. Three replicates were performed on stallion spermatozoa in M-Tyrodes containing 0.02% PVA. Concentrations of NH_4Cl evaluated were 0, 1.7, 3.4, 5, 6.7, 8.4 and 10 mM. Samples were randomized and read at 1, 5 and 30 min intervals after NH_4Cl addition.

Experiment 4: Effects of Glucose Concentration on Stallion Sperm Motility

Glucose interferes with capacitation in bovine sperm but aids hyperactivated motility in human sperm (see Introduction). Three replicates were performed with stallion spermatozoa to evaluate the effect of glucose on the response of equine sperm to NH_4Cl. Sperm were processed in 0.02% PVA M-Tyrodes containing 0, 5, 10, 15 or 20 mM glucose, with or without treatment with 5 mM NH_4Cl. Readings of the treatment samples were randomized and treatments were read at 1, 5 and 30 min intervals after NH_4Cl addition.

Experiment 5: Effects of CO_2 Environment on Response to NH_4Cl

Experiments 1 through 4 were completed in September, 2007. In the spring of 2008, further trials were started to evaluate calcium influx in equine spermatozoa treated with NH_4Cl. However, hyperactivated motility parameters as measured by CASA of NH_4Cl-treated spermatozoa at this time differed markedly from those recorded in 2007. On evaluation of possible reasons for this discrepancy, it was suggested that the CO_2 incubator used (an injection-type incubator without a CO_2 sensor) may not have been properly calibrated in 2007. Measurements of media pH before and at the end of trials in 2007 showed no notable change in pH over time (data not available), however, Fyrite testing of the incubator CO_2 concentration was not performed at that time. Fyrite testing in 2008 showed that this incubator was registering 4.5 to 5% CO_2.

An experiment was performed to evaluate whether exposure to CO_2 affected stallion spermatozoal motility parameters as induced by NH_4Cl. Equine sperm was processed as above, aliquoted, and exposed to NH_4Cl as described in Experiment 2. Three methods of incubation were evaluated: M-Tyrodes incubated in a humidified atmosphere of 5% CO_2 at 37˚C for the length of the trial (CO_2), or M-Tyrodes equilibrated in humidified atmosphere of 5% CO_2 at 37˚C and then placed into an air-only incubator kept at 37˚C, either 30 min prior to semen collection (Air), or >1 hour prior to semen collection (Air+1). Both of these latter treatments were kept in air-only incubation at 37˚C for the length of the trial. Based on the results from the 2007 trials, the concentrations of NH_4Cl used in this study were 0, 5, 10 and 25 mM in each incubation method, and samples were read at 1, 5 and 30 min intervals after NH_4Cl

addition. The order of NH_4Cl addition and initial reading was randomized among treatments, with NH_4Cl levels being read in a pair between CO_2 and Air incubation methods and all NH_4Cl treatments for the Air+1 incubation method read after the initial readings of the other two methods. Three replicates were performed.

Experiment 6: Re-evaluation of Effects of NH_4Cl Concentration on Stallion Sperm Motility in Glucose Modified Medium

Based on the results from Experiment 5, evaluation of the effect of NH_4Cl on stallion motility was repeated. The CO_2 concentration in the incubator was confirmed by Fyrite before each trial replicate. In addition, based on results from Experiment 5, higher concentrations of NH_4Cl and longer assessment times were used.

Three replicates were performed. Stallion spermatozoa were processed with M-Tyrodes with 0.02% PVA and 5 mM glucose and NH_4Cl was added in concentrations of 0, 5, 10, 25, 50 and 100 mM. Order of NH_4Cl addition to samples was randomized; samples were read at 1, 5, 30, 60, 90 and 120 min intervals after NH_4Cl addition.

Polymerase Chain Reaction

To further identify the presence of CatSper1 gene expression in the equine testis, polymerase chain reaction (PCR) and library screening were conducted courtesy of Dr. Nancy Ing and Ms. Cindy Balog Alvarez (Texas A&M University). For cloning of a partial cDNA of equine CatSper1, oligonucleotide primers were designed, using sequences of human CatSper (GenBank Accession Number NM_053054.4). Sense and

antisense primers were synthesized for the initial PCR: (5')TCTTCTGCATCTACGTGGTGGA and (5')CTCTTCTCCAGCCTCAAATGT. Sense and antisense nested primers were also synthesized: (5')GAATTCGGCTTATCCTCATGTTTACCTGCCTCTT and (5')GAATTCGGCTTACAATCTCATCGATGACGGA. Total cellular RNA (5µg), isolated from mature horse testis using TriPure Reagent (Roche, Indianapolis, IN), was reversed transcribed at 42˚C with Superscript II and oligo dT_{20} primers. PCR was performed with Taq DNA polymerase enzyme in a Perkin Elmer 9600 Thermal cycler. PCR reactions were incubated in 35 cycles of 94˚C for 45 seconds, 50˚C for 40 seconds, and 72˚C for 90 seconds. One eighth of the product of the initial PCR was used in subsequent reactions with the nested primers. The expected 496 base product was generated and subsequently cloned into a TA cloning vector pCR2.1 (Invitrogen). The PCR product was gel purified on an 0.8% low melting agarose gel, ligated into the pCR2.1 vector and transformed into One Shot competent cells (TA Cloning Kit, Invitrogen). Recombinant E. coli were grown and plasmid DNA was purified using QIAprep Miniprep kit. Restriction digest of plasmid DNA was performed using the EcoR I enzyme and sequencing analysis confirmed the presence and orientation of the PCR product.

Western Blot Analysis

Procedures for the western blot analysis were performed and provided by Dr. Lawrence Dangott of the Protein Chemistry Lab (Texas A&M University). All proteins

(except commercial human testis extract) were prepared by grinding the frozen tissues into powder under liquid nitrogen and then solubilizing them in a buffer containing 10 mM Tris (pH 7.5), 4% CHAPS. The proteins were solubilized on ice for 1 hour then subjected to centrifugation for 5 min at 13,000 x g at 4˚C. The supernatant materials were analyzed. The human testis extract was a commercial product obtained from ProSci Incorporated (Poway, CA). Equine, murine, bovine, and human proteins were separated on 12% SDS minigels. Proteins were electroblotted from gel to a polyvinylidene fluoride (PVDF) membrane using a CAPS buffer.

For antibody-antigen detection on the membrane, ABC Kits from Vector Laboratories (Burlingame, CA) were used. An alkaline peroxidase system, based on the source of the primary antibody, was used for all blots. All primary antibody dilutions were 1:750 (≈ 1 µg/ml).

Statistical Analysis

Mean values for each sperm motility parameter for each replicate were analyzed using two-way and three-way analysis of variance procedures with Sigma Stat 3.0 statistical software (SPSS Inc., Chicago, IL). The Holm Sidak method was used to examine all pairwise multiple comparisons among variables ($P < 0.05$). The Holm Sidak method was selected because it is a more stringent method, reducing the size of the allowable error for each comparison.

RESULTS

Over a period of 17 months, immunostaining was performed on 29 dates, incorporating 75 separate trials, and hyperactivated motility analyses were performed on 38 dates, incorporating 41 separate trials.

Immunocytochemistry Results

Identification and Localization of CatSper1 on Equine Spermatozoa

The intensity of fluorescence was evaluated using three preset exposure settings on a fluorescent microscope. The intensity of the signal varied among species and between the antibodies used. Using the C-terminus anti-Human CatSper1 antibody, stallion and bull sperm showed CatSper1 expression localized to the principal piece of the flagellum following both the Okunade and Ren procedures (Fig. 1). However, mouse sperm did not show specific staining using this antibody.

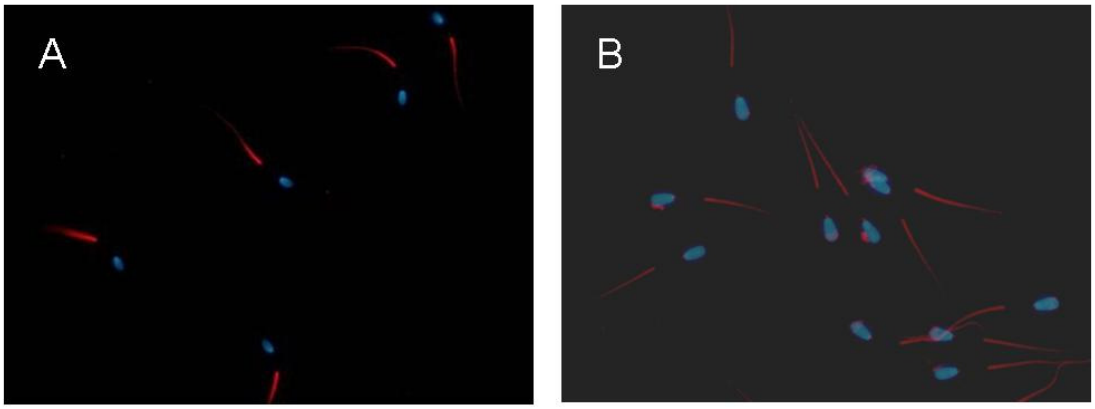

Fig. 1. Localization of CatSper1 to the plasma membrane of the principal piece of equine and bovine sperm. [DAPI- blue; CatSper1 antibody- red]. **A.** Immunostaining of stallion sperm using DAPI and the C-terminus anti-Human CatSper1 antibody following the Okunade procedure at 40x. **B.** Immunostaining of bovine sperm using DAPI and the C-terminus anti-Human CatSper1 antibody following the Okunade procedure at 40x.

