THESIS

ROLE OF α/β HYDROXYLASE DOMAIN CONTAINING PROTEIN 2 IN STALLION SPERM ACTIVATION

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ABSTRACT

ROLE OF α/β HYDROXYLASE DOMAIN CONTAINING PROTEIN 2 IN STALLION SPERM ACTIVATION

Cell signaling pathways involved in stallion sperm activation are not completely understood. Furthermore, failure of equine *in vitro* fertilization is commonly attributed to an inability to successfully capacitate sperm. Sperm activation describes the process by which sperm undergo capacitation, hyperactivation, and acrosome reaction in preparation for interaction with an oocyte. 2-arachidonoylglycerol (2AG) is found in the human sperm membrane and prevents calcium influx through the CatSper channel. The α/β hydroxylase domain containing protein 2 (ABHD2) is also found in the human sperm membrane and functions as a progesterone receptor. When progesterone binds to ABHD2, it removes 2AG from the membrane allowing CatSper to open, which leads to calcium entrance into the cell, resulting in sperm activation. It is unclear if this mechanism holds true in stallion. Experiments were conducted to test the hypothesis that progesterone causes sperm activation through interaction with ABHD2 by 1) determining whether the ABHD2 receptor exists on stallion spermatozoa, 2) determining if progesterone binds to ABHD2 on stallion spermatozoa and 3) demonstrating the role of ABHD2 in sperm activation through correlations between ABHD2 and hyperactivation and/or acrosome reaction. Immunoblotting identified ABHD2 protein in stallion sperm and immunocytochemistry (ICC) localized the receptor to the tail region of stallion spermatozoa. Immunocytochemistry also demonstrated that ABHD2 binds progesterone by restricting fluorescence exhibited by ABHD2 when incubated with progesterone. Stallion sperm were

evaluated for hyperactivation with computer assisted sperm analysis (CASA) following incubation in capacitation medium with either 1) an endogenous activator of sperm; 3 μM progesterone (P4), 2) a positive pharmacological stimulator of hyperactivation not associated with ABHD2; procaine or 3) a known ABHD2-action inhibitor methyl arachidonyl flourophosphatnate (MAFP). MAFP is a serine hydroxylase inhibitor and functions by preventing the removal of 2AG caused by exposure of ABHD2 to progesterone and, thus, limits hyperactivation. Flow cytometry was used to measure the acrosomal status of treated sperm as a subset of the hyperactivation measurements. When MAFP was administered prior to treatment with either P4 and/or procaine the hyperactive movement was inhibited ($p < 0.05$) in the presence of P4 but did not affect procaine induced activity. Results were similar for all ejaculates. The reduced hyperactivation of sperm when incubated with both progesterone and MAFP illustrates a potential connection between ABHD2 and CatSper. No change in acrosomal status was discovered through incubation with P4, procaine, or MAFP. These results indicate 1) that ABHD2 is present on stallion sperm, 2) that progesterone binds to ABHD2 and 3) that progesterone has the potential for causing hyperactivation but does not affect the acrosome reaction.

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CHAPTER 1 – LITERATURE REVIEW

Introduction

For purposes of this review, sperm activation will describe the process by which sperm undergo capacitation, hyperactivation, and acrosome reaction in preparation for interaction with an oocyte. These steps will be discussed in a conventional mammalian approach with specie differences indicated. Although parts of these pathways are known in some species, this may not be what really happens in the equine model. It is difficult to know what causes each step of stallion sperm activation as there is no protocol for successful equine *in vitro* fertilization as exists in human, bovine, or murine models.

Equine *in vitro* Fertilization

Conventional *in vitro* fertilization (IVF) requires penetration of an oocyte by a capacitated spermatozoon. This results in oocyte activation and syngamy. This is common practice in human medicine and livestock production. However there have only been two births of IVF foals. In both cases the sperm were activated with a calcium ionophore and then incubated with oocytes (Bezard et al., 1989; Palmer et al. 1991). Oocytes that underwent cleavage and had 2 polar bodies and/or pronuclei were transferred, along with the culture medium, to recipient mares and resulted in the birth of two live foals (Palmer et al., 1991). Since then, no successful equine IVF protocol has been established (Leemans et al., 2016).

Failure of equine *in vitro* fertilization is due to an inability of sperm to penetrate the zona pellucida surrounding the oocyte likely caused by inadequate sperm capacitation (Leemans et al., 2016). This conclusion is drawn by evidence that *in vitro* matured oocytes can undergo fertilization when transferred to a mare's oviduct and inseminated, but the oocytes are unable to

be fertilized *in vitro* (Hinrichs et al., 2002). Further research found that while sperm could bind to the zona pellucida in an IVF setting, neither capacitation nor acrosome reaction were successfully completed to cause fertilization (Tremoleda et al., 2003). Thus, success of *in vivo* fertilization is attributed to factors within the mare's oviductal environment that supports sperm activation and fertilization. For purposes of this review, sperm activation is used to describe the process by which sperm undergo capacitation, hyperactivation, and acrosome reaction. To date, several substances have been used to initiate sperm capacitation, hyperactivation and/or acrosome reaction yet none have resulted in successful *in vitro* fertilization (Leemans et al., 2016).

A better understanding of the signaling pathways involved in stallion sperm activation is necessary for developing a successful protocol for equine *in vitro* fertilization, as well as potentially treating stallion fertility issues. An understanding of the mechanisms involved in this process will better poise researchers to develop medium that replicates the mare oviductal environment to allow successful fertilization.

Sperm Development

Spermatogenesis is the proliferation and development of sperm cells within a male testis. This process occurs within seminiferous tubules between adjacent Sertoli cells. It can be divided into three phases 1) proliferation, 2) meiosis and 3) differentiation.

The first phase is the proliferation stage where spermatogonia undergo mitotic divisions to replicate in number. The spermatogonia are found in the basal compartment of the seminiferous epithelium (Clermont, 1963). They start as diploid (2N) stem cells. Mitotic divisions result in progression through the many types of spermatogonia. They start as A-spermatogonia and progress as I-spermatogonia to B-spermatogonia. As spermatogonia are

replicated in waves, they reach a point where they can differentiate into primary spermatocytes that enter into meiosis (Dym, 1994). Certain lines of A-spermatogonia maintain the spermatogonial stem cell population and never progress to I-spermatogonia and then B-spermatogonia. Instead they continue to replicate to produce more self-renewing A-spermatogonia. This maintains a continuous supply of spermatogonia (Phillips et al., 2010).

After spermatogonia have completed the necessary mitotic divisions, they result in primary spermatocytes that then cross the blood-testis barrier to enter into the deep adluminal compartment (Clermont, 1963). This barrier is made up of tight junctions between adjacent Sertoli cells (Dym & Fawcett, 1970). There they enter into the meiotic phase. Meiosis I results in genetically diverse secondary spermatocytes that have undergone DNA replication and recombination (Szostak et al., 1983). Meiosis II then results in haploid (1N) spermatids (Johnson, 1991). These spermatids are round in shape and undifferentiated in structure in that they have yet to develop the acrosome or tail (Clermont & Leblond, 1955).

Differentiation is the final phase of spermatozoal development where spermatids transition from spherical structures to differentiated spermatozoa (Clermont & Leblond, 1955). This process is known as spermiogenesis and results in spermatozoa with specialized structures having a head containing the nucleus and acrosome and a tail containing mitochondria and a flagellum. The nucleus transitions from being round to condensed and elongated (Clermont & Leblond, 1955). Proacrosomal vesicles derived from the Golgi apparatus migrate to the apical ridge where they fuse together and form an acrosome cap bound to the plasma membrane (Kierszenbaum et al., 2004). Centrioles move to form the base of the flagellum with mitochondria localized to the middle piece (Clermont & Leblond, 1955). The final release of

spermatozoa into the seminiferous tubule is known as spermiation (O'Donnell et al., 2011). The entire process of spermatogenesis takes roughly 55 days in stallions (Amann, 1981).

Sperm Maturation

After development in the testis, sperm are transported through the seminiferous tubules to the rete testis whereupon they enter the epididymis through the efferent ducts. Sperm maturation and storage take place within the epididymis. Maturation occurs as sperm travel through the epididymis and, once completed, gives sperm the ability to fertilize an oocyte in an *in vivo* environment. This process is typically not completed until sperm reach the cauda epididymis (Holtz & Smidt, 1976). Reabsorption of fluids and proteins occurs as sperm pass through the efferent ducts and caput epididymis (Gatti et al., 2004; Veeramachaneni et al., 1990).

During passage through the corpus epididymis, sperm acquire the ability to be motile which is believed to be due to phosphorylation and de-phosphorylation of proteins on the sperm flagellum (Tash & Bracho, 1994). Though sperm have gained the ability to be motile, they are transported through the epididymis by smooth muscle contractions of the epididymis (Jaakkola & Talo, 1983; Turner, 1991). Sperm undergo further changes in plasma membrane glycoproteins and sterols as part of their maturation process (Retamal et al., 2000). Other changes occur to surface proteins resulting from interaction with fluids and proteins secreted by the epididymis (Gatti et al., 2004). The plasma membrane surrounding sperm experiences elevated proportions of cholesterol due to a loss of phospholipids (Jones, 1998). Increased levels of cholesterol assist in stabilizing sperm membrane for storage in the cauda epididymis where they can maintain viability for several weeks until ejaculation (Moore, 1998; Saether et al., 2003).

