DISSERTATION

IN VITRO CAPACITATION OF STALLION SPERMATOZOA

Submitted by

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WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY BETH SPIZZIRI ENTITLED IN VITRO CAPACITATION OF STALLION SPERMATOZOA BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

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ABSTRACT OF DISSERTATION

IN VITRO CAPACITATION OF STALLION SPERMATOZOA

Equine in vitro fertilization has resulted in limited success, and progress is hindered due to a lack of understanding the molecular and biochemical events involved in stallion sperm capacitation. As no single test exists to determine if a stallion sperm is capacitated, individual events of capacitation can be monitored to determine if treatments can induce *in vitro* changes involved in sperm capacitation. In addition, the limited availability of equine oocytes for experimentation has led to the use of heterologous oocyte assays to determine if various sperm treatments to induce sperm capacitation can result in these sperm fertilizing oocytes *in vitro.*

In experiment 1, sperm plasma membrane cholesterol content of sperm was examined after treatment with capacitation inducing agents. Samples treated with methyl-β-cyclodextrin (MBC) exhibited lower ($p<0.05$) cholesterol content after 3 h incubation (16 $\mu g/10^8$ sperm) than control sperm at 0 h (22 $\mu g/10^8$ sperm). Samples preloaded with cholesterol, after incubation with cholesterol-loaded-cyclodextrin (CLC), contained more cholesterol than control sperm ($p<0.05$).

The second experiment was designed to determine if that protein tyrosine phosphorylation, a component of sperm capacitation, occurs under *in vitro* conditions. Sperm were capacitated *in vitro* in Modified Whitten's (MW) medium alone or with dilauroylphosphatidylcholine (PC12; 40 μm), calcium ionophore A23187 (2 μm), or MBC (1μm) for 0, 30, 90, and 180 min, and the amount of protein tyrosine

phosphorylaton was assessed. PC12-treated sperm exhibited the highest amount of protein tyrosine phosphorylation at time 0 h. Control sperm exhibited the highest amount of protein tyrosine phosphorylation following a 3 h incubation. Tyrosine phosphorylation was negligible with MBC and calcium ionophore A23187 treatments.

The third experiment was designed to adapt detection of protein tyrosine phosphorylation detection of stallion spermatozoa to flow cytometery. When sperm were incubated with nothing (control), PC12 (40 μm), MBC (1 μm), or calcium ionophore A23187 (2 μm) for 0, 10, 20, 30, 45, 60, 90, 120, or 180 min, and then fixed, permeabilized and incubated with a fluorescein isothiocyante (FITC)-labeled monoclonal antibody to phosphorylated protein, no consistent results were obtained using flow cytometry.

Experiment 4 was designed to detect and classify sperm as hyperactive using novel software, minimum square binding ratio (MSBR). Control and CLC-treated stallion spermatozoa were incubated in MW or MW plus 5 mM procaine and then capacitated with PC12 (40 μm) or MBC (1 μm) for 15 min or 3 h. Sperm motility parameters were assessed using both the standard computer assisted sperm analysis (CASA) and the MSBR classification. Procaine treatment only, induced hyperactive motility in CLC-treated PC12-capacitated sperm after 3 h incubation when using standard CASA analysis. MBC- treated spermatozoa exhibited the greatest changes in sperm motion parameters after 3 h. However, MSBR analysis indicated that neither PC12 nor MBC-treated sperm were hyperactive at either time point, although all procaine supplemented samples had higher percentages of hyperactive sperm than control sperm ($p < 0.05$).

Experiment 5 was designed to determine the effects of procaine supplementation on the acrosome reaction of stallion sperm treated with PC12 or MBC. Stallion spermatozoa incubated in MW or MW plus 5 mM procaine were treated with nothing (control), PC12 (40 μm), or MBC (1 μm) for either 15 min or 3 h. The samples were then dual stained with FITC-PNA and propidium iodide (PI) and assessed by flow cytometry. While PC12 and MBC induced acrosome reactions in sperm, procaine had no effect on inducing acrosome reactions in stallion spermatozoa.

Fertilization of bovine oocytes *in vitro,* with PC12-(15 μM) treated stallion sperm resulted in higher cleavage rates (25% ± 3) than untreated sperm (9 % ± 4; $p < 0.05$). The ability of stallion spermatozoa to fertilize bovine oocytes following zona pellucida laser disruption was then addressed. Bovine oocytes given laser treatment exhibited lower cleavage rate when untreated or PC12-treated sperm were co-incubated with them (3 and 4 % ± 2; $p < 0.05$) than zona intact oocytes inseminated with similarly treated sperm (9 vs. 30% ± 2; $p < 0.05$).

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Chapter I

Review of Literature

Introduction

Equine *in vitro* fertilization (IVF) is unsuccessful, and a poor understanding of the mechanisms to capacitate sperm may be responsible for this lack of success. Sperm capacitation and the subsequent acrosome reaction can be induced in vitro in many species (McPartlin et al., 2008), and penetration by "capacitated" equine sperm into zona free and zona intact oocytes has been achieved (Spizziri et al., 2008; Landim-Alvarenga et al., 2001). However, equine sperm capacitation does not routinely lead to offspring produced via IVF. Therefore, a more complete understanding of the events leading to fertilization is necessary.

Both sperm capacitation and hyperactivated motility are required for fertilization (McPartlin et al., 2009). The complexity of capacitation makes a single test for assessing capacitation difficult. Several compounds induce sufficient capacitation for stallion sperm to penetrate oocytes *in vitro*, but we are still unable to produce IVF offspring (McPartlin et al., 2008, Spizziri et al., 2008).

Membrane cholesterol levels, which decrease during sperm capacitation, can be increased by treating sperm with cholesterol-loaded-cyclodextrins (CLC) or decreased by treating cells with methyl-β-cyclodextrins (MBC; Visconti et al., 1999;

Purdy and Graham, 2004; Moore et al., 2005). CLC treatment increases sperm cryosurvival rates but tends to reduce pregnancy rates, likely due to CLC-treated sperm requiring longer capacitation time to remove sufficient cholesterol from the membrane, and by the time they were effectively capacitated, the oocytes were becoming too mature (Spizziri et al., 2010). Stallion and bovine sperm can be capacitated *in vitro* using dilauroylphosphatidylcholine (PC12), a plasma membrane disruptor, as well as MBC, which induces an efflux of cholesterol from membranes. These agents also induce the acrosome reaction, and the treated sperm are able to fertilize and induce cleavage in bovine oocytes. However, sperm treated with these agents fail to fertilize equine oocytes *in vitro* (Spizziri, unpublished observations).

Recently, stallion sperm capacitated in a Modified Whitten's (MW) medium supplemented with 5mM procaine exhibit hyperactivated motility and fertilized equine oocytes (McPartlin et al., 2008, McPartlin et al., 2009).

We are beginning to understand the multifactorial process of stallion sperm capacitation and determine methods to induce each *in vitro*. Previous research in our laboratory (data unpublished) obtained unsuccessful penetration of *in vitro* matured equine oocytes inseminated with stallion sperm capacitated with PC12, MBC, and Calcium ionophore A23187. Therefore, these experiments investigated whether these agents were inducing an actual sperm capacitation rather than simple an acrosome reaction.

Capacitation

Ejaculated mammalian spermatozoa must undergo a series of biochemical and functional modifications, termed capacitation, to acquire the ability to successfully fertilize an oocyte (Austin, 1967; Amann and Graham, 1993; Rathi et al., 2001). Briefly, these modifications include removal of acrosomal stabilizing factor, plasma membrane cholesterol efflux, increased intracellular bicarbonate (HCO^{3-}) and calcium (Ca^{++}) concentrations, protein phosphorylation, acquisition of hyperactive motility, and actin polymerization (Jha et al., 2002; Brener et al., 2003; Breitbart et al., 2005). Collectively, these changes allow the spermatozoa to gain hyperactive motility, bind to the zona pellucida, undergo the acrosome reaction, penetrate the zona pellucida, and initiate gamete fusion (Barbonetti et al, 2008).

Cholesterol efflux ultimately renders the plasma membrane less stable and increases fluidity (Holt and North, 1984; Langlais and Roberts, 1985; Ehrenwald et al., 1988; Amann and Graham, 1993; Visconti et al., 1999). This cholesterol efflux may be due, at least partially, to albumin which is the major protein in female genital tract secretions; this protein binds cholesterol and fatty acids, which induces fatty acid and cholesterol efflux from the sperm plasma membrane (Tulsiani et al, 1997). Reduced membrane stability renders the membrane more permeable to ions, such as calcium and bicarbonate, which initiates a series of transmembrane signaling events that control capacitation (Ehrenwald et al., 1988; Landim-Alvarenga et al., 2001; Travis and Kopf, 2002). Capacitation is likely regulated by a signal transduction pathway involving cross-talk between cyclic adenosine monophosphate (cAMP), protein kinase A (PKA), and tyrosine kinases (Figure 1.1;

Travis and Kopf, 2002). The cAMP/PKA cross-talk pathway is unique to sperm; the increase in cAMP leads to an activation in PKA which then regulates protein tyrosine phosphorylation (Naz and Rajesh, 2004).

Other biochemical changes in sperm that occur during capacitation as a result of the influx of ions (namely calcium and bicarbonate) include adenylate cyclase (AC) activation which increases intracellular cAMP, pH changes, and an increase in protein tyrosine phosphorylation (Parrish et al., 1994; Vredenburgh-Wilberg and Parrish, 1995; Parrish et al., 1999; Visconti et al., 1999). These changes appear to occur in sequence, with the influx of Ca^{++} and bicarbonate ions leading to the activation of AC, which increases cAMP (Breitbart, 2002), and the elevated cAMP activates protein kinase A (PKA), which leads to protein tyrosine phosphorylation (Osheroff et al., 1999; Visconti et al., 1999). The phosphorylated proteins then regulate several sperm processes including sperm capacitation, hyperactive motility, and the acrosome reaction (Grasa et al., 2009).

Capacitation status of sperm cells has been assessed using several staining techniques including chlorotetracycline (CTC) and Merocyanine 540 (M540). CTC, a fluorescent antibiotic, permeates the cell membrane and binds free intracellular calcium, fluorescing when excited with fluorescent light. The CTC-calcium exhibits three staining patterns of presumed spermatozoal status: non-capacitated, capacitated acrosome intact, and capacitated acrosome reacted (Rathi et al., 2001; Gillan et al., 2005). However, there is not a clear molecular understanding of CTC staining patterns with the sperm surface (Rathi et al., 2001).

An alternative stain for capacitation status is M540. M540 is a hydrophobic dye that intercalates into the cell membrane, associated with the lipids and fluoresces differently depending on the "disorder" of the phospholipids in the outer leaflet of the plasma membrane lipid bilayer, a characteristic observed in capacitated sperm (Rathi et al., 2001; Gillan et al., 2005). This altered membrane disorder is believed to occur prior to the calcium influx that is detected with CTC, making this a method to detect the earlier events of capacitation (Gillan et al., 2005). Unfortunately, neither of these methods for assessing capacitation status has led to a successful capacitation treatment selection, resulting in equine IVF offspring. Therefore, a better understanding of the molecular and biochemical events that occur during capacitation is needed to allow the development of an accurate *in vitro* capacitation treatment selection and ultimately, a detection assay.