When the N-terminus anti-Mouse antibody was used as the primary antibody, labeling was seen in the principal piece of mouse spermatozoa, as described by Ren et al. (2001; Fig 2). However, use of this primary antibody resulted in specific staining of the midpiece of stallion spermatozoa. Bull spermatozoa did not show specific staining with this antibody.

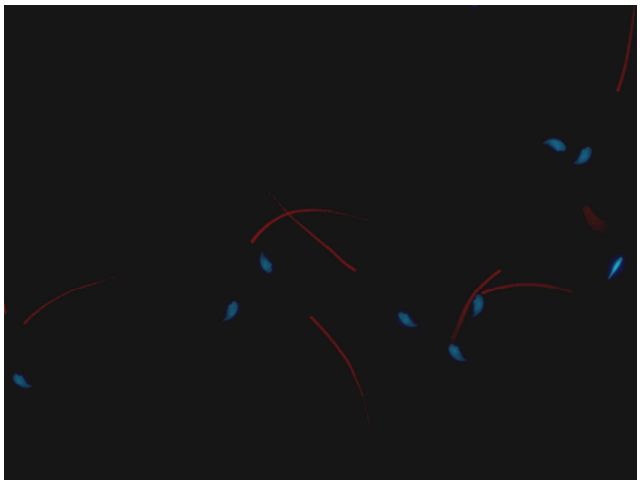

Fig. 2. Localization of CatSper1 to the principal piece of murine sperm. [DAPI- blue; CatSper1 antibody-red]. Immunostaining of mouse sperm using DAPI and the N-terminus anti-Mouse CatSper1 antibody following the Ren procedure at 40x.

Hyperactivated Semen Analysis Results

Preliminary Motility Studies

As noted above, three preliminary trials on fresh bovine spermatozoa and two preliminary trials on fresh stallion spermatozoa were conducted using the M-Tyrodes medium containing BSA, as described by Marquez et al. (2007) for motility analysis. The response of the bovine sperm to the different NH_4Cl concentrations was similar to the results described by Marquez et al. (2007), in that an increase in parameters indicative of hyperactivated motility, VCL and ALH, was seen within 5 min at concentrations of 25 and 50 mM NH_4Cl. However, stallion spermatozoa showed marked head-to-head agglutination after centrifugation in this medium, and motility analyses could not be performed.

In preliminary trials to identify a non-milk based medium that would support multiple centrifugations of stallion spermatozoa without inducing agglutination, use of PVA rather than BSA was found to result in reduced agglutination as assessed both visually and as estimated by evaluating concentration on the CASA system (measured concentration is reduced as sperm agglutinate when evaluated on this system); therefore medium with PVA was used in subsequent motility experiments.

Experiment 1: Effect of NH_4Cl on Hyperactivated Motility of Bovine Spermatozoa

The change in bovine spermatozoa motility parameters VCL and ALH in response to different concentrations of NH_4Cl is presented in Figs 3 and 4. There was no effect ($P > 0.1$) of medium (M-Tyrodes with BSA, 0.1% PVA or 0.02% PVA) on either parameter and there was no interaction of NH_4Cl and medium ($P > 0.1$), therefore data for all media were combined for further analyses.

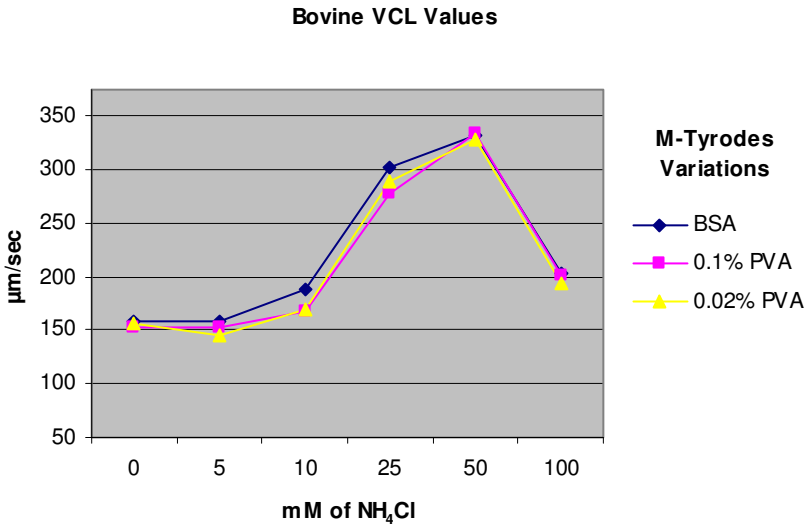

Fig. 3. Effects of NH_4Cl concentration on Curvilinear Velocity (VCL) values of bovine spermatozoa. Readings were taken 5 min after the addition of NH_4Cl.

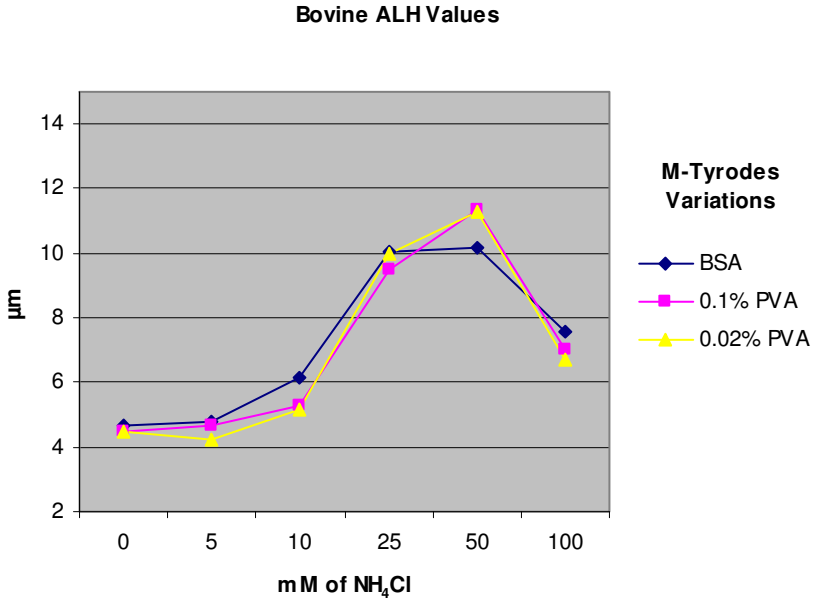

Fig. 4. Effects of NH_4Cl concentration on Amplitude of Lateral Head Movement (ALH) values of bovine spermatozoa. Readings were taken 5 min after the addition of NH_4Cl.

Readings were taken at time 0 (before addition of NH_4Cl) and at 1 and 5 min after NH_4Cl addition. Values at 1 and 5 min were compared to determine the optimum time for evaluation of bovine sperm response to this compound. There was no effect of time (1 vs. 5 min) on VCL or ALH values in the control treatment (0 mM NH_4Cl), but VCL values increased significantly between the 1 and 5 min readings for 25 and 50 mM NH_4Cl treatments ($P < 0.001$ and $P = 0.026$, respectively). Values for ALH did not change significantly from 1 to 5 min for any NH_4Cl concentration.

Because of the significantly higher values of VCL noted at the 5 min readings, this time point was used for analysis of the effects of NH_4Cl concentration. There was no significant difference in VCL between 0 and 10 mM NH_4Cl ($P > 0.1$). There was a significant rise in VCL between 10 and 25 mM NH_4Cl ($P < 0.001$) and also between 25 and 50 mM NH_4Cl ($P < 0.05$). However, VCL values for 100 mM NH_4Cl were significantly lower than those for 50 mM NH_4Cl ($P < 0.001$). Values for ALH showed a significant rise between 0 and 10 mM NH_4Cl treatments, and again between 10 and 25 mM NH_4Cl ($P < 0.001$). However, there was no significant difference between 25 and 50 mM NH_4Cl ($P>0.1$). As found for VCL, ALH values at 5 min for 100 mM NH_4Cl were significantly lower than those for 50 mM NH_4Cl ($P < 0.001$). Thus, the highest values for VCL and ALH in bovine sperm were achieved in the 25 to 50 mM NH_4Cl treatments.

Experiment 2: Effect of NH_4Cl on Hyperactivated Motility of Stallion Spermatozoa

Three replicates were performed assessing stallion spermatozoa in M-Tyrodes with either 0.1% PVA or 0.02% PVA. There was no effect of medium and no interaction of medium with NH_4Cl concentration ($P > 0.1$) on any parameter values.

Medium with 0.02% PVA was chosen for use in subsequent studies, therefore in this study, data for effects of NH_4Cl and time were analyzed in sperm suspended in 0.02% PVA medium. There was a significant effect of time on motility parameters. Values for VCL (Fig 5) in control sperm (0 mM NH_4Cl) increased significantly between 1 and 30 min ($P < 0.05$). In the 5 and 10 mM NH_4Cl treatments, there was a significant increase in VCL values between 1 and 5 min ($P = 0.01$ and $P < 0.001$, respectively), but not between 5 and 30 min ($P = 0.776$ and $P = 0.648$, respectively). Values for VCL in the 25 mM NH_4Cl treatment increased significantly between 1 and 5 min ($P < 0.001$), then significantly decreased between 5 and 30 min ($P = 0.037$).