Capacitation

Sperm activation describes a series of events that sperm must complete as part of the fertilization process. Capacitation is the first step that sperm must complete before they can interact with an oocyte (Austin, 1952; Chang, 1951). Capacitation involves 1) removal of decapacitation factors present on sperm plasma membrane and in seminal plasma, 2) reorganization of the plasma membrane and 3) changes in internal characteristics.

Decapacitation factors are particles present on sperm that prevent capacitation to maintain prolonged viability. These factors become attached to sperm either during epididymal maturation or are present with seminal plasma and coat sperm upon ejaculation (Nixon et al., 2006). Seminal plasma has an inhibitory effect on sperm capacitation which can be removed when the seminal plasma is removed in the female reproductive tract and incubated in the oviduct or a comparable environment (Chang, 1957). Cholesterol is secreted in large amounts in seminal plasma and serves as a decapacitation factor as it incorporates into the sperm membrane (Cross, 1996b; Eliasson, 1966).

During capacitation, the sperm plasma membrane undergoes several changes including an increase in membrane fluidity as well as a reorganization and loss of cholesterol (Martínez & Morros, 1996). The increase in sperm plasma membrane fluidity can be caused by exposure to bicarbonate which is secreted by the uterine epithelium (Gadella & Harrison, 2000; Zhou et al., 2005). Bicarbonate also stimulates soluble adenylyl cyclase activity which leads to an increase in cAMP production (Garty & Salomon, 1987). This, in turn, initiates a redistribution of cholesterol to the apical region on the head of sperm where it accumulates in lipid rafts and a depletion of cholesterol from the plasma membrane in porcine sperm (van Gestel et al., 2005). This redistribution and efflux of cholesterol from the plasma membrane is another step in capacitation

that aids in penetration of the ovum (Ehrenwald et al., 1988a; Ehrenwald et al., 1988b). Cholesterol is the most abundant sterol on the plasma membrane (Lalumière et al., 1976) and, as it is removed, causes an increased responsiveness of sperm cells to both progesterone and calcium ionophores (Cross, 1996a; Zarintash & Cross, 1996). While bicarbonate stimulates a cholesterol removal from the sperm plasma membrane in porcine and murine sperm *in vitro*, this effect is diminished in stallion sperm by the presence of calcium and albumin in the capacitating medium (Macias-Garcia et al., 2015). This suggests a species-specific difference in plasma membrane changes during stallion sperm activation.

The final characteristic of capacitation is a change in sperm internal parameters. Sperm cells experience intracellular alkalinization when undergoing capacitation (Parrish et al., 1989). Bicarbonate, in addition to its role in cholesterol removal, can also serve to increase intracellular pH levels in sperm cytoplasm (Zeng et al., 1996). Oviductal binding can also initiate sperm alkalization likely due to an alkaline microenvironment present along the oviductal epithelium (Leemans et al., 2014). Increased pH levels in sperm, in turn, cause a rise in intracellular calcium levels via the pH-gated calcium channel CatSper (Loux et al., 2013). A final indicator or result of capacitation is protein tyrosine phosphorylation (Visconti et al., 2002), which is dependent upon elevated environmental pH levels (González-Fernández et al., 2012).

In conventional *in vitro* fertilization procedures, sperm cells are separated from seminal plasma and incubated in a capacitating medium containing calcium, bicarbonate, and albumin (Leemans et al., 2019). These factors are known to induce changes in spermatozoa to allow interaction with an oocyte and subsequent fertilization *in vitro* for mice (Visconti et al., 1995) and humans (Osheroff et al., 1999). Some species necessitate specific capacitating factors such as heparin required by bovine sperm (Parrish et al., 1989). Though it is known that complete

capacitation does not occur in stallion sperm with these factors, many experiments still utilize calcium, bicarbonate, and albumin in their capacitating medium (Leemans et al., 2019).

Hyperactivation

Hyperactivation, another step in the sperm activation process, is a form of accelerated motility characterized by asymmetrical flagellar beating which results in a star-shaped pattern of movement *in vitro* (Romero-Aguirregomezcorta et al., 2018). Hyperactivation is necessary for sperm to detach from the oviductal epithelium where they are temporarily stored and penetrate cumulus cells and zona pellucida surrounding the oocyte (Stauss et al., 1995; Suarez & Dai, 1992; Suarez & Pacey, 2006). Initiation of hyperactivation is associated with an influx of calcium into the cytosol of the sperm tail (Suarez, 2008; Suarez et al., 1993) where it binds to calmodulin which is located on the principal piece (Ignotz & Suarez, 2005; Schlingmann et al., 2007). Calcium/calmodulin binding activates CAMKII kinase resulting in hyperactive motility (Marín-Briggiler et al., 2005).

While treatment with a calcium ionophore can increase cytoplasmic Ca^{2+} levels and cause hyperactivation in other species (Schmidt & Kamp, 2004; Suarez et al., 1987; Villaverde et al., 2009), it was found that Ca^{2+} alone could not induce hyperactivated motility in stallions (Loux et al., 2013). Another study found that treatment with a calcium ionophore decreased all forms of stallion sperm motility (Rathi et al., 2001). This matches another experiment that found environmental calcium restricted protein tyrosine phosphorylation caused by bicarbonate on stallion spermatozoa (González-Fernández et al., 2012). Protein tyrosine phosphorylation has been identified as another pathway by which hyperactivation occurs (Visconti et al., 1995). However, it has been reported that sperm cytoplasmic alkalinization increased intracellular Ca^{2+} levels and caused hyperactivated motility (Loux et al., 2013).

In many species, hyperactivation is also associated with soluble adenylyl cyclase stimulated tyrosine phosphorylation of flagellar proteins through generation of cAMP and activation of protein kinase A (Flesch et al., 1999; Visconti et al., 1995). However, stallion sperm can undergo protein tyrosine phosphorylation without initiating hyperactivation (McPartlin et al., 2011). Bovine and murine sperm cells can also experience protein tyrosine phosphorylation and hyperactivated motility independent on one another illustrating a similarity between these species and the equine (Marquez & Suarez, 2007; Olds-Clarke, 1989). cAMP activation of PKA appears to function independently of calmodulin activation of CAMKII (Schlingmann et al., 2007). Together, both pathways can serve to initiate hyperactivation in most species (Figure 1). Currently, signaling pathways by which stallion sperm can undergo hyperactivation are unknown.

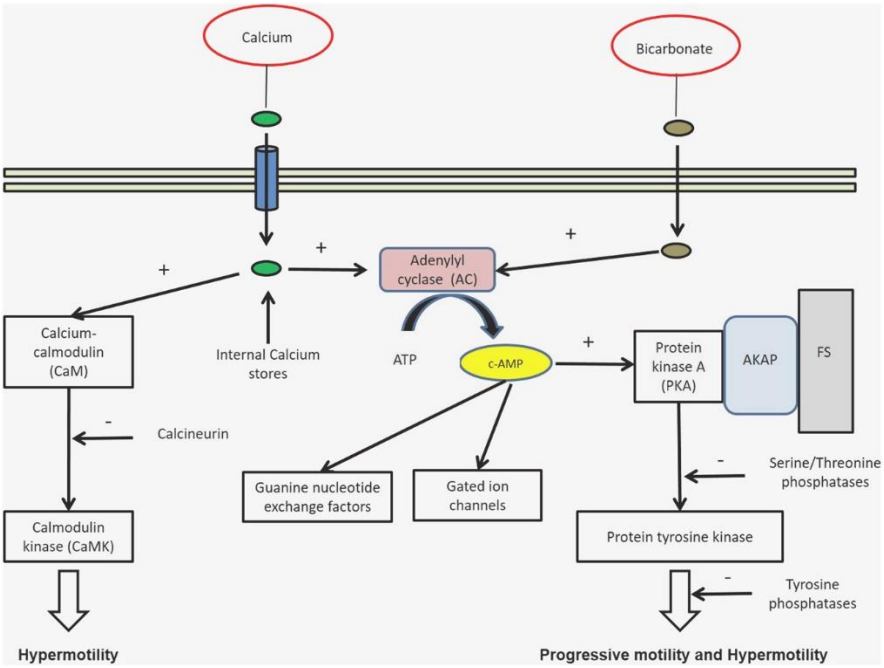

Figure 1. Initiation of Hypermotility

Illustration of pathways involved in initiating hyperactivated motility in mammalian sperm (Leemans et al., 2019). Hypermotility is the result of stimulation of the calmodulin kinase pathway in conjunction with stimulation of the protein tyrosine kinase pathway to higher levels then for progressive motility.

Acrosome Reaction

The acrosome reaction is the final step of sperm activation. It is characterized by fusion of the outer acrosomal membrane and the sperm plasma membrane (Gadella et al., 2001). This fusion releases hydrolytic enzymes from the acrosome that aid sperm in breaking down the zona pellucida matrix surrounding acrosome reacted sperm (Gadella et al., 2001). Exocytosis of acrosomal content relies upon SNARE (Soluble NSF [N-ethylmaleimide-sensitive factor] Attachment protein Receptor) proteins (Tsai et al., 2010) which, in turn, requires an influx of calcium (Roggero et al., 2007). This enables sperm to penetrate the zona pellucida, enter into the perivitelline space, and subsequently bind to the oocyte.