Protein Tyrosine Phosphorylation

Protein tyrosine phosphorylation has been correlated with sperm capacitation in many species (Grasa et al., 2006; Galantino-Homer et al., 1997; Visconti et al., 1995; Pommer et al., 2003). Protein phosphorylation, a post-translational modification, acts as a regulatory mechanism for processes such as cellular growth, cell cycle control, cytoskeleton assembly, ionic current modulation, and receptor regulation (Naz and Rajesh, 2004). Addition or removal of a phosphate group can induce allosteric modifications resulting in conformational changes that lead to protein activation or inactivation, which allows a cell to control signal transduction (Naz and Rajesh, 2004; Grasa et al., 2009). Although phosphorylation

can occur at serine, threonine, and tyrosine residues, the major activator of signal transduction pathways appears to be through phosphorylation of tyrosine residues (Naz and Rajesh, 2004). Multiple phosphorylated proteins and their prospective roles have been identified in sperm of several species (Flesch et al., 1999; Sakkas et al., 2003; Urner et al., 2003; Naz and Rajesh, 2004). The flagellum of the spermatozoon in most species, except the boar, seems to be the primary structure undergoing tyrosine phosphorylation, which is then followed by the midpiece (Naz and Rajesh, 2004). Tyrosine phosphorylation of the flagellum may be responsible for the acquisition of hyperactivated motility (Nassar et al., 1999; Si and Okuno, 1999; Mariappa et al., 2010).

Although not all phosphorylated proteins are present in the sperm from all species studied to date, and there is some discrepancy within species, tyrosine phosphorylation of proteins has extensive involvement in fertilization (Sakkas et al., 2003; Naz and Rajesh, 2004). In addition to proteins phosphorylated in the flagellum, studies report immunolocalization of phosphotyrosine-containing proteins following sperm capacitation in the acrosomal region, and this redistribution of phosphotyrosine residues may be involved in ZP interactions and/or fusion events (Leyton and Saling, 1989; Tardif et al., 2001; Cormier and Bailey, 2003). In human sperm, a number of proteins that interact with the zona pellucida have been identified using an anti-phosphotyrosine antibody (Naz and Rajesh, 2004). Several plasma membrane proteins in boar sperm are phosphorylated and are implicated in ZP binding and the acrosome reaction (Flesch et al. 1999). In human sperm, a 46 kDa protein seems to have an important role in

sperm-ZP binding and capacitation, and cross-talk between 94 and 46 kDa proteins appears to occur during capacitation (Naz and Rajesh, 2004). In mouse sperm, the tyrosine phosphorylation of two chaperone proteins (endeoplasmin and heat shock protein 60) located on the plasma membrane of the sperm head may undergo conformational changes to aid in sperm-ZP binding (Asquith et al., 2004).

Traditional phosphorylation assessment utilizes Western blot procedures (Grasa et al., 2006; Visconti et al., 1995; McPartlin et al., 2009). However, this method is time-consuming, limiting in sample size assessment, and only is semiquantitative (Barbonetti et al., 2008). Recently, flow cytometry has been used to assess tyrosine phosphorylation in several species (Sidhu et al., 2004; Piehler et al., 2006; Barbonetti et al., 2008). Flow cytometry offers a rapid, simple, and reliable alternative for tyrosine phosphorylation studies (Barbonetti et al., 2008).

Hyperactive motility

Oocyte penetration requires a change in sperm motility, termed hyperactivation. Hyperactive motility occurs only in sperm that have undergone capacitation, and the motion characteristics of hyperactivated sperm are markedly different from those exhibited by noncapacitated sperm (Yanagimachi, 1970). Hyperactive sperm exhibit characteristic pronounced flagellar movements in erratic "figure 8" or "star" patterns, and forward progressive motility becomes non-linear (Brewis et al., 2005, Mújica et al., 1994).

In most mammalian species, sperm acquire hyperactivity in the isthmus of the oviduct and progression out the caudal isthmus is seemingly dependent on

sperm hyperactivation (Tulsiani, 1997). Hyperactivation is required for zona pellucida penetration and is implicated in assisting in sperm detachment from the oviductal wall, oviductal transport, and for penetration of the viscous mucoid environments of the female reproductive tract (Suarez et al., 1991; Saurez and Dai, 1992; Tulsiani, 1997; Suarez, 2008; McPartlin et al., 2009). In some species, such as the boar, hyperactive motility is crucial for fertilization as sperm that have not become hyperactive are unable to penetrate the oviductal mucus (Suarez et al., 1992). In addition, hyperactive mouse and hamster sperm are more effective at penetrating highly viscoelastic medium (Suarez et al., 1991; Suarez and Dai, 1992).

Hyperactivation occurs during capacitation; however the pathways coupling these two events have yet to be completely elucidated (Marquez and Suarez, 2008). Hyperactivation appears to involve multiple parameters including increasing cytoplasmic levels of Ca^{++}, cAMP, ATP, and the activation of cation-channel-like proteins (CatSper). Evidence suggests Ca^{++} and cAMP are involved in signaling the onset of hyperactivation (Ho et al., 2002) as increased intracellular Ca^{++} has been observed in hyperactivated hamster and bull sperm (Suarez et al.. 1993; Ho and Suarez, 2001). Increased intracellular cAMP also has been correlated with hyperactivated motility onset during *in vitro* sperm capacitation (White and Aitken, 1989). Additionally, hyperactive motility can be enhanced by treating sperm with membrane permeant cAMP analogues or phosphodiesterase inhibitors (White and Aitken, 1989; Calogero et al., 1998; Yeung et al., 1999).

The mechanism of calcium modulation involved in elevated flagellar Ca^{++} concentrations in hyperactive sperm is unknown (Quill et al., 2003). Extracellular

Ca^{++} is required to maintain sperm hyperactivation, and a proposed activation pathway is that calcium serves as a second messenger to activate calmodulin kinase II (Ho and Suarez, 2001; Suarez, 2008). The primary source of Ca^{++} is extracellular calcium brought through the plasma membrane, but also may come from organelles like the redundant nuclear envelope (Suarez, 2008). Mammalian sperm acrosomes also have been implicated in Ca^{++} storage, where the role of calcium is primarily for acrosomal exocytosis. Intracellular calcium increases have been noted in acrosome-reacted sperm flagella and heads, and the calcium increase may potentially contribute to hyperactivation (Ho et al., 2002; Carlson et al., 2005; Suarez, 2008).

The Ca^{++} release through the CatSper channels involved in hyperactivation begins in the sperm tail and propagates to the head (Olson et al., 2010). Sperm heads exhibited sustained increased Ca^{++} which is necessary for the sperm to acrosome react. In addition, dose dependent Ca^{++} studies in which sperm were incubated in varying Ca^{++} concentrations, resulted in increases in sperm hyperactivation detected by curvilinear velocity, with the highest percentages of sperm hyperactive at 400 nM Ca^{++} (Ho et al., 2002).

Elevated cAMP levels produced by the Ca^{++} and HCO^{3-} activation of soluble adenylyl cyclase (sAC), and are associated with hyperactivated motility (White and Aitken, 1989). Other studies have shown that cAMP is not directly involved in triggering sperm hyperactivation (Ho and Suarez, 2001; Ho et al., 2002; Marquez and Suarez, 2008), but is important for supporting sperm motility in general (Marquez and Suarez, 2008). Additionally, cAMP activation of PKA is necessary for

protein phosphorylation in sperm from hamster, monkey, and humans (Suarez, 2008; Marquez and Suarez, 2004), but protein phosphorylation is not required for bull sperm hyperactivation (Marquez and Suarez, 2004).

A sufficient increase in intracellular ATP is required to produce the flagellar bending characteristics observed in bull sperm hyperactive motility (Ho et al., 2002). Demembranated/nonmotile bull sperm can be reactivated when incubated in medium with supplemental ATP (Ho et al., 2002), and the net ATP consumption is greater in hyperactivated bull sperm than sperm exhibiting progressive motility (Ho and Suarez, 2003). Interestingly, mammalian sperm possess a very high ATP content compared to other cell types (Ho et al., 2002; Ho and Suarez, 2003).

CatSper, specifically CatSper-1 -2 -3 and -4, is required for male fertility (Carlson et al., 2005; Qi et a., 2007). These heterotetrameric, pH, and voltage dependent, sperm specific permeable Ca^{++} ion channels have been localized in the plasma membrane of the flagellum (principal piece) of sperm (Carlson et al., 2005; Olson et al., 2010). The mechanism by which cAMP activates CatSper channels is not completely understood; however, elimination of one or more CatSper proteins leads to decreased sperm motility (Olson et al., 2010). Another important aspect of CatSper activation is that it exhibits increased activation under alkalinized conditions (Kirichok et al., 2006), which biologically occurs as murine sperm transit from the vagina (pH ~5) to the cervical mucus (pH ~8; Qi et al., 2007). This alkalinization may stimulate the axoneme bending seen in hyperactivation (Suarez, 2008; Olson et al., 2010). In addition to sperm motility regulation, targeted gene disruption of these channels prevented hyperactivation (Carlson et al., 2005; Qi et

al. 2007). CatSper activation and Ca^{++} influx are independent of the acrosome reaction, as CatSper null sperm from mice can undergo the acrosome reaction, but do not exhibit hyperactivated motility (Quill et al., 2003). Therefore, the infertility observed in CatSper null mice is due to some mechanism other than failure to capacitate. CatSper null sperm can fertilize oocytes that have had their ZP removed; therefore, the infertility observed by those sperm is due to failure to acquire hyperactive motility which results in ZP penetration problems (Avidan et al., 2003; Quill et al., 2003).

As demonstrated by the hamster, hyperactivation of sperm facilitates zona pellucida penetration (Stauss et al., 1995). When calcium channel blockers were added to capacitated zonae bound hamster sperm, to inhibit hyperactive motility, they did not alter normal sperm motility. However, while these sperm underwent the acrosome reaction, they failed to penetrate the zonae (Stauss et al., 1995). More recent experiments have shown that sperm from mice that were null for CatSper proteins were able to penetrate zonae free oocytes (Quill et al., 2003; Ren et al., 2001). Therefore, sperm capacitation and hyperactivation are synergistic in enabling a sperm to fertilize an oocyte (Suarez et al., 2008).