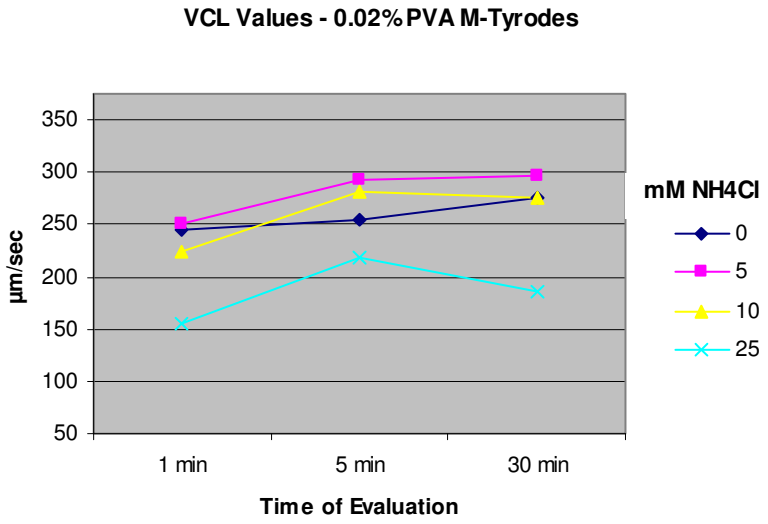

Fig. 5. Effects of time on Curvilinear Velocity (VCL) values of stallion spermatozoa in M-Tyrodes with 0.02% PVA at varying concentrations of NH_4Cl evaluated at 1, 5, and 30 min.

As found for VCL, control values for ALH increased significantly over time ($P < 0.05$; Fig. 6). Highest ALH values for all NH_4Cl concentrations other than 0 were seen at 5 min, although these were not significantly different from those at 1 or 30 min.

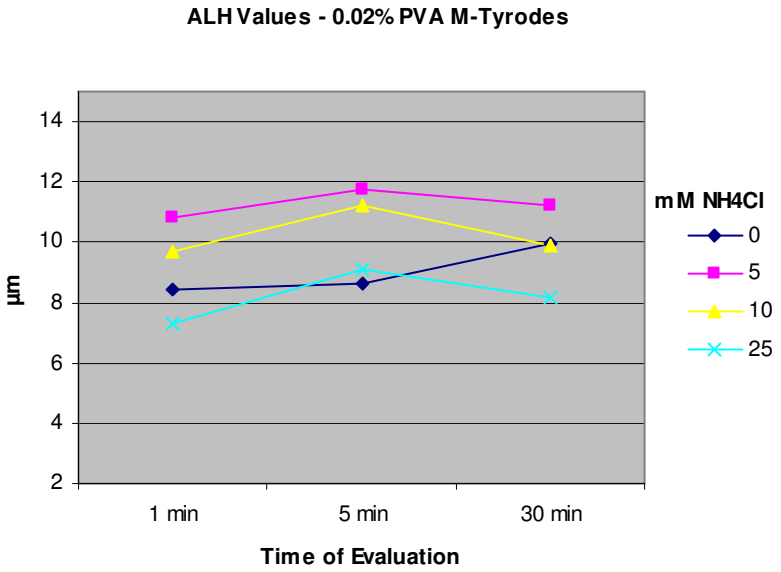

Fig. 6. Effects of time on Amplitude of Lateral Head Movement (ALH) values of stallion spermatozoa in M-Tyrodes with 0.02% PVA at varying concentrations of NH_4Cl evaluated at 1, 5, and 30 min.

Because the highest values for VCL were found at the 5 min assessment, this time period was chosen for evaluation of the effect of NH_4Cl concentration. When assessed at 5 min, the 5 mM NH_4Cl treatment yielded significantly higher VCL values than did the control ($P < 0.05$) and 25 mM NH_4Cl ($P < 0.001$) treatments, however, 5 mM was not significantly different from 10 mM NH_4Cl ($P>0.1$).

Similarly, when evaluated at 5 min, the highest ALH value was achieved in the 5 mM NH_4Cl treatment; this was significantly higher than the values for the control and 25 mM NH_4Cl treatments at this time ($P < 0.001$).

Experiment 3: Further Determination of Optimal NH_4Cl Concentration to Induce Hyperactivated Stallion Sperm Motility

To determine more precisely the concentration of NH_4Cl inducing the highest motility response, an additional trial was conducted to evaluate 6 different concentrations of NH_4Cl between 1.7 and 10 mM, with evaluation of sperm motility performed at 5 min after NH_4Cl addition. Three replicates were performed.

All NH_4Cl concentrations tested resulted in significantly higher motility values than did the control treatment ($P \leq 0.01$; Table 1). There were no significant differences in motility parameters among concentrations higher than 0 mM NH_4Cl ($P>0.1$), with the exception that the VCL value for 3.4 mM NH_4Cl was significantly higher than 10 mM NH_4Cl ($P < 0.05$).

Table 1 Comparison of NH_4Cl concentrations on VCL and ALH values in M-Tyrodes with 0.02% PVA.

	VCL (µm/sec)	ALH (µm)
NH_4Cl Concentration (mM)	5 min	5 min
0	247.47 ± 9.16*	8.47 ± 0.49*
1.7	285.37 ± 25.52	11.13 ± 1.21
3.4	287.97 ± 3.14 [a]	11.40 ± 0.36
5	280.30 ± 4.41	11.27 ± 0.31
6.7	281.57 ± 6.94	11.17 ± 0.67
8.4	277.53 ± 19.33	10.90 ± 1.06
10	264.50 ± 4.79 [b]	10.50 ± 0.3

Average Curvilinear Velocity (VCL) and Amplitude of Lateral Head Movement (ALH) values at 5 min time of evaluation of stallion sperm in M-Tyrodes with 0.02% PVA at varying concentrations of NH_4Cl. Data are shown as mean ± std. dev. a vs b: $P < 0.05$. *, significantly different than all other values for that parameter.

Experiment 4: Effects of Glucose Concentration on Stallion Sperm Motility

Sperm were evaluated for changes in motility parameters 5 min after addition of 5 mM NH_4Cl. Medium glucose concentration did not significantly affect motility parameters before addition of NH_4Cl. Medium glucose concentration had a significant effect on both VCL and ALH values after addition of NH_4Cl.

The presence of glucose increased VCL values after 5 min of incubation with 5 mM NH_4Cl (Fig. 7) at 5 mM ($P = 0.07$) and 10, 15, and 20 mM ($P < 0.05$). Values for VCL for the 5 mM glucose treatment were not significantly different from those for the 10 mM glucose treatment ($P > 0.1$).

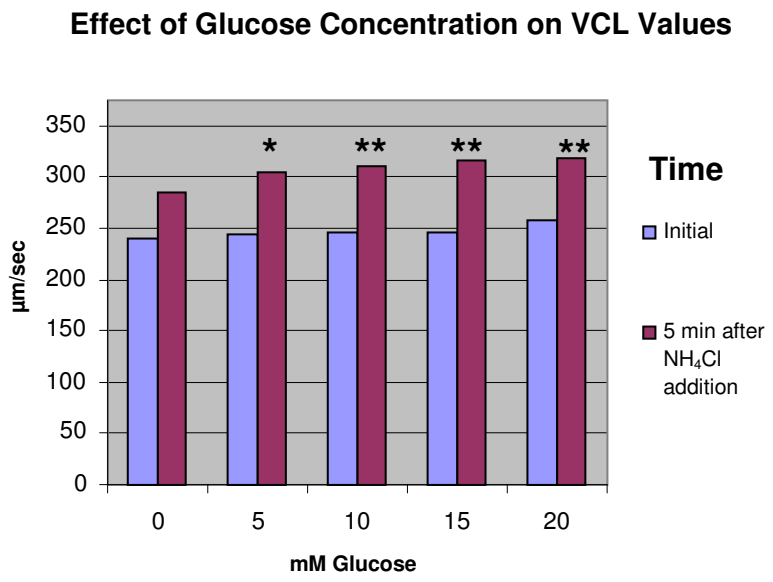

Fig. 7. Average Curvilinear Velocity (VCL) values of stallion sperm in M-Tyrodes with 0.02% PVA at varying concentrations of glucose prior to addition of 5 mM NH_4Cl and 5 min post NH_4Cl addition. Differs from control: * $P = 0.07$; ** $P < 0.05$.

Similar to VCL values, ALH values after NH_4Cl addition (Fig. 8) were significantly higher in 5 mM than in 0 mM glucose ($P < 0.001$). The values for ALH in the 5 mM glucose treatment were not significantly different from those for 10 and 15 mM glucose ($P>0.1$) but tended to be lower than those for 20 mM glucose ($P = 0.06$).

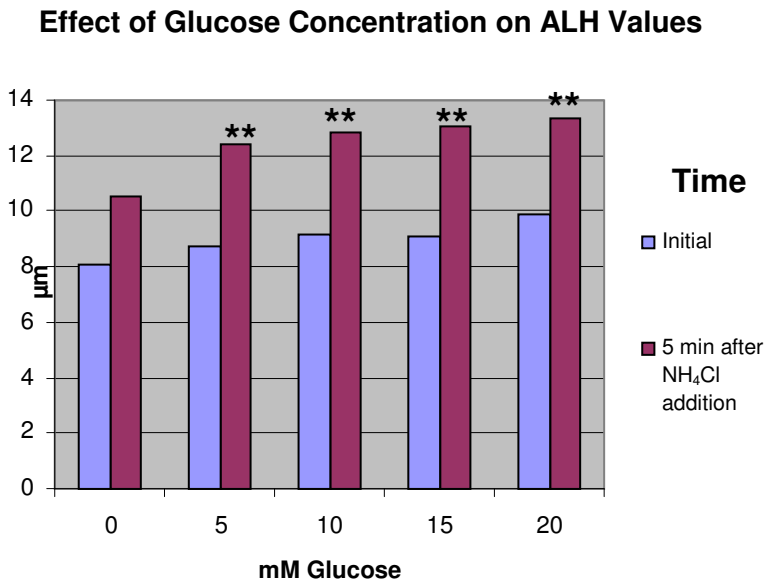

Fig. 8. Average Amplitude of Lateral Head Movement (ALH) values of stallion sperm in M-Tyrodes with 0.02% PVA at varying concentrations of glucose prior to addition of 5mM NH_4Cl and 5 min post NH_4Cl addition. Differs from control: ** $P < 0.001$.