In stallion sperm, the acrosome reaction can be initiated through several means. It can be caused by sperm binding to the zona pellucida (Cheng et al., 1996) or by incubation in the presence of progesterone (Cheng et al., 2002), bicarbonate, or calcium ionophore (Rathi et al., 2001). In these experiments, the acrosome reaction was measured using PNA-FITC staining via fluorescence microscopy with spermatozoa mounted and stained on glass slides. Progesterone causes acrosome reaction by binding to progesterone receptors on sperm plasma membrane (Cheng et al., 1998) and is dependent upon protein kinase C and protein tyrosine kinase (Rathi et al., 2003). This pathway is similar to acrosome reaction initiation following zona binding in that they activate the same protein kinases (Breitbart & Naor, 1999).

For many years, it was commonly accepted that, in an *in vivo* setting, sperm binding to the zona pellucida triggers the acrosome reaction. Furthermore, sperm cells that undergo the acrosome reaction before reaching the zona pellucida were then unable to penetrate the zona pellucida. However it has been found in mice that, when incubated with cumulus-intact oocytes, a greater percentage of sperm began undergoing the acrosome reaction prior to binding to the

zona pellucida then after binding to the zona pellucida (Jin et al., 2011). Further research in mice found that acrosome reacted sperm cells were able to penetrate the zona pellucida of an oocyte, be recovered, and penetrate the zona pellucida of another oocyte and achieve fertilization (Inoue et al., 2011). These findings were possible due to the ability of researchers to utilize *in vitro* fertilization in mice.

Cell Signaling

Sperm activation is the result of multiple signaling pathways, many of which are unknown in stallions. In both humans and mice, hyperactivated motility is dependent upon CatSper, a sperm-specific ion channel (Ho et al., 2009; Qi et al., 2007) located on the flagellum principal piece (Ren et al., 2001; Lishko et al., 2012). The CatSper complex is made up of four α pore-forming subunits that form the transmembrane channel and is accompanied by three auxiliary subunits: β, γ, and δ that (Singh & Rajender, 2015). CatSper1, an α subunit, was the first CatSper protein discovered in mice (Ren et al., 2001) and has since been identified in stallion sperm (Loux et al., 2013). In mice, CatSper responds to intracellular alkalinization caused by capacitation to transport environmental calcium into the cell to initiate hyperactivated motility (Carlson et al., 2003) though, in environments devoid of calcium, CatSper has been found to transport sodium into the cell and cause depolarization leading to loss of motility (Torres-Flores et al., 2011). CatSper channel mutations have been associated with infertility in humans (Avenarius et al., 2009). Differences exist between murine and human CatSper in that progesterone stimulates CatSper in human sperm but not in mice (Lishko et al., 2011). CatSper has been further identified in stallion spermatozoa localized to the principal piece of the tail and responded to increased internal pH with a calcium influx (Loux et al., 2013).

A recent study demonstrated that 2-arachidonoylglycerol (2AG) is present on the plasma membrane of human sperm and prevents calcium influx through the CatSper channel (Miller et al., 2016). This report also identified the α/β hydroxylase domain containing protein 2 (ABHD2) which functions as a progesterone receptor on sperm. When progesterone binds to ABHD2, it acts as a metabolic serine hydrolase and converts 2AG into glycerol and arachidonic acid, depleting 2AG from the sperm plasma membrane (Miller et al., 2016). This removes the inhibition on CatSper allowing it to open causing an influx of calcium and subsequent sperm activation (Figure 2). This pathway operates in a nongenomic manner meaning that progesterone acts on membrane receptors to cause an immediate result instead of the conventional route of steroid signaling by which steroid hormones act through genomic receptors to regulate gene expression. In human sperm, ABHD2 is located in the plasma membrane on the flagellum which enables it to act upon the similarly located CatSper (Miller et al., 2016). CatSper is localized to the principal piece of sperm flagellum in both humans (Cheon et al., 2004) and mice (Ren et al., 2001). In mouse sperm, ABHD2 is localized to the acrosomal region and is not present on the flagellum (Miller et al., 2016). This could explain one of the species differences in that human sperm positively responds to progesterone to stimulate CatSper while murine sperm does not respond to progesterone. The model illustrated by Miller et al. (2016) suggests that 2AG is continuously produced within the plasma membrane to impede CatSper as CatSper activation was reduced when progesterone was removed. While this pathway is only illustrated in sperm, it is possible that ABHD2 responds to progesterone in a genome-independent manner to act as a lipid hydrolase in other tissues given the widespread expression of ABHDs in other tissues (Miyata et al., 2005).

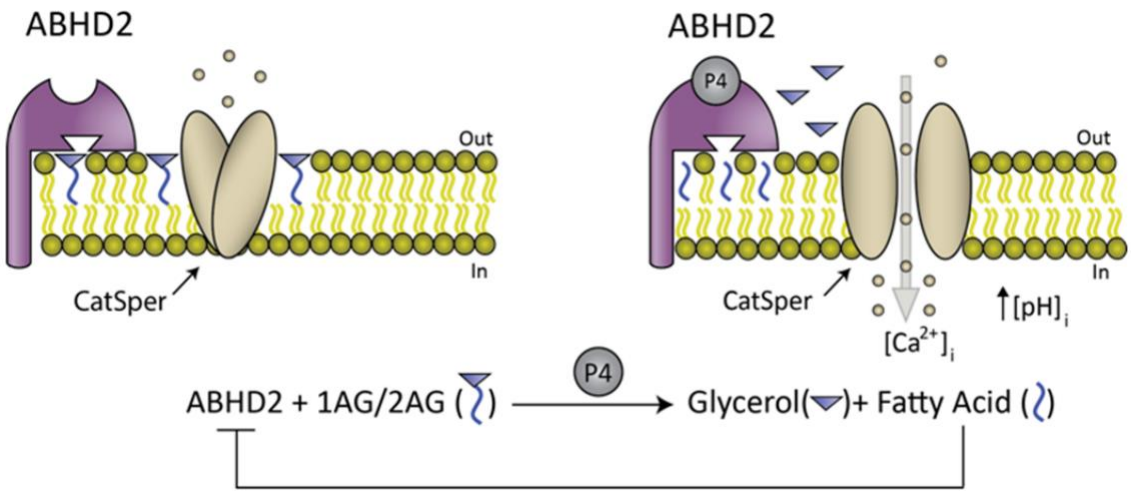

Figure 2. Proposed ABHD2 role in sperm membrane

Progesterone binds to ABHD2 and breaks down 2AG to glycerol and arachidonic acid. This allows CatSper to open causing a calcium influx (Miller et al., 2016).

Sperm signaling pathways have been more readily identified in mammalian species other than horses such as humans and mice due to the ability of researchers to perform *in vitro* fertilization. As *in vitro* fertilization does not work in horses, we investigated one of the mechanisms of sperm activation recently described in humans with hopes that it could be of benefit to future research towards a successful equine *in vitro* fertilization protocol. Although progesterone receptors have been identified on stallion sperm (Rathi et al., 2003) their relationship with CatSper has not yet been demonstrated. We propose to test for an interaction between ABHD2 and CatSper. We hypothesize that progesterone binds ABHD2 on stallion sperm and causes sperm activation. Our specific objectives were to 1) determine presence and location of ABHD2 on stallion sperm, 2) demonstrate that ABHD2 binds progesterone and 3) determine if progesterone bound to ABHD2 activates stallion sperm through hyperactivated motility and acrosome reaction.

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CHAPTER 2 – JOURNAL ARTICLE[1]

Overview

Cell signaling pathways involved in stallion sperm activation are not completely understood. Furthermore, failure of equine *in vitro* fertilization is commonly attributed to an inability to successfully capacitate sperm. Sperm activation describes the process by which sperm undergo capacitation, hyperactivation, and acrosome reaction in preparation for interaction with an oocyte. 2-arachidonoylglycerol (2AG) is found in the human sperm membrane and prevents calcium influx through the CatSper channel. The α/β hydroxylase domain containing protein 2 (ABHD2) is also found in the human sperm membrane and functions as a progesterone receptor. When progesterone binds to ABHD2, it removes 2AG from the membrane allowing CatSper to open, which leads to calcium entrance into the cell, resulting in sperm activation. It is unclear if this mechanism holds true in stallion sperm. Experiments were conducted to test the hypothesis that progesterone causes sperm activation through interaction with ABHD2 by 1) determining whether the ABHD2 receptor exists on stallion spermatozoa, 2) determining if progesterone binds to ABHD2 on stallion spermatozoa and 3) demonstrating the role of ABHD2 in sperm activation through correlations between ABHD2 and hyperactivation and/or acrosome reaction. Immunoblotting identified ABHD2 protein in stallion sperm and immunocytochemistry (ICC) localized the receptor to the tail region of stallion spermatozoa. Immunocytochemistry also demonstrated that ABHD2 binds progesterone by

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restricting fluorescence exhibited by ABHD2 when incubated with progesterone. Stallion sperm were evaluated for hyperactivation with computer assisted sperm analysis (CASA) following incubation in capacitation medium with either 1) an endogenous activator of sperm; 3 μM progesterone (P4), 2) a positive pharmacological stimulator of hyperactivation not associated with ABHD2; procaine or 3) a known ABHD2-action inhibitor methyl arachidonyl flourophosphatnate (MAFP). MAFP is a serine hydroxylase inhibitor and functions by preventing the removal of 2AG caused by exposure of ABHD2 to progesterone and, thus, limits hyperactivation. Flow cytometry was used to measure the acrosomal status of treated sperm as a subset of the hyperactivation measurements. When MAFP was administered prior to treatment with either P4 and/or procaine the hyperactive movement was inhibited ($p < 0.05$) in the presence of P4 but did not affect procaine induced activity. Results were similar for all ejaculates. The reduced hyperactivation of sperm when incubated with both progesterone and MAFP illustrates a potential connection between ABHD2 and CatSper. No change in acrosomal status was discovered through incubation with P4, procaine, or MAFP. These results indicate 1) that ABHD2 is present on stallion sperm, 2) that progesterone binds to ABHD2 and 3) that progesterone has the potential for causing hyperactivation but does not affect the acrosome reaction.