Several local anesthetics as well as Ca^{++} antagonists have been tested for their ability to induce hyperactive motility in sperm (Mújica et al., 1994). Recent research has shown that procaine can induce hyperactive motility in stallion sperm (McPartlin et al., 2009). The mechanism by which procaine induces hyperactive motility is not known; however, procaine is proposed to stimulate Ca^{++} influx via increasing plasma membrane Ca^{++} permeability (Mújica et al., 1994; McPartlin et al.,

2009). This seems reasonable as procaine addition leads to increased cytoplasmic Ca^{++} only when high extracellular calcium levels are maintained (Mújica et al., 1994). Calcium plays a crucial role in procaine induced hyperactivation that may be a separate or divergent pathway from acquisition of acrosomal responsiveness (Marquez and Suarez, 2004). In addition, procaine will induce hyperactive sperm motility regardless of the capacitation status of sperm, since adding procaine to uncapacitated bull and guinea pig sperm induces a rapid induction of hyperactivated motility (Mújica et al., 1994; Ho et al., 2002). Again, these data seem reasonable since bull sperm hyperactivation occurs independently of capacitation, and activating the capacitation signal transduction pathway is insufficient to stimulate hyperactivation. Additionally, bull sperm hyperactivated motility is not associated with increased protein tyrosine phosphorylation, and inhibiting cAMP/PKA has no effect on procaine induced hyperactivation (Marquez and Suarez, 2004). Procaine has seemingly no effect on tyrosine phosphorylation or acrosomal exocytosis, and increased fertilization rates are thought to be attributed only to its ability to induce hyperactive motility (McPartlin et al., 2009).

Computer-assisted semen analysis (CASA) has been used to analyze hyperactivation (Mortimer and Maxwell, 1999; Cancel et al., 2000; Suarez, 2008). The motility parameters used to determine hyperactive motility are species dependent (Mortimer and Mortimer, 1990; Suarez et al.. 1992; Baumber and Meyers, 2006), and until recently these parameters for horse sperm were undefined (McPartlin et al., 2009). Recently, McPartlin et al. (2009) defined motion parameters in equine sperm following incubation in procaine that were consistent

with hyperactive sperm in other species. However, CASA analysis still is limited in classifying hyperactivation (Kaula et al., 2009). Kaula et al. (2009) developed a robust algorithm, termed Minimum Bounding Square Ratio (MBSR), which can accurately detect the motility pattern changes seen as sperm transition from progressive to hyperactive motility. Retrospective MBSR analysis indicated that sperm were correctly classified as hyperactive (>93%) and progressive (98%) sperm whereas traditional sperm curvilinear velocity (VCL) analysis that is traditionally done with CASA results was far less sensitive (<50%; Kaula et al., 2009). This discrepancy indicated that the MBSR classification is a more promising and sensitive method of detecting hyperactive sperm motility (Kaula et al., 2009).

Acrosome Reaction

Following capacitation and the acquisition of hyperactive motility, sperm undergo an exocytotic event known as the acrosome reaction (Austin, 1967; Graham, 1996; Fraser, 1998). The "normal" acrosome reaction occurs only in capacitated sperm and is triggered by the binding of spermatozoon to proteins on the oocyte zona pellucida (ZP). Binding to the ZP initiates the influx of calcium into the sperm that acts as a second messenger to signal transduction pathways, causing plasma membrane fusion with the outer acrosomal membrane at multiple sites. The inner acrosomal membrane becomes continuous with the plasma membrane at the post equatorial segment. This membrane fusion exposes sperm receptors for the oolemma that are located on the inner acrosome membrane, and releases the acrosomal hydrolytic enzymes, which digest a hole through the zona pellucida (Graham, 1996; Gadella et al., 2001; Breitbart, 2002; Neild et al., 2005). The

spermatozoon enters the perivitelline space, binds to and then fuses with the oolemma, finally activating the oocyte (Gadella et al., 2001).

Similar to capacitation, the signaling mechanisms involved in the acrosome reaction are not completely understood. Tyrosine phosphorylation of proteins appears to be important in the acrosome reaction and oocyte penetration as the distribution of phosphorylated proteins changes following the induction of the acrosome reaction (Grasa et al., 2009). The acrosome reaction leads to a loss of phosphorylated proteins on the acrosome and an increase in the post-acrosomal region and the flagellum (Grasa et al., 2009). The altered localization of phosphorylated proteins following the acrosome reaction could activate proteins involved in sperm-oocyte penetration (Grasa et al., 2009).

Oocyte Penetration

Mammalian fertilization includes a series of molecular and cellular events. These events include sperm penetration through the cumulus cell mass, sperm adhesion and binding to the zona pellucida (ZP), sperm acrosomal exocytosis, sperm penetration through the ZP, and finally sperm and oocyte fusion (Hong et al., 2002; Mugnier et al., 2009; Kim et al., 2008). The cumulus cells surrounding an oocyte support oocyte growth and maturation, as well as maintain meiotic arrest until the oocyte achieves adequate cytoplasmic maturation (Goud et al., 1998; Van Soom et al., 2002). Cumulus cells significantly contribute to sperm penetration, as the sperm that pass through the cumulus exhibit normal morphology and acrosome reactions (Hong et al., 2002).

The oocyte's cumulous oophorus is highly viscoelastic and sperm penetration is thought to be assisted by sperm head hyaluronidase activity, but hyperactivation also may play a role (Suarez, 2008; Kim et al., 2008; Kim et al., 2005). The cumulus cells of an oocyte are abundant in hyaluronan, a polysaccharide consisting of repeating disaccharide units of *N*-acetyl-D-glucosamine and D-glucuronic acid (Kim et al., 2005). Mammalian sperm have a GPI-anchored hyaluronidase, PH-20, thought to catalyze hyaluronan degradation, which then allows acrosome intact sperm to penetrate the cumulus (Cherr et al., 2001; Kim et al., 2005; Kim et al., 2008). In stallion spermatozoa, PH-20 expression in ejaculated stallion sperm is significantly different than the expression found in cauda sperm (Meyers, 2001). The altered expression of mature stallion sperm indicates that PH-20 could be involved in cumulus penetration, sperm-egg recognition, and oolemmal fusion (Meyers, 2001). Other research also indicates PH-20's potential role in the acrosome-reacted sperm's binding to the ZP (Kim et al., 2005). However, male mice lacking PH-20 are still fertile and have similar ZP binding (Kim et al., 2005). Epididymal sperm that lack PH-20, display a delayed dispersal of cumulus cells which may be attributed to an additional protein, a 55-kDa hyaluronidase (Hyal5), which is present in epididymal sperm and may serve to aid in cumulus cell penetration (Kim et al., 2005).

Following penetration of the cumulus cells, a capacitated, acrosome-intact sperm binds to the zona pellucida, which induces the acrosome reaction. In equine sperm, the acrosome reaction is induced by ZP3, one of four equine sulfated glycoproteins on the ZP (Kopf, 1990; Wassarman and Litscher, 2008; Mugnier et al.,

2009). Acrosin, a multifunctional protein and inner acrosomal enzyme, begins zona pellucida digestion, allowing for future fusion events (Klemm et al., 1991). Following the acrosome reaction, the sperm binds to ZP2, penetrates the zona pellucida, traverses the perivitelline space, and fuses with the oocyte plasma membrane (Kopf, 1990). Following sperm oocyte fusion, the oocyte undergoes the cortical granule reaction that releases enzymes that modify the zona glycoproteins (Kopf, 1990). These changes collectively form a polyspermy block, which hardens the zona to prevent secondary sperm binding and zona pellucida penetration (Wassarman and Litscher, 2008; Kopf 1990).

Following sperm penetration, a series of events termed "oocyte activation" commences. Oocyte activation is a series of events including cortical granule exocytosis, block to polyspermy, and the release from meiotic arrest (Ajduk et al., 2008; Grasa et al., 2008). In addition, a series of Ca^{++} oscillations are induced that lead to completion of meiosis, pronuclear formation, initiation of DNA synthesis, and embryonic cleavage (Bedford et al., 2004). This activation results from an increase in cytosolic free Ca^{++} concentration within the oocyte which commences 1-5 min following gametic fusion (Jellerette et al., 2000; Knott et al., 2003). Activation likely is initiated due to the sperm-specific phospholipase C zeta (PLCζ; Jellerette et al., 2000; Saunders et al., 2002, Grasa et al., 2008). PLCζ cleaves phosphatidylinositol 4,5-bisphosphate (PIP2) to 1,4,5-inostiol triphosphate (IP_3) and diacylglycerol (DAG). IP_3 then binds to its receptor, likely IP_3R1 located in the endoplasmic reticulum (ER) of the oocyte, which triggers the opening of the Ca^{++} channels and consequent Ca^{++} release and subsequent oscillations into the cytoplasm (Ajduk et al.,

2008). DAG is involved in the activation of PKC which induces downstream signal transduction of a whole host of cytoplasmic enzymes (Tatone and Carbone, 2006).

There is very little research available using equine sperm and equine oocytes due the limited availability of equine oocytes. Therefore, researchers have had to develop heterologous assays to research equine stallion sperm and oocyte interactions (Alm et al., 2001; Sinowatz et al., 2003; Coutinho da Silva et al., 2004; Mugnier et al., 2009). This research has shown that stallion sperm can bind to, acrosome react, and fuse with heterologous oocytes (Sinowatz et al., 2003; Mugnier et al., 2009). The ability of equine sperm to bind to the bovine zona pellucida and acrosome react indicates a partially conserved ZP ligand system between the equine and bovine (Sinowatz et al., 2003). Zona-free hamster and bovine oocytes also can be used to assess the fertilizing capacity of sperm from a variety of heterologous species. However, because the zona pellucida is removed, this assay does not evaluate the ability of sperm to bind to the zona pellucida or undergo the acrosome reaction (Brackett et al., 1982; Sinowatz et al., 2003). Mugnier et al. (2009) found that *in vitro* capacitated equine sperm have a decreased capacity to undergo the acrosome reaction after ZP binding when compared to porcine sperm **using what species of oocyte**. More importantly, the origin of ZP did not determine the ability of equine sperm to acrosome react. Therefore, the decreased ability of an equine sperm to acrosome react once bound to a ZP is likely a result of difficulties with effective induction of *in vitro* sperm capacitation (Mugnier et al., 2009).

In addition to capacitation difficulties, the ZP is another barrier that limits equine IVF success as only zona-free equine oocytes were penetrated by equine

sperm (Mugnier et al., 2009). Additionally, equine sperm were more capable of penetrating zona-free porcine oocytes compared to zona-intact porcine oocytes (Mugnier et al., 2009). The decreased sperm penetration rates of equine sperm into zona intact equine oocytes also may be due to a decreased number and diameter of small pores in the mesh-like structure of the equine ZP (Mugnier et al., 2009). This problem can be circumvented with partial disruption of the ZP by mechanical or chemical techniques (Choi et al., 1994; Mugnier et al., 2009). Clinically, intra cytoplasmic sperm injection (ICSI) is a successful assisted equine reproductive technique used to eliminate sperm binding and sperm penetration problems (Squires et al., 2003).