Experiment 5: Effects of CO_2 Environment on Response to NH_4Cl

This study determined the effect of CO_2 exposure on sperm motility and was conducted after discovery of possible compromise of incubator CO_2 concentrations during performance of the previous studies (see Materials and Methods). Values for VCL for the different NH_4Cl concentrations and CO_2 environments are given in Fig. 9. At 1 and 30 minutes, there was a significant suppressive effect of CO_2 incubation exposure on VCL values in the control treatment (0 mM NH_4Cl; $P < 0.01$).

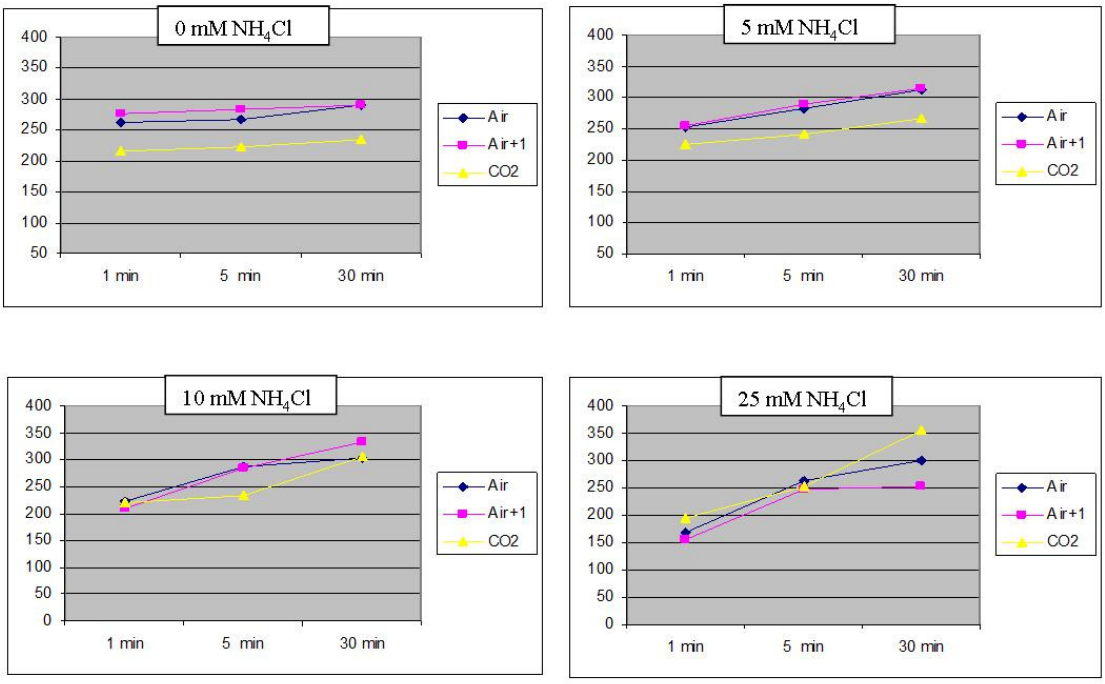

Fig. 9. Curvilinear velocity (VCL) values of stallion sperm in M-Tyrodes with 0.02% PVA and 5 mM glucose sustained in various CO_2 environments with 0, 5, 10, and 25 mM NH_4Cl treatments, at 1, 5 and 30 min after NH_4Cl addition.

At 30 minutes, VCL values for the CO_2 environment rose to become significantly higher than those for the Air+1 and Air incubation methods ($P < 0.001$ and $P < 0.05$, respectively) within the 25 mM NH_4Cl treatment. There was no significant difference in incubation methods within the 5 or 10 mM NH_4Cl treatments at 30 min ($P>0.05$).

Experiment 6: Re-evaluation of Effects of NH_4Cl Concentration on Stallion Sperm Motility in Glucose Modified Medium

The effect of time and NH_4Cl concentration on motility parameters was re-examined (cf. Experiment 2). All recorded motility parameters, including VCL, ALH, LIN, straightness (STR), average path velocity (VAP), straight line velocity (VSL), beat cross frequency (BCF), total motility, progressive motility, rapid motility, medium motility, slow motility, static motility, total cell count, and total concentration were examined at the end of this study to determine if there were any additional measures responsive to NH_4Cl treatment. Graphs of all motility parameters for this study are presented in Figs. 10 to 24. Values for VSL, VAP and progressive motility did show NH_4Cl dose-dependent changes reflective of those for VCL and ALH, but the overall change from control values were not as vivid; therefore we continued to use VCL (Fig. 10) and ALH (Fig. 11) for estimation of changes denoting hyperactivated motility.

Curvilinear Velocity (VCL)

The control treatment (0 mM NH_4Cl) showed no significant change in VCL (Fig 10) over time (1 to 120 min). At the 1 min reading, values for VCL were depressed in a dose-dependent fashion. At 1 min, the VCL values for 25, 50, and 100 mM NH_4Cl treatments were significantly lower than those for the control treatment ($P < 0.001$). Thereafter, there was a dose-dependent increase in VCL, with the 25 and 50 mM treatments achieving the highest values. The VCL values for both 25 and 50 mM treatments rose significantly between 1 and 5 min and between 5 and 30 min ($P <$

0.001), and were significantly higher than control VCL values at 30 min ($P < 0.001$). The VCL values for 25 mM NH_4Cl continued to rise significantly between 30 and 60 min ($P < 0.05$), then remained unchanged to 120 min ($P > 0.1$), whereas values for the 50 mM NH_4Cl treatment decreased significantly between 60 and 90 min ($P < 0.01$). At 60 min, VCL values for 25 mM NH_4Cl were significantly higher than those for 50 mM NH_4Cl ($P = 0.001$). The VCL values for the 100 mM NH_4Cl treatment were significantly lower than those for the control treatment at all time points ($P < 0.01$).

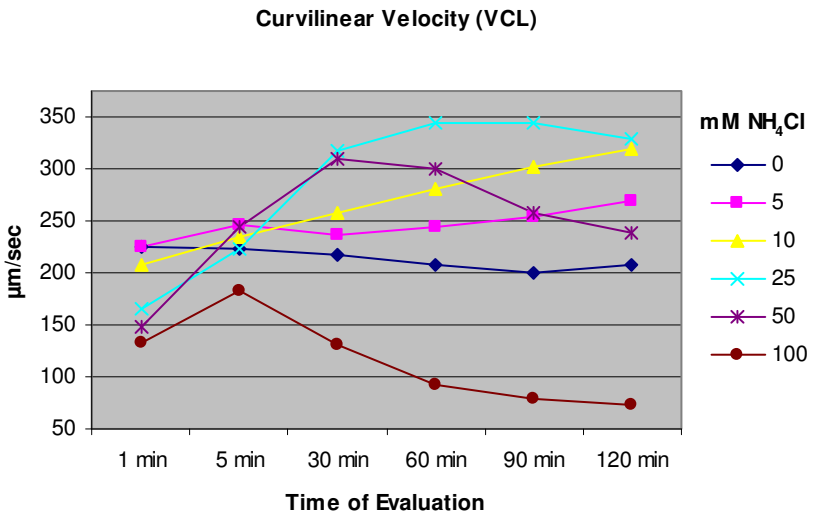

Fig. 10. Response of stallion spermatozoa Curvilinear Velocity (VCL) values to NH_4Cl within M-Tyrodes containing 0.02% PVA and 5 mM glucose.

Lateral Head Movement (ALH)

The control treatment (0 mM NH_4Cl) showed no significant change in ALH (Fig. 11) over time. As seen previously for VCL, there was an initial dose-dependent depression in ALH values. At 1 min the ALH values for both 25 and 50 mM NH_4Cl treatments were significantly lower than those for the control treatment ($P < 0.05$ and $P < 0.001$, respectively), and the 100 mM NH_4Cl treatment was also significantly lower than that for the control treatment ($P < 0.001$). Thereafter, the 25 and 50 mM NH_4Cl treatments achieved the highest ALH values. Values for ALH for both 25 and 50 mM increased significantly between 1 and 5 min and between 5 and 30 min ($P < 0.001$). Both 25 and 50 mM NH_4Cl were significantly higher than the control by 5 min ($P < 0.05$ and $P < 0.001$, respectively). The ALH values for 25 mM NH_4Cl remain unchanged from 30 to 120 min, whereas in the 50 mM treatment, values for ALH fell significantly between 60 and 90 min ($P < 0.01$). At 60 min, ALH values for 25 mM NH_4Cl were significantly higher than those for 50 mM NH_4Cl ($P < 0.05$). Values for ALH in the 100 mM NH_4Cl treatment were significantly lower than the control at all time periods other than 5 min ($P>0.05$).

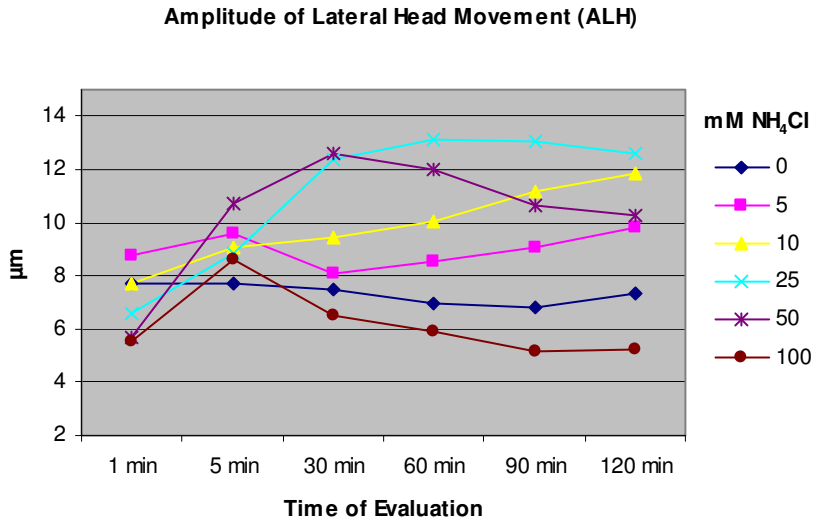

Fig. 11. Response of stallion spermatozoa Amplitude of Lateral Head Movement (ALH) values to NH_4Cl within M-Tyrodes containing 0.02% PVA and 5 mM glucose.