Introduction

Conventional *in vitro* fertilization (IVF) requires capacitated sperm to penetrate an oocyte. This results in oocyte activation and syngamy. Although IVF is successful in human medicine and livestock production, it is currently unsuccessful with equine gametes (Leemans et al., 2016). To date, several substances have been used to initiate capacitation, hyperactivation

and/or acrosome reaction in stallion sperm yet none have resulted in successful *in vitro* fertilization (Cheng et al., 2002; Loux et al., 2013; Rathi et al., 2003).

Spermatozoa utilize several signaling pathways for capacitation. This includes reorganization of the sperm plasma membrane, redistribution and efflux of cholesterol, calcium activation of calmodulin kinase, and bicarbonate induction of tyrosine phosphorylation (Bernecic et al., 2019). In many species, sperm activation is also the result of an influx of calcium through CatSper, a pH-gated calcium channel (Brenker et al., 2012; Ren et al., 2001). CatSper has been identified on stallion sperm (Loux et al., 2013) although how it induces sperm hyperactivation remains unknown. Miller et al. (2016) demonstrated that 2-arachidonoylglycerol (2AG) is present on the plasma membrane of human sperm and prevents calcium influx through CatSper channel. They also identified α/β hydroxylase domain containing protein 2 (ABHD2) which functions as a progesterone receptor on sperm (Miller et al., 2016). When progesterone binds to ABHD2, it acts as a lipid hydrolase and breaks down 2AG, depleting it from the sperm plasma membrane which removes its inhibition on CatSper, allowing the CatSper channel to open causing an influx of calcium and subsequent sperm activation (Miller et al., 2016). Progesterone receptors have been identified on stallion sperm (Rathi et al., 2003) although whether their function is associated with CatSper is not known.

We propose to determine if ABHD2 affects stallion sperm activation. We hypothesize that progesterone bound to ABHD2 activates stallion sperm. Our specific aims were 1) determine presence and location of ABHD2 on stallion sperm, 2) demonstrate that ABHD2 binds progesterone and 3) determine if progesterone bound to ABHD2 activates stallion sperm through hyperactivated motility and acrosome reaction.

Materials and Methods

Horse Care

All animal procedures were approved by the Colorado State University Institutional Animal Care and Use Committee (IACUC# 16-6954A). Some of the stallions (n = 3) were housed at the Colorado State University Equine Reproduction Laboratory in Fort Collins, CO for the duration of this experiment. Another stallion (n=1) was housed at Vista Equine in Windsor, CO. Stallions were of proven fertility and maintained on grass-alfalfa mix hay with free choice salt and mineral supplement.

Semen Collection and Initial Evaluation

Semen was collected from stallions for each experiment using a Colorado State University model artificial vagina. Stallions were collected on a phantom in the presence of a tease mare in estrus. Each ejaculate was assessed for gel-free volume, concentration using a 591B Equine Densimeter (Animal Reproduction Systems, Ontario, CA), and motility with computer assisted sperm analysis (CASA; AndroVision, Minitube, Verona, WI) at 4 fields of view with a minimum of 200 cells prior to use.

Western Blot

Stallion sperm (n = 3 stallions, 1 ejaculate each) were centrifuged at 5,000 x g for 20 min at 23°C in a 5417R Centrifuge (Eppendorf North America, Hauppauge, NY). The seminal plasma was discarded and the sperm pellet resuspended in RIPA lysis buffer (0.484 g Tris pH 8.0, 1.6 g NaCl, 20 mL Glycerol, 2 mL Nonidet P-40, 0.2 g Sodium Dodecyl Sulfate, 1.0 g Deoxychlorate, 0.117 g Ethylenediamine Tetraacetic Acid, bring to 200 mL with H_2O at a pH of 8.0) and sonicated at 30 second intervals for 5 minutes at 50% output and 40% duty control on a Branson Sonifier 250 (Branson Ultrasonics, Danbury, CT). The suspension was then centrifuged

at 10,621 x g for 10 minutes at 4°C in a 5417R Centrifuge (Eppendorf North America, Hauppauge, NY). The protein-rich supernatant was collected and stabilized with protease inhibitor cocktail (PIC; Thermo Fisher, Waltham, MA) and 10 mM phenylmethane sulfonyl fluoride (PMSF; Thermo Fisher, Waltham, MA), both at a 1X concentration. Aliquots were stored in 1 mL amounts in 1.7 mL Optimum Tubes (Life Science Products, Frederick, CO) at -20°C. The amount of protein in each sample was quantified with Pierce BCA Protein Assay Kit (Thermo Fisher, Waltham, MA). The crude cell lysate was mixed with 6x SDS loading buffer (3.75 mL 1 M Tris-HCl pH 6.8, 6 mL Glycerol, 1.2 g SDS, 0.93 g DTT, 6 mg bromophenol blue, adjusted to total volume of 10 mL with H_2O) and loaded into a 10% gradient Mini-Protean TGX SDS-PAGE gel (Bio-Rad, Hercules, CA) at a 50 µg per well. Protein from equine brain tissue (30 µg) was prepared in a similar fashion and used as a positive control for expression of ABHD2. Protein from ovine caruncle tissue (30 µg) was prepared in a similar fashion and used as a negative control for expression of ABHD2. The protein was separated by electrophoresis at 80V for 30 minutes and then 160V for approximately 60 minutes. The protein was transferred onto a nitrocellulose membrane at 100V for 80 minutes. The blot was blocked in 5% milk in 1x TBST. Antibodies were prepared in the blocking solution. The blot was probed with anti-ABHD2 (Aviva, San Diego, CA) primary antibody and incubated overnight at 4°C. After washing, the blot was incubated with HRP-conjugated secondary antibody (Santa Cruz Biotechnology, Dallas, TX). The membrane was developed with SuperSignal West Dura kit (Thermo Fisher, Waltham, MA). Precision Plus Protein Standards, Dual Color (Bio-Rad, Hercules, CA) was used as a ladder.

Immunocytochemistry

This method was taken from Miller et al., (2016). Spermatozoa from three stallions (20µL at approximately 300 x 10^6 cells/mL) were plated onto glass slides and allowed to adhere for 30 minutes. The cells were fixed in 4% paraformaldehyde in 1x PBS for 10 minutes, washed twice in 1x PBS for 4 minutes each and allowed to dry. The cells underwent further fixation in 100% methanol cooled to -80°C. Samples were washed twice in 1x PBS for 4 minutes each and allowed to dry. Cells were permeabilized in 0.5 µg/mL saponin in 1x PBS for 5 minutes followed by two washings in 1x PBS for 4 minutes each. The cells were then blocked in 10% bovine serum albumin in 1x PBS for 1 hour at room temperature and washed twice in 1x PBS for 4 minutes each and allowed to dry. Cells were incubated in rabbit based primary antibodies for ABHD2 (Aviva, San Diego, CA) in blocking solution overnight at 4°C. Samples were washed three times in 1x PBS for 4 minutes each and allowed to dry. Cells were incubated in Alexa-Fluor 488 goat anti-rabbit secondary antibodies (Thermo Fisher, Waltham, MA) in blocking solution for 1 hour at room temperature. Samples were washed three times for 4 minutes each in 1x PBS and allowed to dry. Coverslips were mounted using ProLong Gold Antifade reagent with DAPI (Thermo Fisher, Waltham, MA). Images were acquired with a Zeiss LSM 800 microscope equipped with differential interference contrast and coupled to an XCite fluorescence source.

In a separate experiment, stallion sperm samples were incubated in progesterone prior to treatment with antibodies to detect altered binding of ABHD2 antibodies to the epitope within the binding site. Spermatozoa from two stallions (20µL at approximately 50 x 10^6 cells/mL) were plated onto glass slides and allowed to adhere for 30 minutes. Sperm cells were incubated in 3µM progesterone (Abcam, Cambridge, MA) in water for 2 hours at room temperature. Control samples were incubated in water for 2 hours at room temperature. Samples were

processed and imaged as previously described for immunocytochemistry utilizing the same primary and secondary antibodies.