The relationship between spermatozoal zona binding and fertility is controversial. Braundmeier et al. (2002) were unable to correlate the relationship between the zona binding ability of sperm and the fertility of bulls using a competitive assay. In addition, Braundmeier et al. (2002) stated that correlations between human fertility and zona pellucida binding assays are poor due to the difficulty of collecting accurate fertility data. However, Liu et al. (2003) stated that zona pellucida binding of human sperm correlated well with in vitro fertilization rates as non motile sperm from subfertile men are unable to bind to the zona pellucida as efficiently as motile sperm from fertile men. Human IVF laboratories have utilized the zona pellucida binding assay to evaluate spermatozoal fertilizing ability (Muller, 1992). In humans, failure of sperm-zona pellucida binding and

penetration is usually attributed to sperm morphological abnormalities (Liu and Baker, 2000). Therefore, this assay can be used to measure simultaneous sperm attributes as an indication of fertility (Graham, 1996).

Capacitation Inducing Agents

The calcium ionophore A23187, a divalent cationic ionophore, induces both capacitation and the acrosome reaction in bull and stallion sperm by causing calcium entry into the cell (Triana et al., 1980; Christensen et al., 1996; Graham, 1996; Landim-Alvarenga et al., 2001). Decreased cell viability has been reported with the addition of A23187, potentially due to the ATPase effects that high intracellular calcium concentrations have on cellular ATPases. One example is the calcium-ATPase located in the spermatozoal plasma membrane that pumps calcium out of the cell to maintain low intracellular calcium levels. The large calcium influx induced by A23187 may be too great for the calcium-ATPase to handle, resulting in high intracellular calcium levels in the spermatozoal head and midpiece. These high calcium concentrations also may affect mitochondrial function directly, causing a loss in cell viability (Landim-Alvarenga et al., 2004).

Dilauroylphosphatidylcholine (PC-12) is a fusogenic lipid induces capacitation and the acrosome reaction by altering sperm plasma membrane physiology in bull, ram, and stallion sperm (Graham, 1996; Graham, 2001; Landim-Alvarenga et al., 2001; Landim-Alvarenga et al., 2004). Graham et al. (1987) reported that PC-12 liposomes induced the acrosome reaction in stallion sperm,

enabling them to penetrate zona-free oocytes. Sperm may undergo a PC-12-induced acrosome reaction due to the ability of PC-12 to induce lipid scrambling in the plasma membrane, and by creating ion pores in the plasma membrane lipid bilayer, increasing membrane permeability to ions (Nolan et al., 1992). Following membrane incorporation, PC-12 alters membrane physiology by reorganizing cholesterol, inducing a fusion between the plasma membrane and the outer acrosomal membrane. This is likely caused by PC-12 accumulation in the outer membrane leaflet, which induces cholesterol to shift from the inner to outer leaflet, leaving an unstable inner leaflet that makes the entire membrane susceptible to fusion (Nolan et al., 1992).

Cyclodextrins are water-soluble cyclic heptasaccharides consisting of β (1-4)-glucopyranose units that can promote cholesterol efflux from a variety of somatic cells, including sperm (Colas et al., 2008). Their cholesterol-binding efficiency correlates directly with their ability to increase protein tyrosine phosphorylation in sperm. Methyl β cyclodextrins are the most potent cyclodextrins for removing cholesterol from membranes, a step in sperm capacitation, because of their high affinity for cholesterol (Tulsiani et al, 1997). Alternatively, cyclodextrins can be preloaded with cholesterol (CLC's), and in this case, they will transfer cholesterol down a concentration gradient into the cell plasma membrane, resulting in increased plasma membrane cholesterol levels (Klein et al., 1995; Purdy and Graham, 2004; Moore et al., 2005).

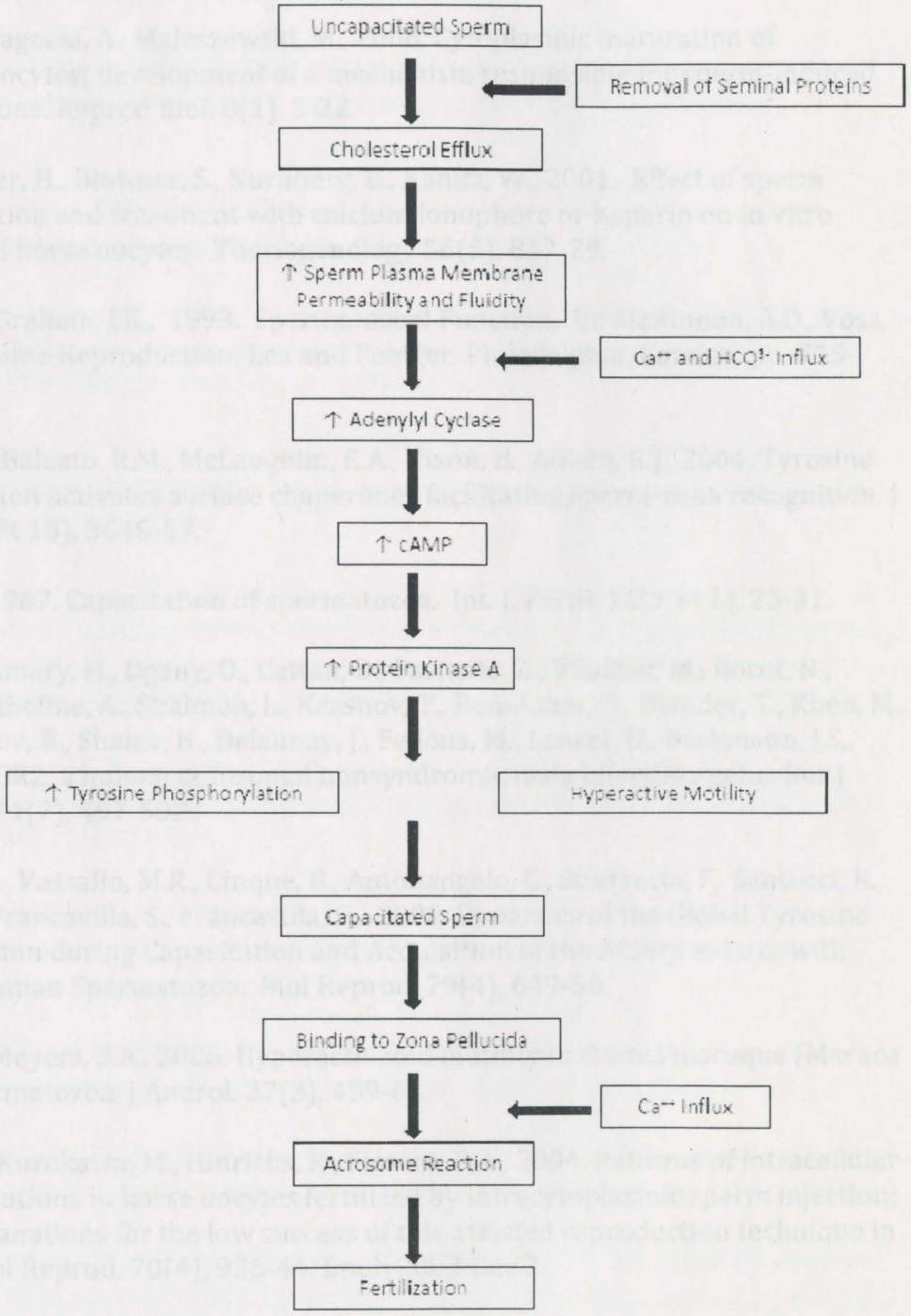
Conclusions

From this review, it is evident that sperm capacitation events are complex, and our limited understanding of such events precludes success with equine IVF. Several attempts have been made to capacitate stallion sperm and fertilize equine oocytes *in vitro*, but as of yet, routine fertilization with equine IVF does not occur. Sperm plasma membranes can be disrupted using a number capacitation agents that induce downstream signaling events associated with stallion sperm capacitation. Our understanding of the induction of protein tyrosine phosphoryaltion, hyperactive motility, decreased plasma membrane cholesterol content, and the acrosome reaction involved in sperm capacitation, should aid in selecting a method of successful *in vitro* stallion sperm capacitation. This knowledge, coupled with the use of bovine oocytes to determine how treated stallion sperm interact with the zona pellucida, and subsequently fertilize the oocyte should increase our knowledge of sperm capacitation, as well as assist in developing a technique to capacitate stallion sperm sufficiently to result in successful equine IVF.

The studies presented in subsequent chapters were conducted to determine if sperm capacitated *in vitro,* using PC12, MBC, or A23187, will exhibit cholesterol depletion, protein tyrosine phosphorylation, hyperactive motility, and finally fertilize *in vitro* matured bovine oocytes. Studies also were performed to adapt protein tyrosine phosphorylation detection on the flow cytometer as a more rapid and reliable method of analysis of stallion sperm capacitation. The effect of PC12 and MBC on stallion sperm hyperactive motility was assessed using a novel method to distinguish hyperactive motility from progressive motility. Since equine oocytes

are only available in limited quantities, bovine oocytes were used as the method of discerning successful fertilization following *in vitro* capacitation of stallion sperm. Results from these studies will enhance our understanding of these capacitation agents' abilities to induce significant sperm physiology changes, which will allow for the selection of a successful sperm treatment for use in equine IVF.

Figure 1.1. Flow chart illustrating potential steps and components involved in the signal transduction mechanism of sperm capacitation, binding to the zona pellucida, the acrosome reaction, and fertilization.



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Chapter II

Induction of Stallion Sperm Capacitation Utilizing Capacitation Agents

Introduction

Capacitation, a poorly understood series of biochemical and functional modifications, is hallmarked by changes in sperm including increased protein phosphorylation, acquisition of hyperactive motility, and plasma membrane cholesterol efflux, and removal of acrosome stabilizing factor (Rathi et al., 2001; Jha et al., 2002; Brener et al., 2003; Pommer et al., 2003; Breitbart et al., 2005; Grasa et al., 2006). The complexity of these events makes assessment difficult, which also illustrates the need for a more complete understanding of the events involved in fertilization.

Sperm plasma membrane cholesterol efflux occurs during capacitation, leading to increased membrane fluidity and decreased membrane stability (Holt and North, 1984; Langlais and Roberts, 1985; Ehrenwald et al., 1988; Visconti et al., 1999). This decreased membrane stability results in ion influx, which in turn initiates a series of transmembrane signaling events that control capacitation (Ehrenwald et al., 1988; Travis and Kopf, 2002). To this end, sperm plasma membrane cholesterol levels can be increased *in vitro* by treating cells with cholesterol-loaded-cyclodextrins (CLC) or decreased using methyl-β-cyclodextrin (Moore et al., 2005; Visconti et al., 2005).