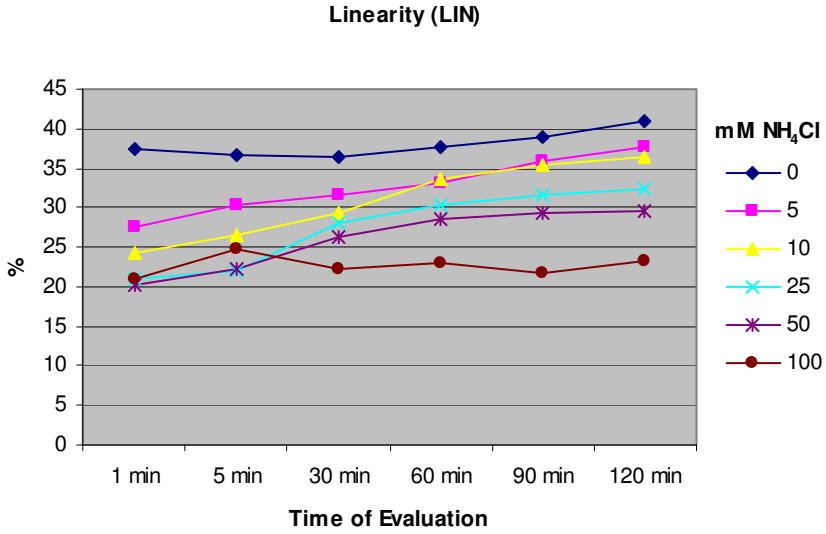

Fig. 12. Response of stallion spermatozoa Linearity (LIN) values to NH_4Cl within M-Tyrodes containing 0.02% PVA and 5 mM glucose.

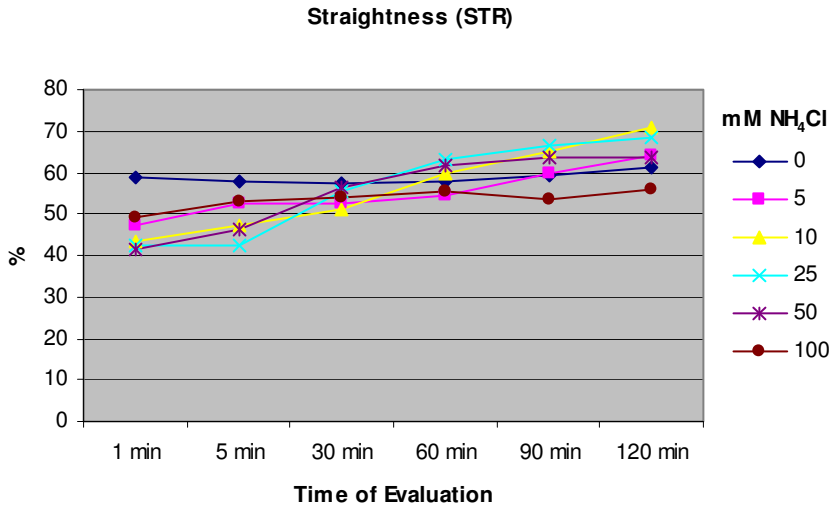

Fig. 13. Response of stallion spermatozoa Straightness (STR) values to NH_4Cl within M-Tyrodes containing 0.02% PVA and 5 mM glucose.

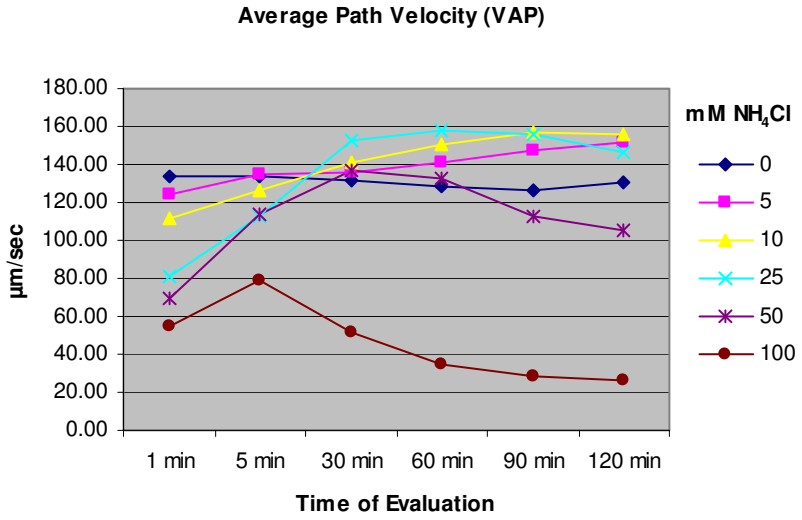

Fig. 14. Response of stallion spermatozoa Average Path Velocity (VAP) values to NH_4Cl within M-Tyrodes containing 0.02% PVA and 5 mM glucose.

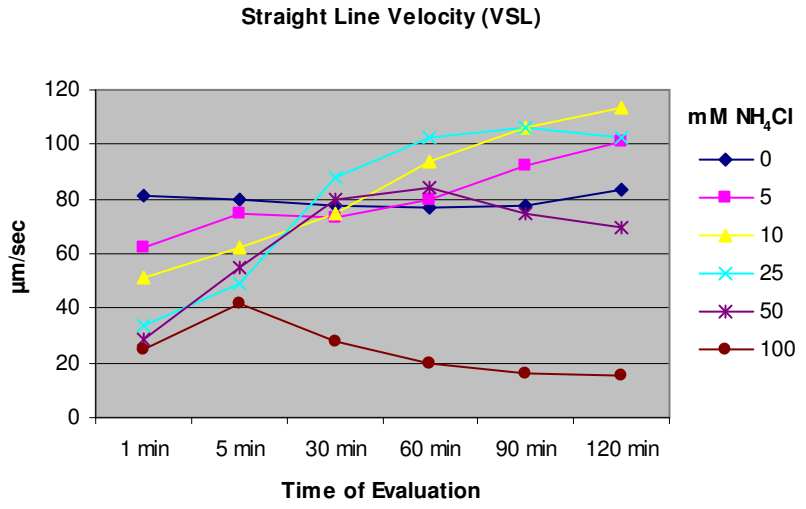

Fig. 15. Response of stallion spermatozoa Straight Line Velocity (VSL) values to NH_4Cl within M-Tyrodes containing 0.02% PVA and 5 mM glucose.

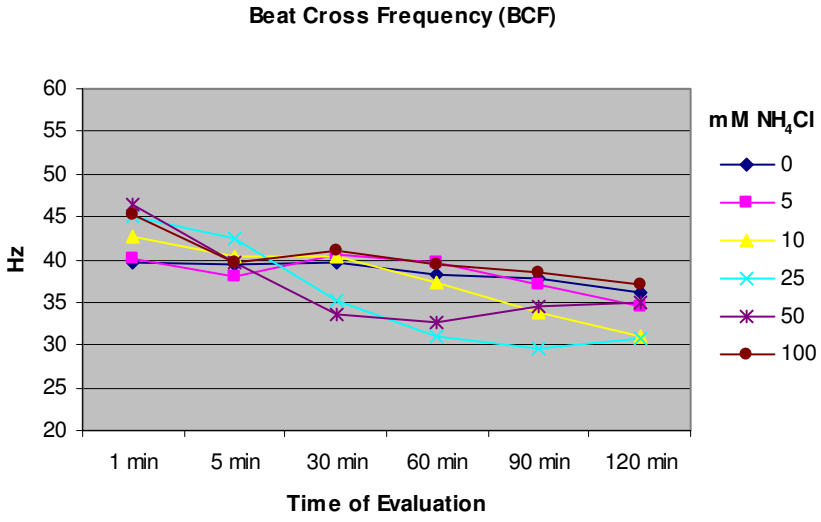

Fig. 16. Response of stallion spermatozoa Beat Cross Frequency (BCF) values to NH_4Cl within M-Tyrodes containing 0.02% PVA and 5 mM glucose.

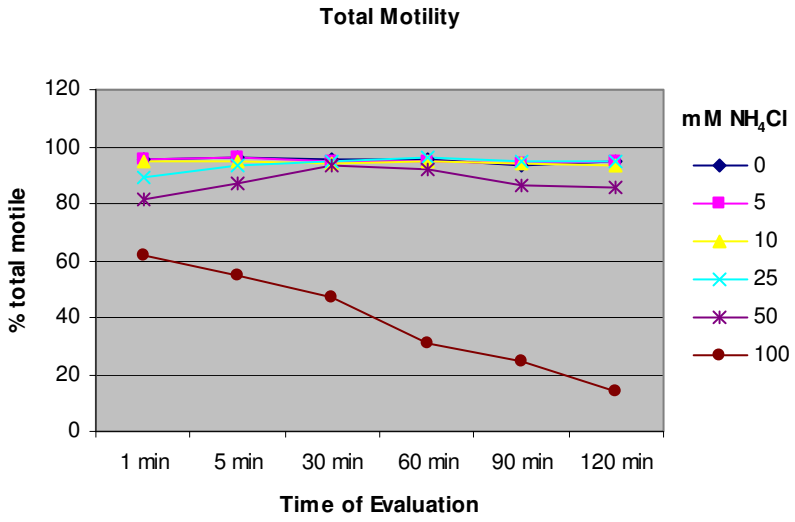

Fig. 17. Response of stallion spermatozoa Total Motility values to NH_4Cl within M-Tyrodes containing 0.02% PVA and 5 mM glucose.