Sperm Hyperactivation Analysis

Stallion spermatozoa (n = 4 stallions, 1 ejaculate each) were centrifuged at 1600 RPM for 5 minutes in a CL2 Centrifuge (Thermo Fischer Scientific, Waltham, MA) and then extended in a Modified Whittens solution (5.84 g/L NaCl, 0.357 g/L KCl, 0.257 g/L $CaCl_2$, 0.207 g/L KH_2PO_4, 0.293 g/L magnesium sulfate, 0.991 g/L glucose, 5.236 g/L HEPES, 0.022 g/L pyruvate, 3.68 mL/L Lactate, 2.106 g/L $NaHCO_3$, 7 g/L bovine serum albumin) at approximately 7.35 pH which was used as the capacitation medium to a concentration of 50 x 10^6 cells/mL. Sperm aliquots were incubated with 3µM progesterone (Abcam, Cambridge, MA) a concentration meant to mimic physiologic concentrations of progesterone, 5mM procaine (Abcam, Cambridge, MA), or 2µM methyl arachidonyl fluorophosphatnate (MAFP) (Abcam, Cambridge, MA). Procaine served as a positive control for hyperactivation, as it causes hyperactivated motility through a pathway different to CatSper. MAFP, a serine hydrolase inhibitor which prevents the breakdown of 2-arachidonylglycerol, was used as a negative control. Cells were incubated at 37°C for 90 minutes with measurements recorded prior to adding progesterone, procaine, or MAFP and then at 30 minute intervals. Figure 3 illustrates separation of the samples that were evaluated at each time point.

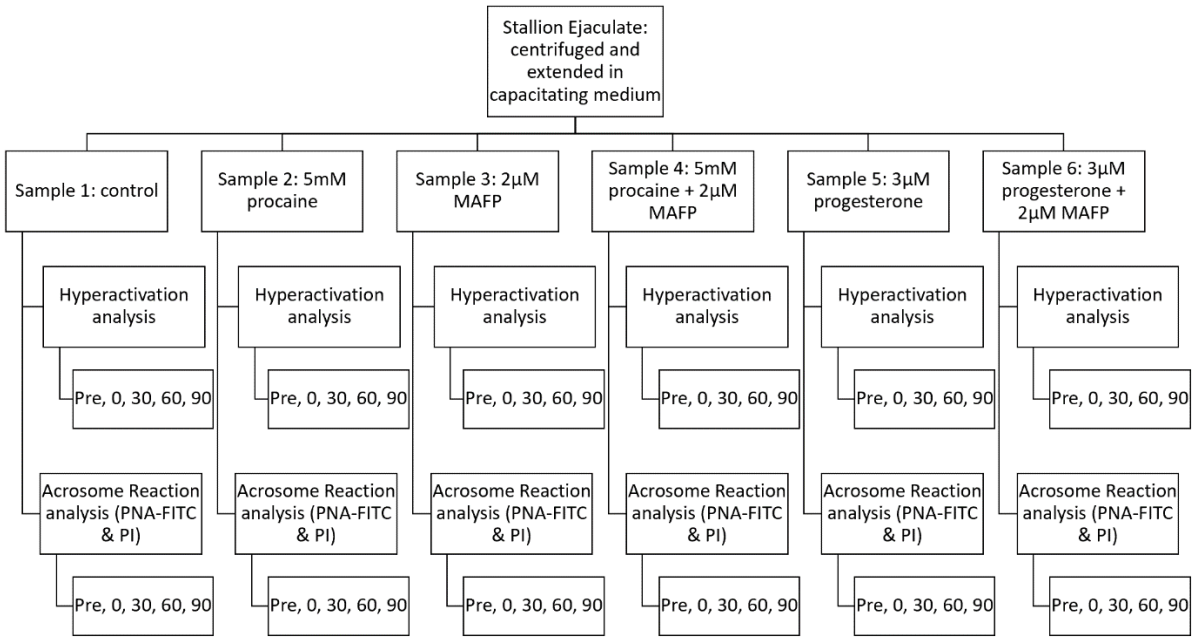


Figure 3. Sample preparation for hyperactivation and acrosome reaction analysis

Equine sperm samples were divided into aliquots and incubated in capacitating medium at 37°C with either 1) control, 2) 5mM procaine, 3) 2µM MAFP, 4) 5mM procaine + 2µM MAFP, 5) 3µM progesterone, or 6) 3µM progesterone + 2µM MAFP. "Pre" measurements were taken prior to adding any chemical agents. Time 0 measurements were taken immediately following addition of chemical agents. Times 30, 60, and 90 measurements were taken 30, 60, and 90 minutes respectively following addition of chemical agents.

Sperm motility was analyzed using computer assisted sperm analysis (CASA; AndroVision, Minitube, Verona, WI). CASA measured amplitude of lateral head movement (ALH), average path velocity (VAP), and linearity of movement (LIN) through which it calculated the percentage of hyperactivated cells. Hyperactivated sperm motility was optimized for stallion spermatozoa by manufacturer settings and was defined as sperm that exhibited ALH > 7.5 µm, VAP ≥ .75 µm/s, and LIN < 50%. Data was corrected for total motility and compared to the control at each time point. A minimum of 4 fields of view and 200 cells were analyzed for each sample at every time point.

Acrosome Reaction Analysis

Acrosome status and sperm viability were examined by staining cells with 1µg/mL PNA-FITC (Sigma-Aldrich, St. Louis, MO) and 1µM propidium iodide (Abcam, Cambridge, MA). PNA-FITC stains for acrosomal membrane disruption (L'Hôte et al., 2007) and propidium iodide (PI) stains for sperm plasma membrane disruption (Riedl et al., 2011). This was performed simultaneously with the sperm hyperactivation analysis utilizing the same sperm samples and treatments. Every time a sample was measured utilizing CASA, an aliquot was extracted and PNA-FITC and propidium iodide were added. Aliquots were incubated in the dark at 37°C for 5 minutes and the samples evaluated by flow cytometry (Accuri C6, Becton Dickinson, San Jose, CA) at a rate of 200 cells/second for 10,000 events. Fluoresce from PNA-FITC and propidium iodide allowed us to determine four sperm populations: (i) live sperm with intact plasma and acrosomal membranes ($PNA\text{-}FITC^{-}/PI^{-}$); (ii) dead sperm with damaged plasma and acrosomal membranes ($PNA\text{-}FITC^{+}/PI^{+}$); (iii) live acrosome reacted sperm with intact plasma membranes, but disrupted acrosomal membrane ($PNA\text{-}FITC^{+}/PI^{-}$); and (iv) dead sperm with damaged plasma membrane but an intact acrosomal membrane ($PNA\text{-}FITC^{-}/PI^{+}$).

Statistical Analysis

Proportions of hyperactivated sperm cells were checked for normal distribution and subsequently, arcsin transformed. Models included the variables: treatment, time, and the interaction term treatment*time. Data were analyzed using repeated measures analysis (PROC MIXED of SAS; SAS Institute, Cary, NC, USA) and significant differences by treatment and time point were determined using the Tukey-Kramer test. Treatments were considered different at $P < 0.05$. After completion of the analyses, the results were back-transformed to be reported in the original scales. These models were protected by an F-test for difference in treatment, time,

and interaction of treatment by time which allowed for means separation evaluation. Proportions of acrosome reacted sperm cells were similarly analyzed.

Results

Western Blot

Antibodies against ABHD2 detected protein in stallion spermatozoa at approximately 48 kDa (Figure 4). ABHD2 expression was also demonstrated in equine brain tissue which was used as a positive control. No ABHD2 expression was evident in ovine caruncle tissue which was used as our negative control.

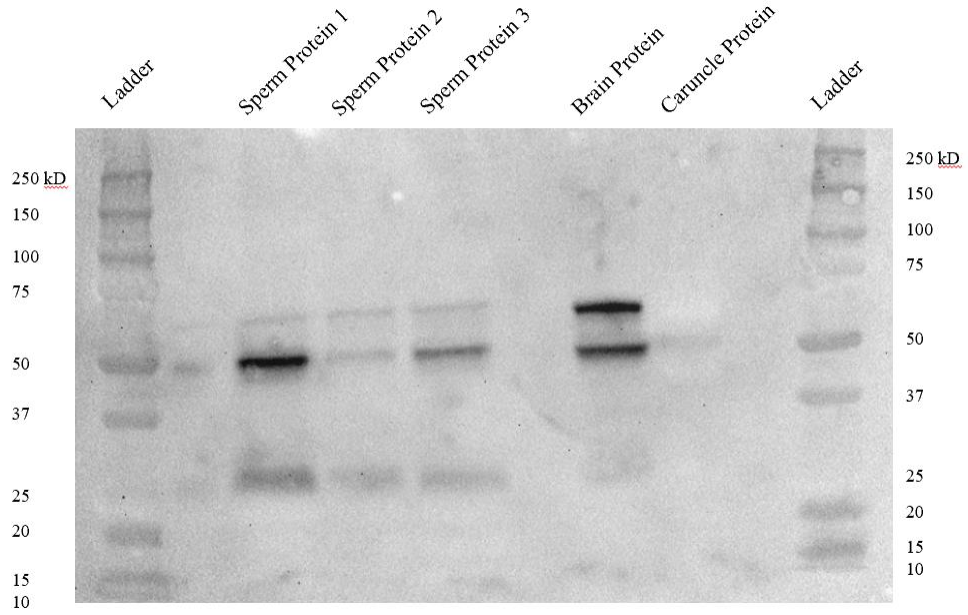


Figure 4

Western Blot with ABHD2 expression in Sperm Protein 1, Sperm Protein 2, Sperm Protein 3, and Brain Protein. No ABHD2 expression was demonstrated in Caruncle Protein. Sperm Protein 1, 2, and 3 each represent protein isolated from spermatozoa from a different stallion.