Protein phosphorylation can induce allosteric protein modifications that result in conformational changes which can activate or inactivate protein function (Naz and Rajesh, 2004). Tyrosine residues of proteins are a major activator of signal transduction pathways in sperm capacitation (Visconti et al., 1995; Galantino-Homer et al., 1997;). Additionally, the proteins in the spermatozoal flagellum are the primary structures that undergo tyrosine phosphorylation, and these phosphorylations may, therefore, be responsible for the sperm acquiring hyperactivated motility during the capacitation process (Naz and Rajesh, 2004). Multiple tyrosine phosphorylated proteins are involved in sperm capacitation and ZP binding, and have been identified in several species (Flesch et al., 1999; Naz and Rajesh, 2004).

Historically, protein phosphorylation assessment has been performed using Western blot procedures (Grasa et al., 2006; Visconti et al., 1995; McPartlin et al., 2009). However this method has limitations; Western blotting analysis is time-consuming, is only semiquantitative, and provides a population estimate, not an estimate of individual cells (Barbonetti et al., 2008). Therefore, flow cytometery recently has been adapted for phosphorylation assessment, and is a highly attractive alternative as it is rapid, reliable, and evaluates individual cells (Sidhu et al., 2004; Piehler et al., 2006; Barbonetti et al., 2008).

Hyperactive motility in spermatozoa is typically confirmed with computer-assisted semen analysis (CASA; Mortimer and Maxwell, 1999; Suarez, 2008). McPartlin et al. (2009) recently defined a series of motility parameters that are potentially attributed to capacitation in stallion sperm, following incubation in a

Modified Whitten's media (MW). However, although these capacitated sperm appear to be hyperactive based on CASA assessment, little progress has been made in producing equine IVF offspring. More recently, Kaula et al. (2009) created a robust algorithm, termed the Minimum Bounding Square Ratio (MBSR), which more effectively detects and classifies sperm tracks obtained from CASA as hyperactive, transitional, or progressive, as compared to classical CASA measurements.

In previous reports, stallion sperm capacitated in MW medium supplemented with 5 mM procaine exhibited hyperactivated motility and were able to fertilize equine oocytes (McPartlin et al., 2008, McPartlin et al., 2009). Therefore, this medium was selected to be the control treatment for the following experiments.

The objectives of these experiments were to 1) determine plasma membrane cholesterol levels for sperm treated with capacitation agents; 2) quantify the protein tyrosine phosphorylation of sperm treated with capacitation inducing agents; 3) adapting flow cytometry as a method of assessing protein tyrosine phosphorylation; 4) quantify hyperactive motility of *in vitro* capacitated sperm utilizing both traditional CASA methods as well as MBSR; and 5) confirm the use of capacitation inducing agents to induce the acrosome reaction.

Materials and Methods

Unless otherwise specified, all chemicals were of reagent grade (Sigma Chemical Co., St. Louis, MO).

Preparation of Medium

Modified Whitten's medium (MW) was used to dilute all ejaculates for all semen preparations. This medium was supplemented with 0.7% fatty acid-free bovine serum albumin (BSA) and 2.5 mM Calcium Chloride, as described by McPartlin et al. (2008) to enhance sperm capacitation.

Semen Collection and Processing

Semen was collected from light horse stallions housed at the Colorado State University Equine Reproduction Laboratory maintained under the guidelines approved by the Colorado State University's Animal Care and Use Committee. Ejaculates were collected using a CSU Model Artificial Vagina (Animal Reproduction Systems, Inc., Chino, CA), and immediately following collection, spermatozoal concentrations were determined using a densimeter (Animal Reproduction Systems, Inc., Chino, CA). A small sample was diluted to 50 x 10^6 sperm in EZ-Mixin® Basic Formula Extender (Animal Reproduction Systems, Inc., Chino, CA), and the percentages of total and progressively motile spermatozoa were determined using computer assisted sperm analysis (CASA; IVOS, Hamilton Thorne Biosciences, Beverly, MA, USA). Ejaculates containing less than 50% total motility were discarded. Each ejaculate was diluted 1:1 (v:v) in MW and centrifuged through a 30% Percoll® gradient as described above.

Sperm Processing for Capacitation

After centrifugation through 30% Percoll®, the resultant pellet was diluted in MW to a final concentration of 50 x 10^6 spermatozoa/ml.

Preparation of Percoll® Gradient

A 30% Percoll® solution was prepared by diluting 90% Percoll® (0.67 ml) with MW (1.33 ml). Fresh spermatozoa (2 ml) were then layered over 2 ml of 30% Percoll® solution in 15 ml conical tubes (Fisher Scientific, Houston, TX) and centrifuged for 5.5 min at 400 x g. The supernatant was discarded and the sperm in the resulting pellet were used in the experiments.

Preparation of Capacitation and Acrosome Reaction Agents

Liposomes composed of dilauroylphosphatidylcholine (PC-12) were prepared as described by Nolan et al. (1992). Briefly, PC-12 was dissolved at 10 mg/ml in chloroform and the solvent removed with nitrogen gas. The lipids were suspended in a HEPES-buffered saline medium (S-MEDIA; 37 mM NaCl, 10 mM KCl, 1 mM KH_2PO_4, 36 mM $NaHCO_3$, 2.4 mM $MgSO_4$, 1.7 mM $CaCl_2 \cdot 2H_20$, 84 mM fructose, 5.5 mM glucose, 110 µM Na-pyruvate, 33 mM Na-lactate, 0.3% BSA, Hepes-buffered ; pH 7.3; Wilhelm et al., 1996) put into suspension using a Branson Sonifier® equipped with a CE Converter 102C (Branson Ultrasonics Corporation, Danbury, CT) for 5 min. The liposomes were stored in 0.5 ml aliquots at -70°C until further use. An ammonium ferrothiocyanate assay was used to determine the phospholipid concentration (µM) of the liposome preparation, as described by Stewart (1980).

A calcium ionophore (A23187) stock solution was prepared by diluting the ionophore in dimethylsulfoxide (DMSO) to 9.5 mM. This ionophore solution was stored at 5°C until further use. Prior to addition of A23187 to spermatozoa, a working solution of 250 μM A23187 was prepared by diluting the stock solution in DMSO.

Methyl-β-cyclodextrin was loaded with cholesterol as described by Purdy and Graham (2004). Briefly, 1 g of methyl-β-cyclodextrin was dissolved in 2 ml of methanol in a glass test tube. In a second glass test tube, 200 mg of cholesterol was dissolved in 1 ml of chloroform and a 0.45 ml aliquot of this cholesterol solution was added to the methyl-β-cyclodextrin solution. The solution of cyclodextrin and cholesterol was mixed, and the solvents, removed using a stream of nitrogen gas. The resulting crystals were stored at 22°C until further use. A working solution of CLC was prepared by adding 50 mg of CLC to 1 ml S-MEDIA at 37°C. This CLC working solution was vigorously vortexed prior to obtaining aliquots.

Sperm Processing for Western Blotting

After incubation, protein was extracted from whole sperm using a modified protocol described by Pommer et al. (2003). Briefly, samples removed at each time point were centrifuged at 10,000 x g for 1 min. The supernatant was discarded and the sperm pellets were suspended with 300 μL radioimmunoprecipitation assay (RIPA) lysis buffer and 6 μL of dithiothreitol (DTT). Samples were heated on a heating block (73°C; 20 min) and then sonicated using a Branson Sonifier® equipped with a CE Converter 102C (Branson Ultrasonics Corporation, Danbury, CT)

on ice (2 x 1 min). Following sonication, the samples were mixed for 5 min using a vortex mixer and heated on the heating block (73°C) for 5 min. The samples were then centrifuged (10,000 x g for 3 min), and 2 μL of β-mercaptoethanol (βME) was added to new 500-μL microcentrifuge tubes, along with the supernatant while the pellets were discarded. The samples were then mixed, heated (73°C) for 5 min, and centrifuged for 1 min. The protein concentration of each sample was determined using a Bradford Assay, diluted with 6 X sample buffer [300 mM Tris-HCl (pH 6.8), 60% glycerol, 30mM DTT, 6% SDS], mixed, and then heated (73°C)for 5 min before being stored at -20°C until use. Prior to SDS-PAGE, the samples were thawed, mixed, heated (73°C) for 5 min, and centrifuged (10,000 x g for 1 min). The supernatant of each sample was then loaded onto the gel.

Flow Cytometric Analysis of Acrosome Reaction

Flow cytometry was used to evaluate capacitation status by evaluating acrosomal integrity for procaine-treated and untreated control sperm when challenged with capacitation inducing agents for 20 min. Sperm were centrifuged through Percoll® and incubated to capacitate the cells. Subsamples (1 ml) from the incubating sperm were removed and stained with both fluorescein isothiocyanate (FITC)-PNA (1 mg/ml) and propidium iodide (PI; 1 mg/ml) for 10 min prior to analysis using flow cytometry (MoFlo™ High-Performance Cell Sorter; Dako-Cytomation, Fort Collins, CO) as described by Rathi et al. (2001). Data from 50,000 cells from each sample were analyzed using Summit software (Dako-Cytomation, Fort Collins, CO). The flow cytometer was gated to only evaluate fluorescence from

particles having the light scatter and size of spermatozoa. The cells were categorized by the amount of PI and FITC-PNA that accumulated for each. Cells were then classified in one of three categories: live acrosome intact, live acrosome reacted, or dead. The percentages of live acrosome reacted sperm from each assessment were used for statistical analysis.

Hyperactive Motility

Hyperactive motility was assessed using HMTM IVOS computer assisted sperm analysis system (Hamilton-Thorne BioSciences, Beverly, MA). To assess the percentage of motile spermatozoa using CASA, a 5 µL drop of spermatozoa (50 x 10^6 sperm/ml), from each treatment, was placed on a preheated slide (37°C) and a minimum of 200 spermatozoa per sample were analyzed. System parameters for these analyses were: 45 frames acquired at 60 frames per second; minimum contrast = 70, minimum cell size = 4 pixels; lower VAP cut-off = 20 µm/s; lower VSL cut-off = 0 µm/s; VAP cut-off for progressive cells = 50 µm/s and straightness = 75%. An algorithm developed by N. Kaula (2009) was also used to analyze the spermatozoal tracks using MBSR, which classifies each sperm as having hyperactive, transitional, or progressive motility.

Experiment 1. Determination of plasma membrane cholesterol content after capacitation agent challenge.

Optimal sperm capacitation treatments were selected (40 µM PC12; 1 µM MBC) based off preliminary experiments analyzing sperm capacitated with PC12

(15, 20, and 40 μM) or MBC (0.5, 1, or 2 μM) on the flow cytometer assessing percentages of acrosome reacted sperm.