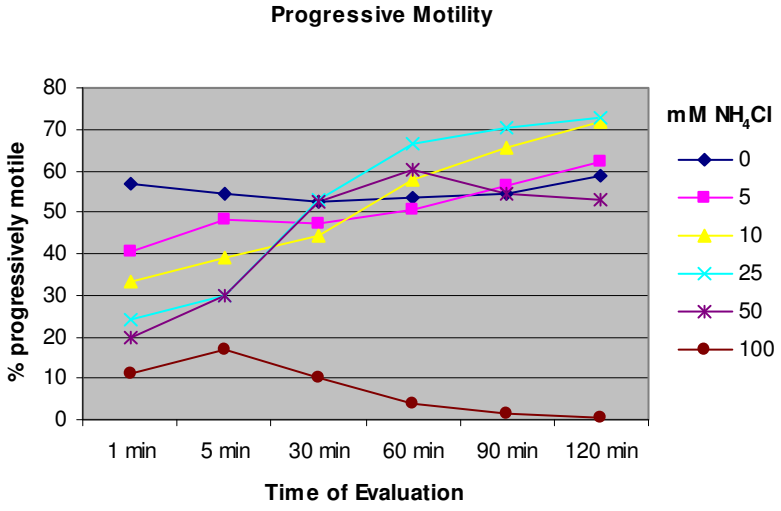

Fig. 18. Response of stallion spermatozoa Progressive Motility values to NH_4Cl within M-Tyrodes containing 0.02% PVA and 5 mM glucose.

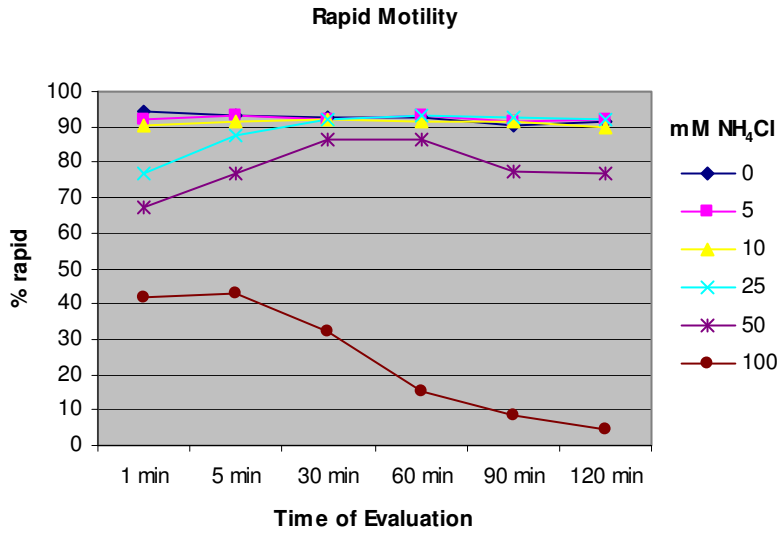

Fig. 19. Response of stallion spermatozoa Rapid Motility values to NH_4Cl within M-Tyrodes containing 0.02% PVA and 5 mM glucose.

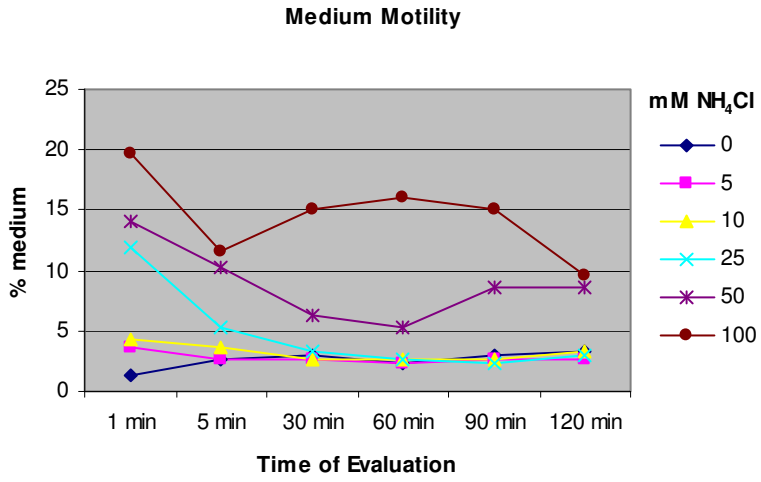

Fig. 20. Response of stallion spermatozoa Medium Motility values to NH_4Cl within M-Tyrodes containing 0.02% PVA and 5 mM glucose.

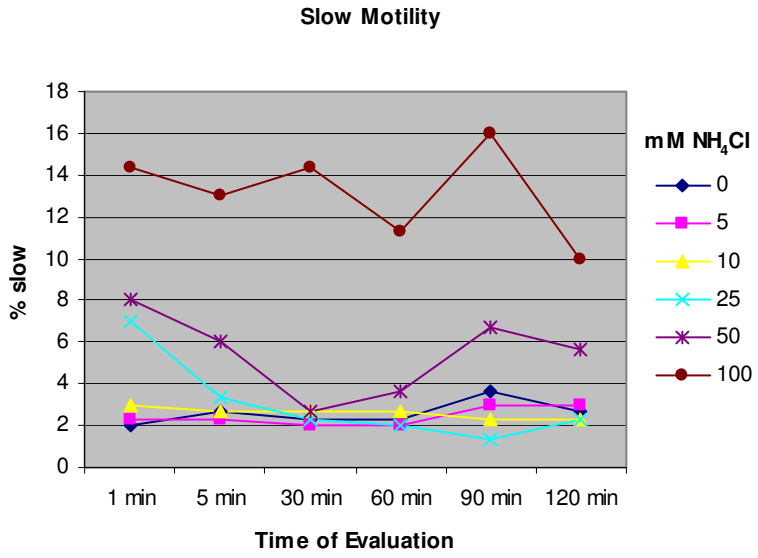

Fig. 21. Response of stallion spermatozoa Slow Motility values to NH_4Cl within M-Tyrodes containing 0.02% PVA and 5 mM glucose.

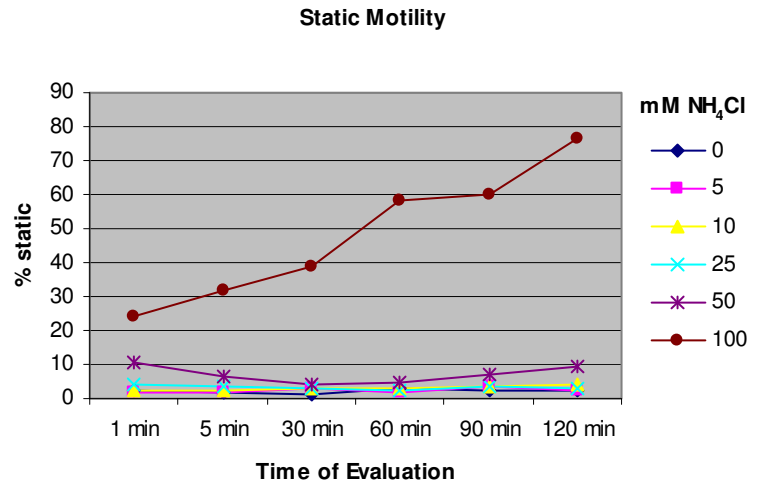

Fig. 22. Response of stallion spermatozoa Static Motility values to NH_4Cl within M-Tyrodes containing 0.02% PVA and 5 mM glucose.

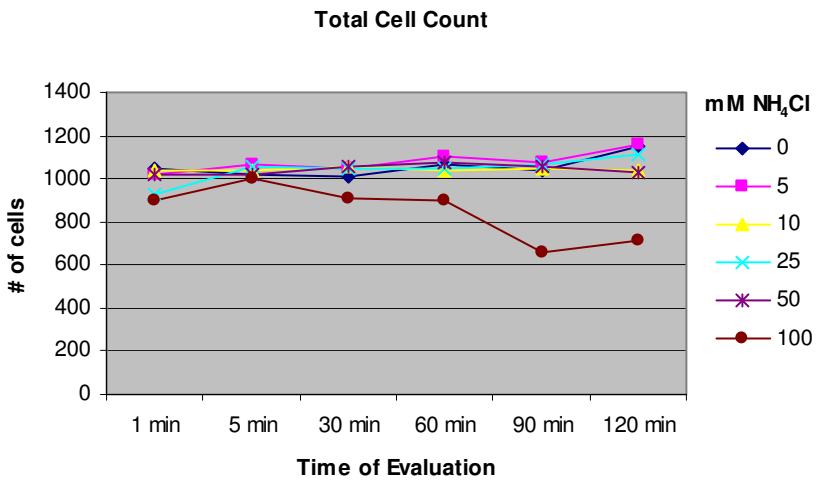

Fig. 23. Response of stallion spermatozoa Total Cell Count values to NH_4Cl within M-Tyrodes containing 0.02% PVA and 5 mM glucose.