Immunocytochemistry

ABHD2 location in equine spermatozoa was demonstrated through the use of immunofluorescence staining for antibodies against ABHD2 (Figure 5A) compared to vehicle-matched controls (Figure 5B). ABHD2 expression was localized to the tail of spermatozoa.

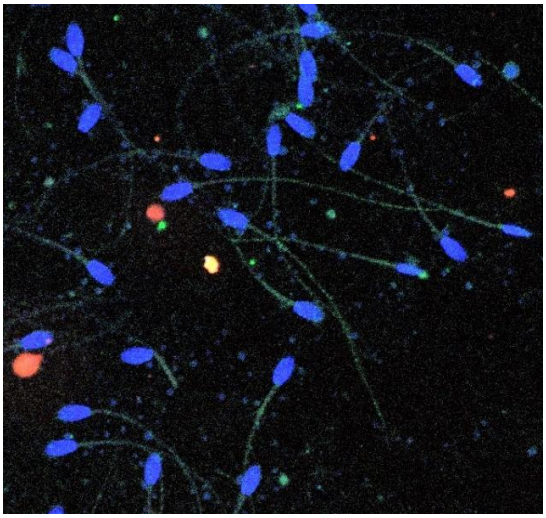
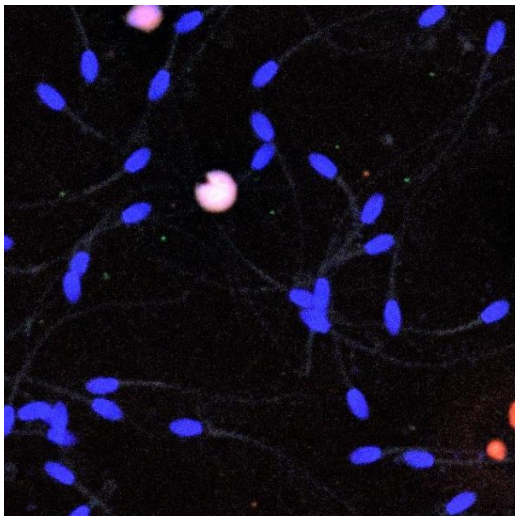
A  **B** 

Figure 5. ABHD2 expression in stallion spermatozoa

Immunofluorescence of stallion spermatozoa. Nuclei were stained blue by DAPI. Green fluorescence indicates expression of ABHD2. (**A**) Sperm cells incubated in primary antibodies against ABHD2 (**B**) Sperm cells incubated in absence of primary antibodies.

Progesterone treated sperm cells (Figure 6A) demonstrated less fluorescence staining for antibodies against ABHD2 compared to non-treated sperm cells (Figure 6B). This suggests binding of progesterone to ABHD2 changing the binding ability of antibodies against ABHD2.

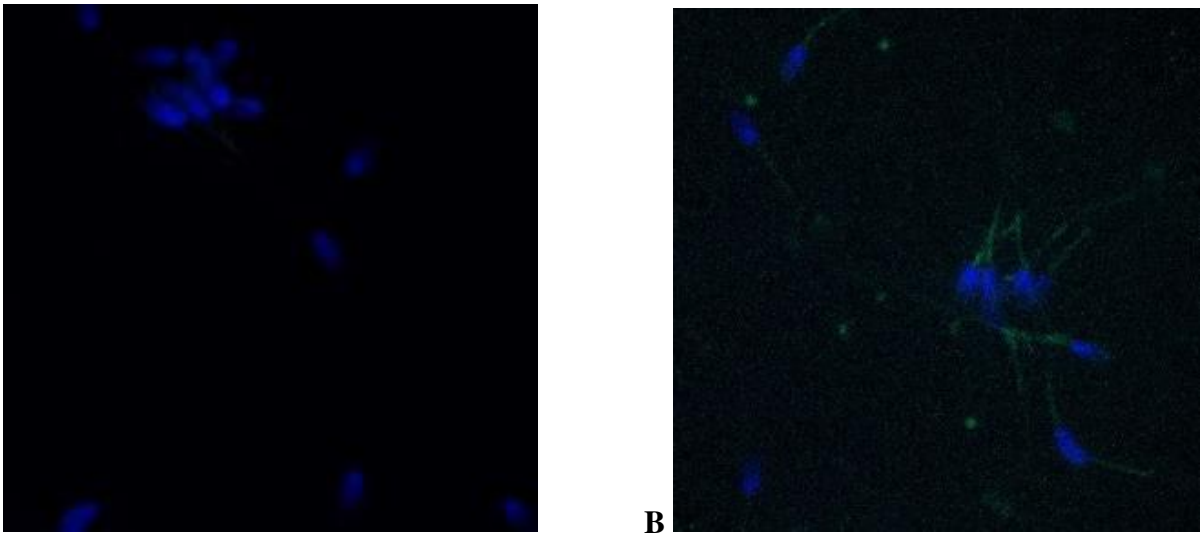
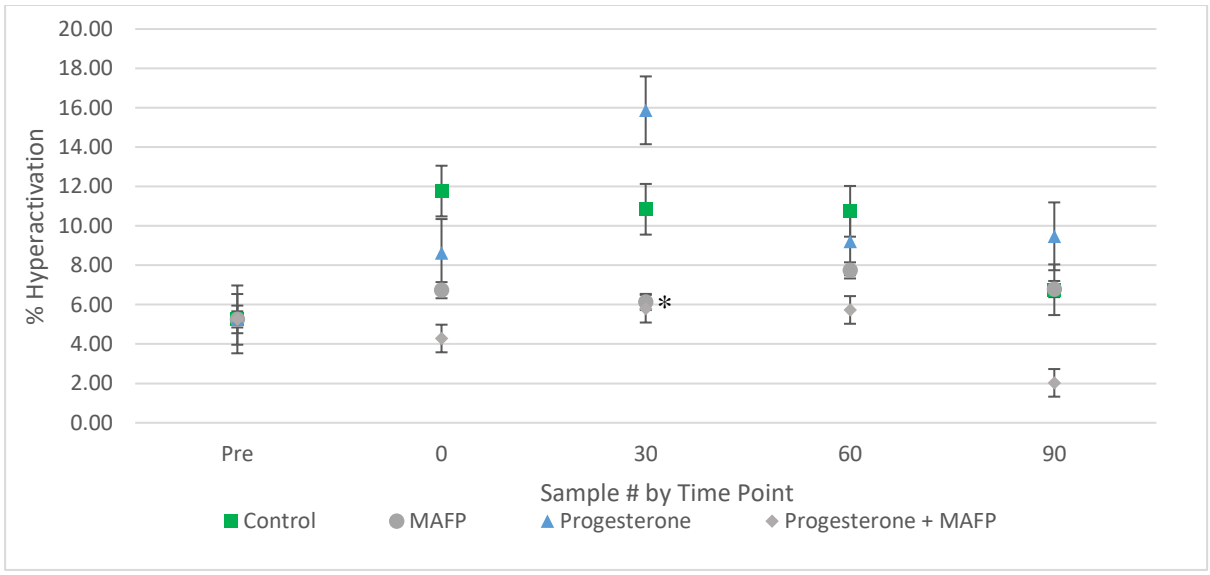


Figure 6. Progesterone binds to ABHD2 in stallion spermatozoa

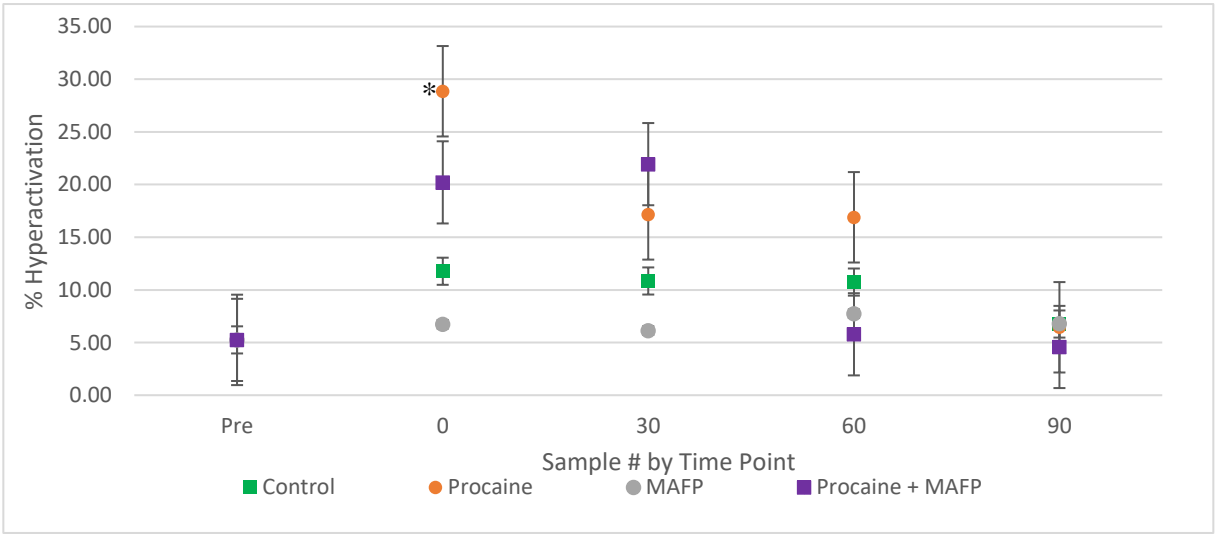
Immunofluorescence of stallion spermatozoa. Nuclei were stained blue by DAPI. Green fluorescence indicates presence of ABHD2. (**A**) Progesterone treated sperm cells. (**B**) Vehicle matched sperm cells.