The cholesterol content of control and CLC-treated sperm (1.5 mg CLC/120 x 10^6 total sperm) from six stallions was determined using mass spectroscopy. CLC-treated and control sperm were subsequently treated with 1) nothing; 2) 40 μM PC12; or 3) 1 μM MBC for 15 min and 180 min. Samples were centrifuged (1000 x g; 9min) and the lipids from sperm samples were extracted using a modified Folch extraction, as described by Graham and Hammerstedt (1992). Briefly, approximately 155 ± 28 million sperm were suspended in 1 ml methanol to which 2 ml chloroform were added and the samples incubated at 5°C overnight. The samples were then filtered through a glass wool column, 1.5 ml water were added to each, and the samples mixed. The samples were centrifuged (70 x g for 1 min), and the aqueous phase discarded. The chloroform was then removed using nitrogen gas, and the samples suspended in 350 μL toluene (Fisher Scientific; Fair Lawn, NJ). The amount of cholesterol in each sample was determined using a Waters Quattro Micro GC-MS/MS system operated in the electron ionization (EI) positive mode. The carrier gas was ultra high purity helium at a head column pressure of 69 kPa. Injections were made using an Agilent 7683B autosampler in the split mode (10:1) onto a DB-5ms capillary column (30mm X 0.25mm I.D., 0.25um film thickness; J&W Scientific, Santa Clara, CA). The cholesterol was detected using an oven temperature profile of 200 to 280°C at 30°C/min then to 300°C at 5°C/min and held for 4 min. The inlet temperature was 280°C and the GC-mass spectrometer interface temperature was set at 300°C. The mass spectrometer was operated in single ion

monitoring (SIM) mode targeting m/z 275 (qualification) and m/z 301 (quantitation). Plotting the peak areas for m/z 301 against those of standard amounts of cholesterol using linear regression allowed the amount of cholesterol in each sample to be quantified.

Experiment 2. Detection of Tyrosine Phosphorylation using Western Blotting.

Following their initial preparation (centrifugation through Percoll ® and resuspension to 50 x 10^6 spermatozoa per ml), samples from two stallions were exposed to five treatments that aid in capacitating the sperm: MW (control); PC12 (40 µm); A23187 (2 µm); MBC (1 µm); or PKA inhibitor H-89 (60 µM; N-[2-(p-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide-dihydrochloride; Calbiochem; La Jolla, CA); and incubated for 0, 30, 90, and 180 min at 37°C in 5% CO2. Subsamples (1.5ml) were removed at each time point and protein extracted as described above.

A total of 20 µg protein, from each treatment and time point, was loaded into wells of a 10% Tris-HCL precast gel (Bio-Rad; Hercules, CA). Electrophoresis was performed at 110 volts for 75 min, and the protein was transferred onto a nitrocellulose membrane (GE Healthcare; Piscataway, NJ , 200 mA, 55 min). The membrane was then washed in Tris-buffered saline containing 0.1% Tween 20 (TBST). The membrane was blocked for 2 h at room temperature with 2% BSA in TBST, washed with TBST (4 x 15 min) and incubated with a mouse anti-phosphotyrosine 1° antibody (4G10 mAB; 1:1000; Catalog # 05-1050; Millipore; Bedford, MA) in TBST overnight on an orbital shaker at room temperature. The blot

was then washed 4 x 15 min with TBST and then incubated with a 2° antibody conjugated to horseradish peroxidase (goat anti-mouse; Catalog # 170-6516;1:1000; Bio-Rad; Hercules, CA) in TBST on an orbital shaker for 2 h at room temperature. After washing 4 x 15 min with TBST, bound peroxidase activity was visualized by the ECL-Plus chemiluminescence system using a Storm mager (GE Healthcare; Piscataway, NJ) according to the manufacturer's instructions. The intensity and relative molecular weights of observed bands were determined (ImageQuant TL; Amersham Biosciences; Piscataway, NJ). The anti-phosphotyrosine blots were then stripped at 60°C with 60 mM β-mercaptoethanol for 45 min. After stripping, membranes were washed 4 x 15 min with TBST and blocked with 2% BSA for 2 h at room temperature and then reprobed with mouse anti-actin 1° antibody (1:10,000; Catalog # sc47778; Santa Cruz Biotechnology, Inc; Santa Cruz, CA). After washing in TBST, blots were incubated with an anti-mouse 2° antibody conjugated to horseradish peroxidase (goat anti-mouse; Catalog # 170-6516;1:1000; Bio-Rad; Hercules, CA), and immunoreactive bands were visualized by chemiluminescence. The experiment was conducted using sperm from two stallions each replicated twice. The β-actin binding control experiment was replicated three times.

Experiment 3. Detection of Tyrosine Phosphorylation Utilizing Flow Cytometry.

Sperm from three stallions were initially prepared and capacitated as described in Experiment 2. Briefly, sperm were collected and diluted in MW medium 1:1 (v:v) and centrifuged through 30% Percoll® for 4 min . The resultant

pellet was diluted to 50 x 10^6 spermatozoa/ml and divided into four treatments (Untreated Control, 40 μM PC12, 1 μM MBC, or 2 μM A23187). Samples incubated in 5% CO2 in air for 0, 10, 20, 30, 45, 60, 90, 120, and 180 min. Sperm were prepared for flow cytometric assessment using a modified protocol described by Barbonetti et al. (2008). Briefly, subsamples of sperm (2 x 10^6) were fixed in ice-cold 1% formaldehyde in PBS (pH 7.2) for 30 min at 4°C. Spermatozoa were then centrifuged (1,000 x g; 4 min) and permeabilized in 0.1% Triton-X 100 in PBS for 10 min at room temperature. Sperm were then stained for 1 h with a fluorescein isothiocyanate (FITC)-labeled monoclonal antibody (4G10; Catalog #16-104; Millipore, Billerica, MA; 10 μg/mL). Stained samples were analyzed using flow cytometry (MoFlo™ High-Performance Cell Sorter; Dako, Fort Collins, CO). Data from 50,000 events from each sample were analyzed using Summit software. The proportion of spermatozoa with low and high fluorescence intensities was determined.

Experiment 4. Detection of hyperactive motility utilizing novel software.

Ejaculates sperm from four stallions were split and diluted 1:1 (v:v) with MW or MW containing 5 mM procaine. Samples were centrifuged through 30% Percoll® for 5.5 min (400 x g) and the resultant pellets were suspended in MW or 5 mM Procaine MW. Samples were then capacitated as described above, by incubating CLC-treated and control sperm with MW, PC12 (40 μM), or MBC (1 μM) for 15 min or 180 min. At each time point, subsamples were removed and the

motility parameters were assessed using both the standard CASA and MSBR (Kaula et al., 2009) methods.

Experiment 5. Determination of Procaine Effects on Acrosome Reaction

Ejaculates from six stallions were split, and one half of the ejaculate incubated with CLC (1.5 mg/120 x 10^6 spermatozoa; Moore et al., 2005) for 15 min. Following cholesterol incorporation into the plasma membrane, samples were diluted 1:1 (v:v) with MW or MW with 5mM procaine. The samples were centrifuged through 30% Percoll® for 5.5 min (400 x g) and the resultant pellets were suspended in either MW or MW with 5mM procaine as before. The CLC-treated and control sperm were then treated with MW, PC12 (40 μM), or MBC (1 μM), for either 15 min or 180 min. Following incubation, samples were stained with FITC-PNA (1 mg/ml) and PI (1 mg/ml), incubated for 5 min, and analyzed for the percentage of live-acrosome reacted sperm by flow cytometry.

Statistical Analysis

Data for Experiments 1 and 4 were analyzed by an analysis of variance (ANOVA; SAS Institute Inc., 1985). Treatment means for Experiment 1 were separated by the Student-Newman Keuls (SNK) mean separation technique (SAS Institute Inc., 1985). All percentage data for Experiment 4 (Table 2.2) were transformed by Arsin prior to running analysis. Means presented in Table 2.2 are untransformed data.

Results

Experiment 1.

Sperm treated with CLC contained more cholesterol than control sperm ($p<0.05$; Table 2.1). In addition, control sperm and sperm treated with MBC had lower cholesterol content after 180 min incubation than they did at 15 min ($p<0.05$; Table 2.1).

Experiment 2.

When 20 µg of total protein was loaded in each lane, several protein bands increased in phosphorylation intensity over the 180 min incubation for control sperm (lanes 3,4,5,6; Figure 2.1). Specifically, proteins at kDa 240, 227, and 218 exhibited increased intensity for phosphorylation over the 180 min incubation. Conversely, PC12 treated cells exhibited the greatest phosphorylation intensity at 0 min and this intensity then decreased over the 3 h incubation (lanes 7, 8, 9, 10; Figure 2.1). In addition, sperm samples capacitated in MW for 180 min, in the presence of PKA inhibitor H-89 (60 µM) displayed low intensities of these phosphorylated proteins. Sperm treated with MBC and A23187 did not display distinguishable tyrosine phosphorylation bands (Data not shown). The tyrosine phosphorylation data were normalized (Figure 2.2) with β-actin expression and the PC12 teated sperm had significantly higher tyrosine phosphorylation at 0 min and

30 min, while having decreased phosphorylation at 180 min compared to the control samples.

Experiment 3.

We were unable to obtain consistent results for the same treatment regarding the adaption of tyrosine phosphorylation detection utilizing the flow cytometer (Figure 3.2).

Experiment 4.

The addition of procaine to sperm did not affect any sperm motion parameters when standard CASA analysis was conducted (data not shown), except for CLC-treated sperm subsequently capacitated with PC12 at 180 min (Table 2.2). Control sperm and MBC-treated sperm exhibited the greatest changes in motion parameters indicative of hyperactivity after 180 min incubation when analyzed using standard CASA parameters (Table 2.2).

These same treatments, when analyzed using MSBR, showed that control, PC12-treated, and MBC-treated sperm did not undergo hyperactive motility at either 0 or 3 h (Table 2.3; $p>0.05$). However, all procaine supplemented samples exhibited higher percentages of hyperactive sperm than those incubated without procaine ($p<0.05$; Table 2.4).

Experiment 5.

There were no differences in mean acrosome reactions at 15 min or 180 min for any treatment; therefore, the data were pooled for determining the effects of procaine supplementation on acrosome reactions (Table 2.5; $p<0.05$). Although procaine induced hyperactive sperm motility with MBSR analysis (Table 2.4), it did not induce the acrosome reaction in stallion sperm ($p>0.05$; Table 2.5). The tested levels of PC12 and MBC were capable of inducing an acrosome reaction in control and procaine-treated spermatozoa ($p <0.05$; Table 2.5). Since procaine did not affect the acrosome reaction of sperm, the data were pooled to test the effects of PC12 and MBC treatment on inducing the acrosome reaction for both control and CLC-treated spermatozoa (Table 2.6). CLC-treated sperm when challenged with MBC had significantly less live cells when compared to CLC-treated control sperm or CLC-treated PC12 challenged sperm (Table 2.7). Procaine did not decrease the percent live cells in any treatment (Table 2.8).

Discussion

Spermatozoal capacitation is a poorly understood process that is necessary for a spermatozoon to fertilize an oocyte (Austin, 1967; Amann and Graham, 1993; Rathi et al., 2001). There are many steps involved in sperm capacitation including the loss of cholesterol from the sperm plasma membrane, the phosphorylation of tyrosine residues on several proteins, acquisition of hyperactive motility, and the induction of the acrosome reaction (Jha et al., 2002; Brener et al., 2003; Breitbart et al., 2005). To date, no assay exists that can evaluate spermatozoa capacitation

completely; therefore, several of the sperm attributes that define capacitation were individually analyzed.