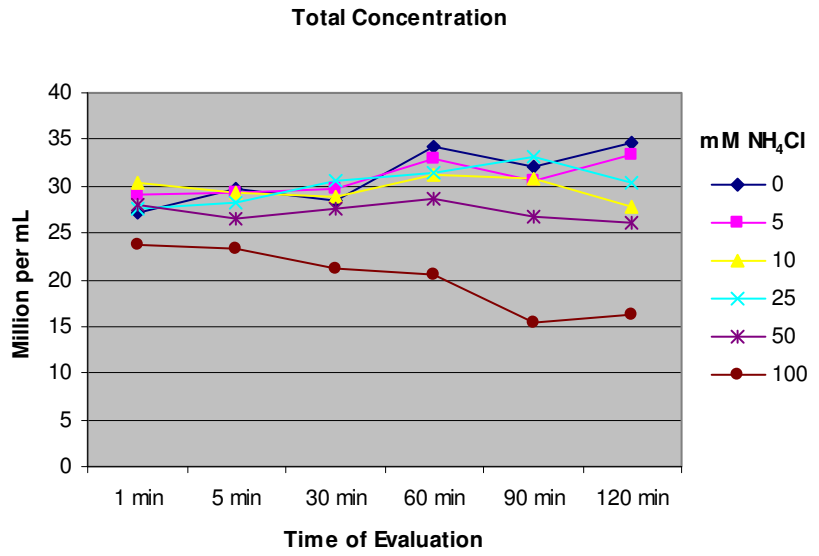

Fig. 24. Response of stallion spermatozoa Total Concentration values to NH_4Cl within M-Tyrodes containing 0.02% PVA and 5 mM glucose.

Polymerase Chain Reaction Results

In order to further document the presence of CatSper in equine testis tissue and equine spermatozoa, PCR was conducted. Testing showed mRNA for CatSper1 was found in the stallion testis. Sequence for the horse CatSper cDNA was 89%, 87%, 85%, and 80% identical to the canine, bovine, human and murine sequences. Amino acid CatSper sequences were similar with residues identical at 90%, 87%, 83% and 76% identical to the canine, bovine, human and murine protein sequences.

Western Blot Analysis Results

A control blot served as a positive control on human testis lysate tissue with the C-terminus anti-Human CatSper1 antibody. The C-terminus anti-Human CatSper1 antibody produced a single band of ~70 kDA on equine testis lysate and equine sperm lysate as seen in Figs 25 and 26. Blot analysis of equine heart and liver extracts showed no reaction to the antibodies.

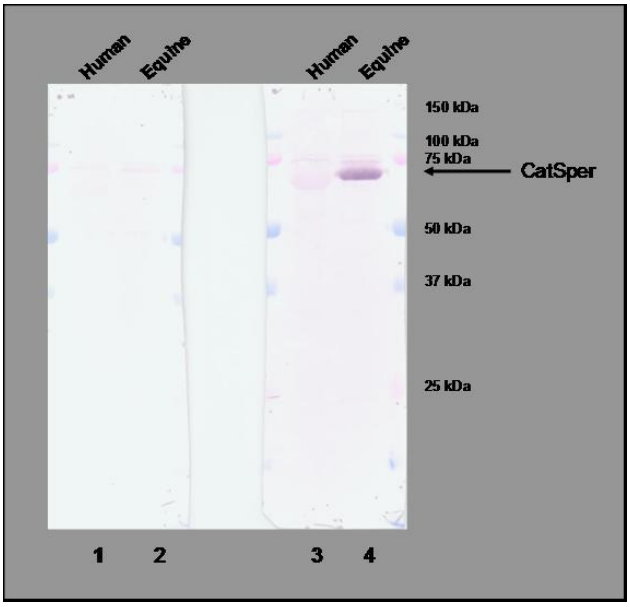

Fig. 25. The restricted expression pattern of CatSper1 in testis and sperm: Human and Equine Testis Lysate. Western blot of CatSper1 protein using anti-human CatSper antibody. Lanes 1 and 2 used as a control blot containing no primary antibody loaded with human and equine testis lysate. Lanes 3 and 4 were loaded with human and equine testis lysate, respectively.

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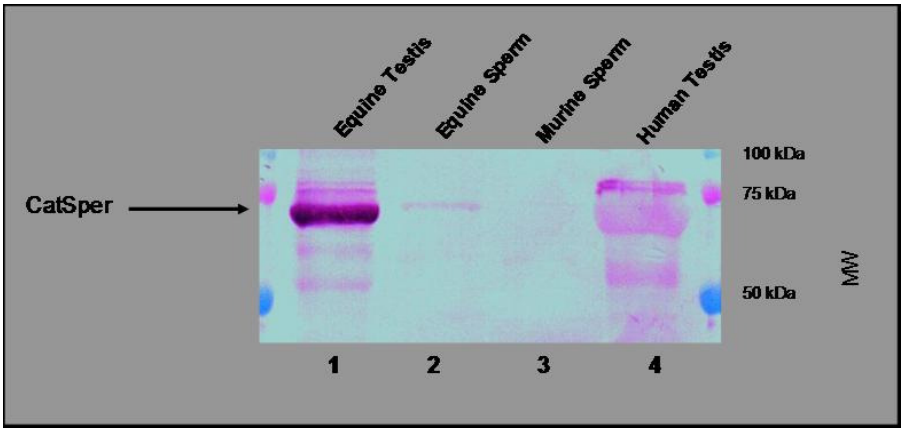

Fig. 26. The restricted expression pattern of CatSper1 in testis and sperm: Human and Equine Testis Lysate and Equine and Murine Sperm Lysate. Western blot of CatSper1 protein using anti-human CatSper antibody applied to all lanes. Lanes 1 through 4 were loaded with equine testis lysate, equine sperm lysate, murine sperm lysate and human testis lysate, respectively.

DISCUSSION

These experiments are the first to establish the existence of the CatSper1 protein in equine spermatozoa, and to identify factors affecting the induction of hyperactivated-like motility in these sperm. The results of this research demonstrate that CatSper1 protein is present and located on the principal piece of equine spermatozoa, that equine sperm show hyperactivated-like motility patterns after treatment with NH_4Cl to induce intracellular alkalinization, and that equine sperm hyperactivation appears to have different kinetics than does that of bovine sperm.

Our immunocytochemistry studies showed that the localization of CatSper1 in equine sperm is consistent with that in other species, such as the mouse and bull. Both the stallion and bull sperm labeled strongly and specifically in the principal piece using the C-terminus ant-Human antibody, whereas mouse sperm labeled similarly with the N-terminus anti-Mouse antibody raised against a GST-fusion protein consisting of the first 150 amino acids of mouse CatSper1. Mouse sperm stained more clearly with the protocol used by Ren et al. (2001), whereas in the bull and stallion, staining was similar when either the Okunade or Ren protocol was employed.

The difference in antibody labeling results may be clarified using the National Center for Biotechnology Information Basic Local Alignment Search Tool (NCBI BLAST). When querying the translated equine genome with the human CatSper protein, the putative equine CatSper aligns well with the human protein, including the C-terminus to which the anti-Human antibody is directed. However, when the translated

equine genome is queried with the mouse CatSper protein, there is no alignment of the putative equine CatSper with the N-terminus region of the mouse protein. The anti-Mouse antibody is directed at the first one hundred and fifty amino acids of the mouse protein and thus does not recognize the equine protein.

Our preliminary motility studies showed that the Tyrodes medium utilized by Marquez et al. (2007) was not an appropriate medium for equine sperm centrifugation and evaluation of motility, due to the head-to-head aggregation of sperm, especially after the second centrifugation. Aggregation of sperm cells was noted both visually (microscopically) as well as by CASA, on which sperm concentration decreased markedly over time. This agglutination of the sperm severely hindered the ability to evaluate motility or hyperactivity. Aggregation of the sperm cells after centrifugation has been noted previously (D. Varner and C. Love, personal communication, 2008) and is typically avoided by use of skim-milk containing media. However, skim-milk components may affect other aspects of induction of hyperactivated motility and its evaluation. Therefore, defined alternative media were explored, leading to our use of PVA in place of BSA within M-Tyrodes. Equine spermatozoa centrifuged in medium containing PVA showed markedly less agglutination and maintained motility and concentration when evaluated by CASA.

A bovine hyperactivated motility study was conducted based on the methods used by Marquez et al. (2007) to validate the procedure in our laboratory and to determine whether exchange of PVA for BSA affected response of bovine sperm to NH_4Cl. The rise in VCL and ALH values with 25 mM NH_4Cl in our study was

comparable to the results achieved by Marquez et al. (2007). However, whereas the previous authors noted that maximum values were achieved with 25 mM, in our study there was a slight but significant rise in VCL values between the 25 and 50 mM NH_4Cl treatments. The 0 mM NH_4Cl control treatment in our studies possessed lower baseline values than those reported by Marquez et al. (2007), resulting in a greater degree of rise in motility parameter values.

Marquez et al. (2007) evaluated bovine sperm within 2 min of NH_4Cl addition, which is equivalent to our 1 min reading, as this was read after 1 min incubation. In contrast, we found significantly higher VCL values were achieved at the 5 min evaluation than at the 1 min, although ALH values showed no significant difference between the 1 and 5 min times of evaluation. Similar to the findings of Marquez et al. (2007), ALH showed peak values at 25 mM NH_4Cl in our study.

The similar response of bull spermatozoa to NH_4Cl in BSA and PVA-containing media validated the use of PVA-containing medium for assessment of stallion sperm response. We found in Experiment 2 that stallion sperm appeared to require a markedly lower dosage of NH_4Cl, i.e, 5 mM, than that needed by bovine sperm (25 mM) to elicit maximal motility parameter values. However, the change in motility values for the stallion sperm were not as great as those we found for bovine sperm in Exp. 1. For each concentration of NH_4Cl studied in stallion sperm in Exp. 2, maximum values were obtained at the 5 min reading; values decreased at the 30 min reading. For this reason, 5 min was chosen as the time for reading of motility in further studies. Interestingly, in this study, we found a significant increase in the control (0 mM NH_4Cl) VCL values

between 1 and 30 min times of evaluation, suggesting an increase in hyperactivated-like motility of the stallion spermatozoa over time in media without addition of NH_4Cl.