Sperm Hyperactivation Analysis

Stallion spermatozoa demonstrated a potential for increased hyperactivation when treated with progesterone compared to the control sample (Figure 7A). The percentage of hyperactivated sperm cells due to incubation with progesterone was decreased ($P < 0.05$) with the addition of methyl arachidonyl fluophosphatnate (MAFP; Figure 7A). This demonstrates that progesterone is associated with hyperactivation. Procaine was used as a positive control for hyperactivation. Procaine treatment caused increased hyperactivated motility ($P < 0.05$) compared to the control sample initially and then dropped below the control after 1 hour (Figure 7B).



A



B

Figure 7. Hyperactivated rates in stallion spermatozoa

Equine sperm samples were divided into aliquots and incubated in capacitating medium at 37°C with either 1) control, 2) 5mM procaine, 3) 2µM MAFP, 4) 5mM procaine + 2µM MAFP, 5) 3µM progesterone, or 6) 3µM progesterone + 2µM MAFP. "Pre" measurements were taken prior to adding any chemical agents. Time 0 measurements were taken immediately following addition of chemical agents. Times 30, 60, and 90 measurements were taken 30, 60, and 90 minutes respectively following addition of chemical agents. Measurements indicate percent of hyperactivated sperm cells based upon total motility calculated by computer assisted sperm analysis. (**A**) Significance indicates difference from progesterone treatment sample *P < 0.05. (**B**) Significance indicates difference from control sample *P < 0.05. N = 4

Total motility was maintained similarly across treatment and time (Figure 8).

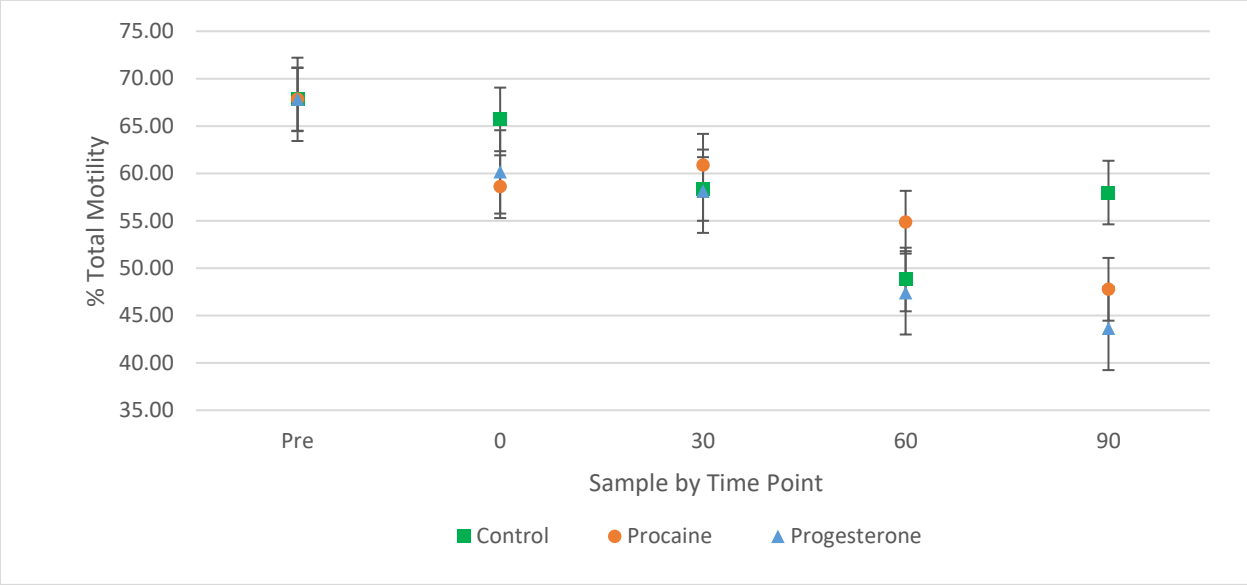


Figure 8. Total motility of stallion spermatozoa

Equine sperm samples were divided into aliquots and incubated in capacitating medium at 37°C with either 1) control, 2) 5mM procaine, 3) 2µM MAFP, 4) 5mM procaine + 2µM MAFP, 5) 3µM progesterone, or 6) 3µM progesterone + 2µM MAFP. "Pre" measurements were taken prior to adding any chemical agents. Time 0 measurements were taken immediately following addition of chemical agents. Times 30, 60, and 90 measurements were taken 30, 60, and 90 minutes respectively following addition of chemical agents. Measurements indicate percent of total motility of sperm cells. Significance indicates difference from control sample *P < 0.05.
N = 4

Acrosome Reaction Analysis

Stallion spermatozoa did not exhibit a difference in acrosomal status with incubation with progesterone (Figure 9). This demonstrates that progesterone does not play a role in the acrosome reaction in equine spermatozoa that we were able to identify. Furthermore, no difference was evident with either procaine, MAFP, or a combination of either.

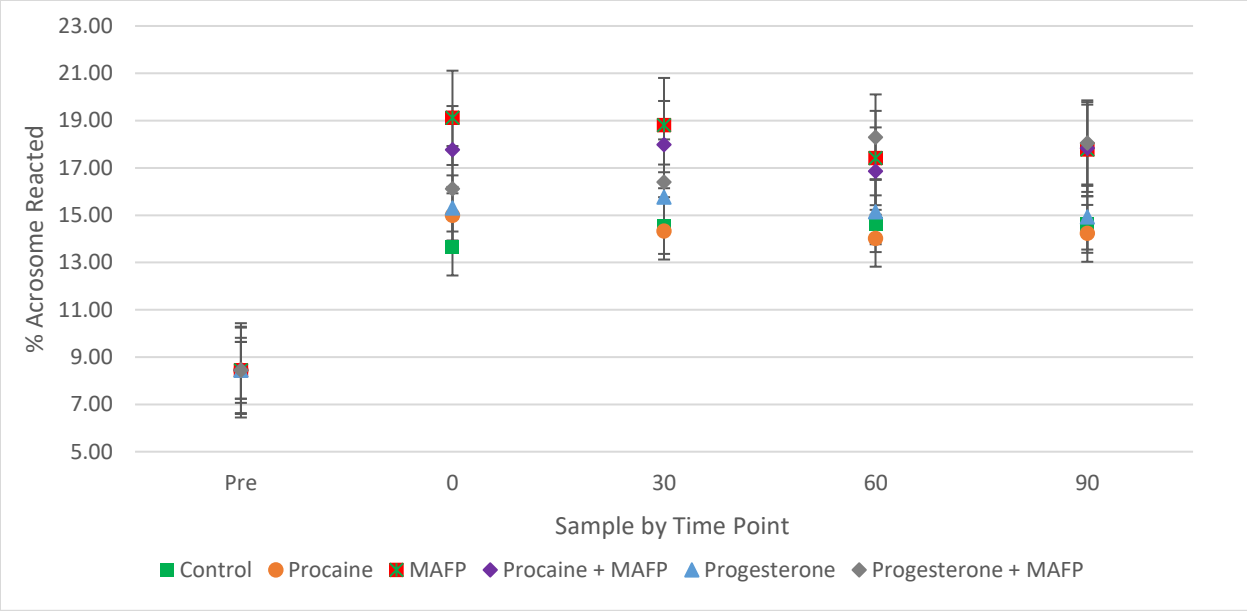


Figure 9.Effect of progesterone on acrosomal status

Equine sperm samples were divided into aliquots and incubated in capacitating medium at 37°C with either 1) control, 2) 5mM procaine, 3) 2µM MAFP, 4) 5mM procaine + 2µM MAFP, 5) 3µM progesterone, or 6) 3µM progesterone + 2µM MAFP. "Pre" measurements were taken prior to adding any chemical agents. Time 0 measurements were taken immediately following addition of chemical agents. Times 30, 60, and 90 measurements were taken 30, 60, and 90 minutes respectively following addition of chemical agents. Measurements indicate percent of acrosome reacted sperm cells calculated by flow cytometry. Significance indicates difference from control sample *P < 0.05. N = 4

Discussion

ABHD2 was identified on stallion sperm localized to the tail where it serves as a progesterone receptor. This is comparable to where ABHD2 was found on human sperm (Miller et al., 2016) and suggests a function of ABHD2 in acting on CatSper in stallion sperm similar to human sperm. CatSper is localized to the mid-piece of the tail on stallion spermatozoa which is necessary for it to influence hyperactivated motility through calcium influx (Loux et al., 2013). In our hyperactivation measurements, we report a potential for sperm cells treated with progesterone trending towards increased hyperactivation (P = 0.26) compared to non-treated sperm cells. Though the increase in hyperactivation of progesterone treated sperm cells

compared to non-treated cells is not significant, it is possible that a peak in hyperactivation was missed in the interval between measurements at Time 0 and at 30 minutes. This possibility is supported by the slight decrease in total motility demonstrated by progesterone and procaine treated samples compared to non-treated samples. This suggests that treated spermatozoa enter hyperactivation in a swift fashion that is easily missed in our 30 minute intervals. The increased hyperactivation rates caused by progesterone ($P = 0.26$) were significantly ($P < 0.05$) decreased by the presence of MAFP. This supports our theory that progesterone is exerting its influence on CatSper through the hydrolyzation of 2-arachidonylglycerol by ABHD2. MAFP prevents breakdown of 2AG and, when it was combined with progesterone, MAFP significantly reduced the hyperactivation caused by progesterone. Procaine served as the positive control for hyperactivation and our results support the conclusion of Loux et al. (2013) that procaine causes hyperactivation through a mechanism independent of CatSper.