Addition of cholesterol, using CLCs, to sperm plasma membranes increases membrane stability, and this increases the number of sperm that survive cryopreservation (Moore et al., 2005). However, fertility of CLC-treated stallion spermatozoa was not better than untreated sperm, even though more viable sperm were inseminated into each mare (Spizziri et al., 2008). A potential that may have affected these CLC-treated spermatozoa experienced a delay in the timing of capacitation since these sperm have 3 times more cholesterol than untreated sperm, and therefore by the time they were effectively capacitated, the oocytes were becoming too mature.

Several compounds, including PC12, MBC, and A23187, can be used to "capacitate" sperm *in vitro.* In addition, each of these compounds induces different aspects of spermatozoa capacitation with different efficiency. These compounds were selected because they also induce capacitation through different mechanisms, and thereby may provide a more complete understanding of what is important for sperm capacitation. PC12 is a fusogenic lipid that disrupts normal sperm plasma membrane structure, thereby rendering the membrane permeable to various ions that can induce downstream signaling events (Graham, 1996; Graham, 2001). MBC efficiently removes cholesterol from the sperm plasma membrane, making the membrane less stabile and leading to increased membrane permeability to ions (Colas et al., 2008). A23187 is a cationic pore that permits calcium entry into the

cell, which signals downstream signaling events (Triana et al., 1980; Christensen et al., 1996; Graham, 1996).

Initial investigation in our laboratory of tyrosine phosphorylation with these agents indicated that MBC and A23187 were incapable of inducing tyrosine phosphorylation at the investigated concentrations. A23187 was excluded from other experiments due to poor fertilization rates of bovine oocytes when used for IVF (data not shown). Previous research indicated that procaine did not alter tyrosine phosphorylation, and treating sperm with procaine was therefore excluded from tyrosine phosphorylation experiments (McPartlin et al., 2009). We detected tyrosine phosphorylated proteins for both control and PC12 treated cells. However, the proteins we observed to be phosphorylated (247, 224, and 218 kDA) were different from those observed by previous investigators. It is unknown why we were unable to reproduce the same set of proteins observed by McPartlin et al. (2009) or Pommer et al. (2003). One possible reason for these differences may be differences in the amount of protein loaded. We were unable to detect sufficient protein tyrosine phosphorylation with the amount of protein loaded by Pommer et al. (2003), and the amount of protein loaded by McPartlin et al. (2009) was not reported. In addition, the medium used to capacitate the stallion spermatozoa was different than that used by Pommer et al. (2003). Our research confirmed the findings by Pommer et al. (2003) and McParlin et al. (2009) that the PKA inhibitor H-89 inhibited phosphorylation in control cells and PC12-treated cells, indicating that the phosphorylated proteins we detected are mediated by a signaling pathway regulated by PKA.

The flow cytometer would be an attractive method for studying tyrosine phosphorylation due to the short processing time required, simple techniques, the increased reliability of the data, and the ability to collect data on individual cells, not just cell populations (Barbonetti et al., 2008). However, to accurately assess phosphorylation status, the cells must be fixed to "freeze" the phosphorylation status of proteins, and then the cells must be permeabilized to allow antibody access for binding to intracellular proteins (Krutzik and Nolan, 2003). Cells are typically fixed in formaldehyde and glutaraldehyde and then permeabilized with saponin, alcohols and/or detergents (Krutzik and Nolan, 2003). Each of the mentioned reagents has benefits and drawbacks. Formaldehyde is a better reagent for fixation, since glutaraldehyde increases both background and nonspecific binding (Krutzik and Nolan, 2003). In addition, triton permeabilization decreases the number of cells lost to detergent lysis and yields improved signal intensity compared to other permeabilizers (Krutzik and Nolan, 2003). Unfortunately, we were unable to obtain consistent results using flow cytometry. Very few studies have attempted to use flow cytometry to assess protein tyrosine phosphorylation in stallion spermatozoa. One potential limit to our analysis was not being able to optimize cell fixing and permeabilization. Studies indicate that results can vary dramatically depending on the fixation and permeabilization utilized and
that different cell types require different fixing and permeabilization methods to provide reliable data (Krutzik and Nolan, 2003). Therefore, additional research needs to be conducted to optimize this procedure for stallion spermatozoa .

The signaling pathways involved in induction and maintenance of hyperactive motility are poorly understood. The induction of hyperactive motility appears to be independent of sperm capacitation and protein tyrosine phosphorylation (McPartlin et al., 2009). Procaine has been investigated for its effects on hyperactive motility (McPartlin et al., 2009; Mújica et al., 2004); its mechanism for doing this is thought to be by increasing plasma membrane permeability to calcium. Stallion sperm capacitated in MW supplemented with 5 mM procaine exhibited hyperactivated motility and fertilized equine oocytes (McPartlin et al., 2008, McPartlin et al., 2009). Procaine appears to exert its effect immediately (McPartlin et al., 2009), which was confirmed with our MBSR analysis, and the decreases in the percentages of hyperactive sperm over time likely is due to decreased cell viability.

Historically assessment of hyperactive motility is performed using CASA parameters (Mortimer and Maxwell, 1999; Suarez, 2008). Recently, McPartlin et al. (2009) defined motility parameters that are potentially involved in sperm capacitation for stallion sperm incubated in MW. As stallion sperm capacitation requires prolonged incubation, two parameters were selected as hallmark indicators of hyperactivity after a 6 h incubation: stallion sperm with ≤50.9 μm/sec straight-line velocity (VSL) and ≤20.9 μm/sec linearity (LIN) were considered hyperactivated (McPartlin et al., 2008; McPartlin et al., 2009). Although we were able to repeat these particular values with several of our capacitation treatments, they were not different from measurements obtained from control sperm.

Using these CASA assessments, we were unable to detect the differences reported by McPartlin et al. (2009) in our experiments, and even though several treatments had ≤50.9 μm/sec VSL, none was significant. A potential reason for the dissimilarity in CASA analysis is stallion variability. However, the inability to repeat the experimental results and conclusions of McPartlin et al. (2009), suggests limitations in CASA analysis for detecting hyperactive motility. CASA detection of hyperactive motility only was confirmed after 3h incubation in our experiments, and not in all treatments (Table 2.2). Our investigation determined significant changes in multiple parameters after a 3h incubation: motility, progressive motility, average path velocity (VAP), VSL, curvilinear velocity (VCL), beat cross frequency (BCF), and straightness (STR). The limits of the CASA hyperactivation detection indicate a need for a more sensitive method of detection.

However, utilizing the novel MBSR algorithm, adding 5mM procaine certainly induced sperm hyperactivity. McPartlin et al. (2009) reported ~ 73% and 55% hyperactive sperm after 0 and 6 h incubations, respectively. Our results indicated 72% and 58% hyperactive sperm when 5 mM procaine was added to samples after 0 and 3 h of incubation, respectively. Sperm treated with PC12 and MBC exhibited higher percentages of hyperactive sperm than those reported by McPartlin et al. (2009) after capacitation by incubation for an extended time period; however, our capacitation treatments were not significantly greater than our control treatments.

Analyzing sperm using MBSR is more accurate for classifying sperm as hyperactive or not than are the classical CASA parameters (Kaula et al., 2009). The greatest percentages of sperm exhibiting hyperactive motility were detected for

sperm treated with 5 mM procaine for 0 h, which is similar to the results reported by McPartlin et al. (2009). In addition, MBC-treated and control sperm incubated for 180 min with 5 mM procaine had significantly lower hyperactivity than the 15 min treatment. None of the compounds that induce "capacitation" *in vitro*, PC12, MBC, or A23187 by themselves resulted in hyperactive sperm motility as detected by MBSR.

As previously mentioned, procaine may alter membrane permeability to calcium and calcium influx into sperm is involved in the acrosome reaction. However, results from Experiment 5 confirmed that if procaine does cause an influx of Ca^{++}, it has no effect on the induction of the acrosome reaction. CLC-treated and control spermatozoa that were treated with either PC12 or MBC exhibited higher percentages of live acrosome-reacted sperm. In addition, sperm treated with both PC12 and 5mM procaine exhibited higher percentages of acrosome reacted sperm, while MBC treatment of CLC-treated sperm also resulted in a significant decrease in percentage of live cells when compared to CLC-treated sperm. Sperm treated with procaine alone did have an increase in percentages of acrosome reacted sperm or percentages of live cells.

Conclusions

One limit to successful equine *in vitro* fertilization is the inability to sufficiently capacitate the sperm. Several of the steps involved in sperm capacitation, including membrane cholesterol depletion, protein tyrosine phosphorylation, acquirement of hyperactive motility, and the acrosome reaction,

can be studied to determine the most effective method to induce stallion sperm capacitation *in vitro.*

The ability to alter plasma membrane cholesterol content can be accomplished, either by increased membrane cholesterol with CLC or depleting it with MBC. Since cholesterol loss is one step in sperm capacitation, removing cholesterol *in vitro* using MBC may be effective in inducing the downstream events in capacitation. In our studies, MBC was able to induce an acrosome reaction in sperm, but was not able to induce noticeable protein tyrosine phosphorylation. Our western blotting analysis indicated that PC12-treated sperm exhibited the highest amount of protein tyrosine phosphorylation. Flow cytometric analysis confirmed that PC12 was also capable of inducing the acrosome reaction. The hyperactive motility studies yielded conflicting results. Although our initial study, using CASA parameters indicated several motility parameters could be altered by MBC treatment, the addition of procaine did not induce the changes reported by McPartlin et al. (2009). However, analysis using the MBSR algorithm did indicate that procaine induced hyperactive motility. Unfortunately, none of the compounds that capacitate sperm induced hyperactive motility in the absence of procaine. The increased tyrosine phosphorylation, acquisition of hyperactive motility with procaine incubation, and induction of the acrosome reaction suggest the use of PC12 may be able to more accurately induce *in vitro* capacitated stallion spermatozoa leading to a successful IVF sperm capacitation treatment.

Tables

Table 2.1. Mean cholesterol content (µg/ 10^5 sperm) of Control or Cholesterol-Loaded-Cyclodextrin (CLC)-treated sperm following incubation with Dilauroylphosphatidylcholine (PC12) or Methyl-β-Cyclodextrin (MBC) after either 15 min or 180 min incubation (n=6).

Treatment	15 min	180 min
Control	0.022 + 0.004[a]	0.017 + 0.003[b]
PC12 Treated	0.018 + 0.002[a]	-
MBC Treated	0.018 + 0.003[a]	0.016 + 0.002[b]
CLC	0.106 + 0.028*	0.059 + 0.009*
CLC + PC12	0.064 + 0.011*	-
CLC + MBC	0.057 + 0.009*	0.054 + 0.009*

[a,b] denote row differences between 15 min and 180 min ($p<0.05$).
*** indicates mean is different from Control at 15 min ($p<0.05$).**

Table 2.2. CASA mean motility parameters at for capacitated sperm following a 15 min or 180 min incubation (n=4).