Experiment 3 was conducted to determine more precisely the optimum level of NH_4Cl for maximum response of hyperactivated-like motility parameters. Since this study showed that there was no significant difference in response among 1.7 to 8.4 mM NH_4Cl treatments, and that all concentrations of NH_4Cl induced significantly higher VCL and ALH values than those for controls, we continued to use 5 mM NH_4Cl for Experiment 4.

Results of Experiment 4 showed that glucose addition did not affect hyperactivated-like motility directly, but did significantly enhance the response of equine sperm to NH_4Cl. While the effects of glucose on equine capacitation have yet to be determined, the presence of the compound appears to enhance hyperactivated-like motility in the stallion.

Experiments 1 to 4 were concluded in the early Fall of 2007. In late spring 2008, we planned to compare pH-induced hyperactive motility in stallion spermatozoa to that induced by calcium ionophore A23187. However, when we attempted to assess motility using our original protocol (5 mM NH_4Cl in medium containing 5 mM glucose, assessed at 5 min after NH_4Cl addition) as a control for the ionophore studies, we were unable to replicate the results of the previous year; ALH and VCL values barely changed in the treated medium. To determine the cause of variation between experiments from 2007 and 2008, several procedural checks were run to ensure quality control among trials. Factors evaluated included centrifugation methods, semen handling and processing, and

CASA settings. Stallion variability was ruled out by using sperm from the same stallion used in 2007 studies. Further emphasis was placed on the media, M-Tyrodes; several stock and working batches were reconstructed all delivering the same uncharacteristic results. New media components, including NH_4Cl and sodium lactate, were purchased to rule out effects from compound contamination and expiration. New pipettes were also used along with sterile pipette tips to ensure accurate measurement of NH_4Cl and to eliminate possible contamination. Lastly, the CO_2 incubator at the Equine Pavilion was examined through temperature testing and Fyrite analysis. The temperature and CO_2 concentrations were within acceptable limits (36 ^{o}C and 4.5 to 5% CO_2).

Because the incubator was an injection-type (without CO_2 censor) and there had been some question on the integrity of the incubator door in 2007, it was hypothesized that the CO_2 incubator may have been the source of the discrepant results between Experiments 2 through 4 in 2007, and the preliminary data in 2008. Experiment 1, with bull sperm, would not have been affected by any malfunction of the incubator at the Pavilion, as media for this experiment were maintained the in CO_2 incubator in the Equine Embryo Laboratory until the arrival of the bull semen to the laboratory, then the trial was run immediately afterward. However, in the horse sperm trials, after equilibration in the incubator at the Equine Embryo Laboratory, the medium was brought to the Pavilion and placed, open, in the incubator for a variable period of time until the semen was collected from the stallion, and then kept in the incubator for additional time during semen processing, for a total of up to 2 hours before the trials were run, then for up to 2 hours during the running of the trials. Medium pH values

were obtained before and after the experiments in 2007 to check on adequate pH maintenance during the experiments, as we worried that the multiple openings and closing of the incubator door could either cause CO_2 loss, or alternatively CO_2 overload as the incubator attempted to quickly restore CO_2 levels. The pH measurements showed no notable change in pH over the course of the experiments. However, the incubator was not tested with a Fyrite in 2007.

Experiment 5 was conducted to evaluate whether performance of the NH_4Cl dose-response study in the presence or absence of CO_2 could affect results. This experiment yielded vividly different results from prior experiments. The three replicates in this trial presented very similar data, whereas there had been a large amount of variability among replicates in Experiments 2 to 4. The baseline values for VCL and ALH in the CO_2 environment were lower than those seen previously (e.g. ALH of 7.7, vs. 8.1 at 1 min in Experiment 4) and the response to NH_4Cl was higher than that achieved in the 2007 experiments (e.g. maximum of 287 and 11.4 for VCL and ALH in Experiment 4, vs. 351.4 and 13.6 for these parameters in Experiment 5). Motility parameters for the control treatment did not change over time in the CO_2 environment, whereas they had increased over time in Experiments 2 to 4. In addition, we found that the requirements for inducing maximal hyperactivated motility differed in the CO_2 treatment, in that the highest values of ALH and VCL were achieved with the maximum concentration used, 25 mM NH_4Cl, and were seen at the latest assessment time, 30 min after addition of this compound. Due to this discovery it was decided to repeat earlier trials on response of equine sperm to NH_4Cl, using a tested CO_2 environment, higher

amounts of NH_4Cl, and a longer time interval, to ensure an accurate evaluation of the effects of NH_4Cl on equine hyperactivated-like motility.

In Experiment 6, several CASA-evaluated motility parameters including average path velocity (VAP) and straight line velocity (VSL) changed over time. Values for VAP demonstrated a significantly higher value at 25 mM NH_4Cl from the control at 30 min, however, the changes in these parameters were less vivid and did not show a clear dose-dependent response, in contrast to the findings for VCL and ALH. LIN should decrease as hyperactivated motility increases, and was used by Marquez et al. (2007) as an indicator of hyperactive motility. However, it was not possible to differentiate a decrease in LIN, or a low LIN value, caused by cell death (as seen for the 100 mM treatment in this study, which suppressed all sperm motility) from that which could be attributed to hyperactive motility.

Data from Experiment 6 demonstrated that the greatest values for hyperactivated-like motility occurred at 25 mM NH_4Cl at 60 min, and this is our recommendation for the most appropriate treatment to assess hyperactivated motility in equine spermatozoa in future studies.

CatSper1 gene expression was shown in the equine testis by polymerase chain reaction. The sequence of the horse CatSper cDNA (496 bases; GenBank Accession Number EU864033) was most similar to the dog, bovine, and human sequences. The inferred amino acid CatSper sequence had similar homologies.

Specificity of the C-terminal anti-human antibody for equine CatSper1 was shown by Western Blot. The antibody specifically labeled CatSper1 in equine testis and sperm producing a single band of ~70 kDA on equine testis lysate and equine sperm lysate. Blot analysis of equine heart and liver extracts showed no reaction to the antibodies and did not recognize similar-size proteins, confirming that the resulting band was in fact equine CatSper1. Interestingly, when comparing the resulting bands on tested human testis lysate and equine testis lysate, using the anti-Human CatSper1 antibody, the equine band was slightly larger and seemed to be more intensely stained. Similar to results published by Ren et al. (2001), when comparing blot analysis of equine testis extract to equine sperm extract, resulting band signals were stronger in the testis extract thereby producing a better signal.

CONCLUSION

In conclusion, the results of this study show the CatSper1 protein is present on the principal piece of equine spermatozoa. Equine spermatozoa respond to a treatment (NH_4Cl) that causes intracellular alkalinization by exhibiting increased values for motility parameters shown in other species to be associated with hyperactivated motility. To our knowledge, this is the first study documenting the induction of hyperactivated-like motility in equine spermatozoa. Further studies are required to validate these measures of hyperactivated motility in equine sperm with those supporting in vitro fertilization.

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APPENDIX A

Rathi Protocol: R-Tyrodes (in distilled water)

Components	**Sigma Number**	**Grams/500 mL**	**mM**
NaCl	S5886	2.805	96
KCl	P5405	0.116	3.1
$CaCl_2$	C7902	0.147	2
$MgSO_4$	M2643	0.024	0.4
KH_2PO4	P5655	0.0204	0.3
Hepes	H4034	2.383	20
Glucose	G7021	180.2	5
Sodium Lactate	L4263	112.1	21.7
Sodium Pyruvate	P4562	110	1
$NaHCO_3$	S5761	84.01	15
BSA	A3803	3.5	7 mg/mL
Gentamycin	Invitrogen	250 µl	25 µg/mL
pH	**7.4**		

APPENDIX B

Marquez Protocol: M-Tyrodes (in distilled water)

Components	**Sigma Number**	**Grams/500 mL**	**mM**
$CaCl_2$	C7902	0.147	2
KCl	P5405	0.116	3.1
$MgCl_2$	M4880	0.053	1.1
NaH_2PO_4	S5011	0.024	0.4
$NaHCO_3$	S5761	1.050	25
NaCl	S5886	2.890	99
Hepes	H4034	1.190	10
Sodium Lactate	L4263	1.423	25.4
Sodium Pyruvate	P4562	0.055	1
0.02% PVA	P8136	0.4 mg	
Gentamicin	Invitrogen	50 µg/mL	
pH	**7.6**		

APPENDIX C

Blocking Buffer Medium (in PBS)

Components	**Sigma Number**	**%**	**Grams/50 mL**
Goat Serum	Jackson Lab	5%	2.5 mL
BSA	A3803	1%	0.5
Sodium Azide	Mallinckrodt	0.01%	0.005
Gelatin	G2500	0.1%	0.05

VITA

Kristin Rose Rolke graduated from Jersey Village High School in May of 2002 and went on to attend Texas A&M University in College Station, Texas. She received her Bachelor of Science degree in agricultural development with a minor in business administration in May of 2006 and immediately started her graduate career under the guidance of Dr. Katrin Hinrichs in the area of equine reproductive physiology. She received her Master of Science degree in veterinary physiology and biomedical sciences in December of 2008 from Texas A&M University. Her research interests include equine sperm motility and ion channel localization and their possible effects on fertilization.

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