We were unable to identify an effect upon the acrosome reaction with any of our treatments. This result is opposite of what multiple studies found, that progesterone induces the acrosome reaction in stallion sperm (Cheng et al., 1998; Rathi et al., 2003). This raises questions about what could be restricting the acrosome reaction in our study or facilitating the acrosome reaction in other studies. It is possible that our method of using flow cytometry was not as effective as the fluorescence microscopy utilized by the experiments just referenced for measuring acrosome reaction.

We believe that identification of this progesterone receptor allows us to illustrate another component of the sperm activation process. These results will better prepare us and other researchers to make further advancements in understanding the sperm cell signaling pathways

involved with sperm activation. This, in turn, will be beneficial for research towards successful equine *in vitro* fertilization as well as address stallion infertility issues.

Conclusion

In conclusion, this study demonstrated that ABHD2 serves as a progesterone receptor located on the tail of stallion spermatozoa. It also demonstrated that progesterone creates potential for hyperactivated motility of stallion spermatozoa through binding to ABHD2. We believe that this may remove the inhibition on CatSper allowing it to open resulting in an influx of calcium and subsequent hyperactivation as described by Miller et al. (2016) in human sperm. This combines to create a better understanding of one of the pathways involved in stallion sperm activation.

Conflict of Interest

We wish to confirm that there is no conflict of interest with this publication and there has been no significant financial support for this work that could have influenced the outcome. We confirm that the manuscript has been read and approved by all named authors and that there are no other persons who satisfied the criteria for authorship but are not listed. We further confirm that the order of the authors listed in the manuscript have been approved by all of us.

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CHAPTER 3 – DISCUSSION

Discussion

Success of the Project

In conclusion of this project, there were multiple successes. We identified α/β hydroxylase domain containing protein 2 (ABHD2) on stallion spermatozoa localized to the tail. We also demonstrated that ABHD2 functions as a progesterone receptor and potentially induces hyperactivated motility (P = 0.26). We saw an increase in hyperactivation within stallion sperm cells treated with progesterone compared to non-treated cells (P = 0.26). This increase occurred in every stallion but was not significant compared to the control. This is likely due to the 30 minute intervals between sample evaluations causing us to miss a peak in hyperactivated motility.

One of the highlights of the research by Miller et al. (2016) is that they identified ABHD2 as acting in a non-genomic signaling fashion. In conventional steroid signaling, steroid hormones, such as progesterone, easily pass through cell plasma membrane where there they bind to genomic receptors and regulate gene expression in a process that takes hours for an effect to be realized (Lösel & Wehling, 2003). In non-genomic signaling, a steroid hormone can function independently of a genomic receptor to cause an effect in a matter of seconds (Lösel & Wehling, 2003). As ABHD2 operates in a rapid, non-genomic fashion in human sperm, it is possible that equine spermatozoa respond to stimulation by progesterone similarly and that hyperactivation was induced in a manner of seconds. This potential rapid initialization of hyperactivated motility would have been missed within our 30 minute time samples and could explain the lack of significance between progesterone treated hyperactivation and control

hyperactivation rates. Treatment with progesterone caused a 5% increase in hyperactivation compared to control cells which is substantially lower then procaine which caused a 19% increase in hyperactivation. We really expected to see a greater increase in the difference in hyperactivated motility but that could have occurred without our knowledge during the time frame between Time 0 and Time 30.

The small sample size used in our hyperactivation analysis (N = 4 stallions) likely also contributed to lack of significance found between progesterone treated sperm cells and control sample. A larger sample size would likely have provided us with significant differences between progesterone treated sperm cells and control cells.

Progesterone induced hyperactivation possibly occurs through the breakdown of 2-arachidonylglycerol (2AG) which removes an inhibitor on CatSper allowing it to open and calcium to enter into the cell. This model is supported by the significant decrease in progesterone induced hyperactivation by the addition of methyl arachidonyl flourophosphatnate (MAFP) which prevents the breakdown of 2AG.

Finally, we found that progesterone does not initiate the acrosome reaction. This is not surprising based upon the location of ABHD2 on stallion sperm. In mice, ABHD2 is located on the acrosome and, upon progesterone binding, initiates the acrosome reaction but does not affect hyperactivation (Miller et al., 2016). This demonstrates a location-specific effect of ABHD2 that was also evident in our experiments. Furthermore, it provides additional support for the necessity of sperm binding to the zona pellucida to induce the acrosome reaction as seen in other experiments (Cheng et al., 1996).

These conclusions will be beneficial to further research involving stallion sperm activation as well as research towards the goal of successful equine *in vitro* fertilization. Failure of equine *in vitro* fertilization is attributed to an inability of sperm to fully capacitate in order to penetrate the zona pellucida (Leemans et al., 2016). A better understanding of the signaling mechanisms involved in stallion sperm activation are required towards this end but we feel that the information gained from this research creates another step forward.

Possibility for Advancement

Further research is necessary to discover the requirements for activation as well as the complete signaling pathways involved in stallion sperm activation. A better understanding of these mechanisms is necessary for creating a successful protocol for equine IVF. A replication of our sperm motility and acrosomal status experiments with the addition of incubation with cultured oviductal cells and a zona pellucida would be beneficial in creating an environment that more closely resembles the mare's reproductive tract. It would also demonstrate the effect of zona pellucida binding on the acrosome reaction, both with and without incubation in the presence of progesterone.

Additional research would be beneficial to demonstrate that progesterone significantly induces hyperactivated motility in stallion sperm. A replication of our experimental design with a modification of evaluating samples with computer assisted sperm analysis (CASA) every 5 minutes instead of every 30 minutes could demonstrate this. This would also be useful for better describing changes in total motility that follow an increase in hyperactivation. An increase in sample size to 10 stallions instead of the 4 stallions that we used would contribute to a greater significance as well.

Further research is necessary to determine the mechanisms by which procaine causes hyperactivation. Procaine causes hyperactivated motility independent of the CatSper channel as well as in environments low in extracellular calcium demonstrating that hyperactivation can occur without an influx of calcium (Loux et al., 2013). I believe that an understanding of this alternative pathway would create new opportunities for manipulating stallion spermatozoa.

Additional investigations would be valuable to measure calcium influxes into stallion sperm cells based upon treatment with progesterone. This could further validate the model that progesterone bound ABHD2 acts upon CatSper to allow calcium to enter the cell. The results from this test could also be correlated to measurements of total motility at different time points to see if they support each other. Flow cytometry could be used to measure calcium levels within spermatozoa though a stain would have to be used whose fluorescence was not quenched by any of our sample agents. A potential stain that could be used is Fluo-4 AM (Thermo Fisher, Waltham, MA) as Miller et al. (2016) used this stain for fluorescent microscopy of calcium levels in human sperm cells treated with MAFP and did not experience any fluorescent quenching due to an interaction of the two agents. Loux et al. (2013) used this stain to measure intracellular calcium levels in stallion spermatozoa with fluorescent microscopy.

Progesterone was not found to have an effect on acrosome reaction in stallion sperm cells when tested using flow cytometry. These results oppose other studies which were tested using fluorescence microscopy and found that progesterone initiates acrosome reaction in stallion sperm (Cheng et al., 1998; Rathi et al., 2003). It would be beneficial to replicate our acrosome reaction tests while simultaneously following the protocols described by Cheng et al., (1998) and Rathi et al. (2003). Rathi et al. (2003) utilized PSA-FITC to measure acrosome reaction in stallion sperm instead of the PNA-FITC that we utilized so repeating our experiments with PSA-

FITC as our stain might produce different results for the effect of progesterone on acrosome reaction.

One of the issues that we experienced with our experiment is that there is not an appropriate substance to use as a control for initiating acrosome reaction. Exposure of the acrosomal contents can occur through rapid freezing of stallion sperm in liquid nitrogen but that is not representative of the physiologic methods by which sperm undergo acrosome reaction. This method can be used in future experiments though for illustrating dead, acrosome reacted sperm cells for setting gates on the resulting dot plots.

This project has potential for further advancement towards a successful equine IVF protocol. As technologies advance to allow researchers to better replicate the environment of a mare's oviduct, our research can be utilized to a better understanding of the signaling mechanisms involved in stallion sperm activation.

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