Treatment	MOT (%)		PROG (%)		VAP (μm/sec)		VSL (μm/sec)		VCL (μm/sec)		BCF (Hz)		STR (%)	
	15 min	180 min	15 min	180 min	15 min	180 min	15 min	180 min	15 min	180 min	15 min	180 min	15 min	180 min
Control	81±5	61±3*	17±2	8±2*	102±11	69±5*	57±3	40±3*	198±22	148±6	31±3	35±2	60±2	64±2
CLC	76±8	69±7	15±3	10±2	117±12	100±13	64±2	51±2*	230±21	209±19	33±3	32±1	57±3	57±3
PC12	80±3	64±4*	17±4	12±3	108±22	89±19	58±8	50±10	216±41	190±31	34±1	36±2	59±4	58±5
CLC PC12	82±4	74±4	19±2	13±2*	133±25	92±17	71±3	48±2	260±34	196±31*	33±1	35±2	58±4	59±5
CLC PC12 Procaine	62±8	51±9	16±5	8±2	100±11	73±5	58±6	39±4*	215±25	181±7	39±1	43±1*#	59±6	54±5
MBC	70±9	61±3	13±3	6±1	106±11	75±6*	56±5	41±1*	224±19	165±12*	35±6	38±1*	54±1	63±3*

*** indicate that these values are different from time 0h at $p<0.05$.**
 # column mean is different than control at $p<0.05$. Values listed as mean ±SEM.

Table 2.3. The percentages of MSBR classified hyperactive sperm after control, PC12, and MBC treated stallion sperm were incubated for 15 min and 180 min incubation with PC12 and MBC after CASA analysis (n=24).

	15 min	180 min
Control	54 ± 5	46 ± 5
PC12	52 ± 5	52 ± 5
MBC	46 ± 5	44 ± 4

Table 2.4. The percentages of MSBR classified hyperactive sperm when control, PC12, and MBC treated stallion sperm incubated in MW or 5mM Procaine supplemented MW for 15 min and 180 min (n=12).

	Procaine	
	-	+
Control 15 min	$35^{a} \pm 4$	$72^{b} \pm 4$
PC12 15 min	$37^{a} \pm 6$	$66^{b} \pm 6$
MBC 15 min	$28^{a} \pm 4$	$63^{b} \pm 4$
Control 180 min	$33^{a} \pm 5$	$58^{b*} \pm 5$
PC12 180 min	$41^{a} \pm 6$	$63^{b} \pm 6$
MBC 180 min	$33^{a} \pm 5$	$55^{b*} \pm 5$

*** indicate column differences from control $p<0.05$. n=12.**
^{a,b} indicate row differences at $p<0.05$.

Table 2.5. The percentages of live acrosome reacted (AR) sperm after incubation in MW and MW with 5mM Procaine (n=12).

		% Live AR	% Live AR w/ Procaine
No CLC	Control	26	41
	40 µM PC12	47*	57
	1 µM MBC	56*	57
	SEM	8	7
CLC	Control	9	34
	40 µM PC12	63*	64*
	1 µM MBC	51*	42
	SEM	8	7

*** indicate column differences at p<0.05.**

Table 2.6. The percentages of live acrosome reacted sperm in MW and MW with 5mM procaine following treatment with PC12 or MBC(n=24).

	Control	CLC
Control	28	37
PC12	55*	60*
MBC	53*	50*
SEM	5	5

*** indicate column differences at p<0.05.**

Table 2.7. The percentages of live cells of control and CLC-treated stallion spermatozoa when challenged with PC12 and MBC (n=6).

	Control	CLC
Control	40	65
PC12	40	57
MBC	30	45*
SEM	4	3

*** indicate column differences at p<0.05.**

Table 2.8. The percentages of live control and CLC-treated stallion spermatozoa when incubated in MW or MW with 5mM procaine and challenged with PC12 and MBC (n=6).

	Control		CLC	
	Procaine		Procaine	
	-	+	-	+
Control	41	38	67	64
PC12	43	36	58	57
MBC	31	28	39*	52
SEM	4	3	4	4

*** indicate column differences at p<0.05.**

Figures

Figure 2.1. Western blot of detergent extracted sperm following incubation with PKA Inhibitor H-89 (lane 1), ladder (lane 2), control cells at 0 min (lane 3), 30 min (lane 4), 90 min (lane 5), 180 min (lane 6), dilauroylphosphatidylcholine (PC12) treated sperm at 0 min (lane 7), PC12 at 30 min (lane 8), PC12 at 90 min (lane 9), or PC12 180 min (10), and a β-actin binding control.

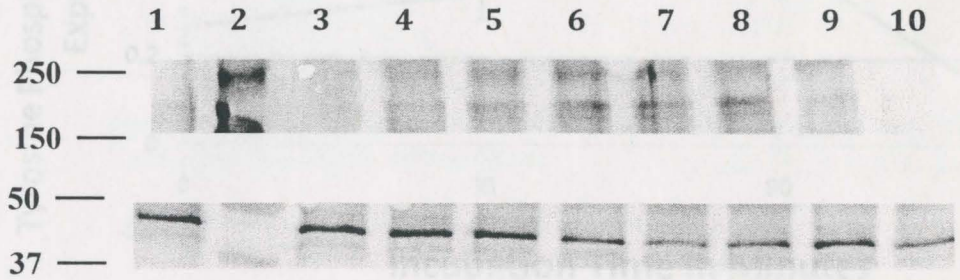


Figure 2.2. Protein tyrosine phosphorylation:β-actin expression for Control sperm, Dilauroylphosphatidylcholine (PC12) treated sperm, and sperm treated with PKA Inhibitor H-89 at 0, 30 min, 90 min, and 180 min incubation n=3. * indicate mean is different Control Sperm at corresponding time point ($p<0.05$).

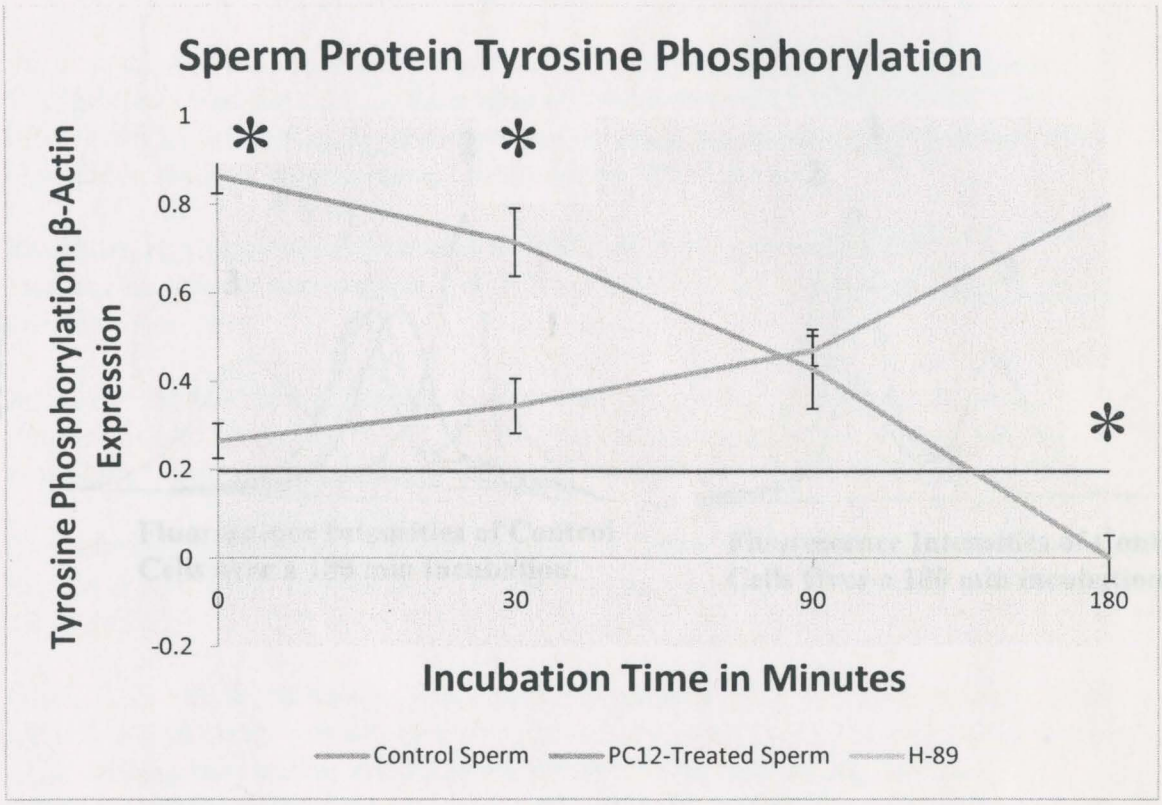
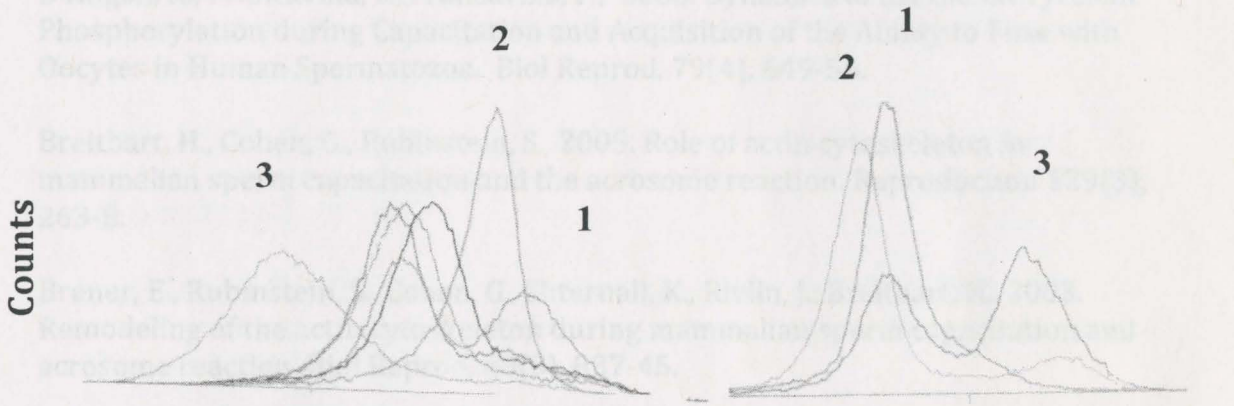


Figure 2.3. Fluorescence Intensities of Control cells at 0 min (1) 30 min (2) and 180 min (3).



Fluorescence Intensities of Control Cells over a 180 min incubation.

Fluorescence Intensities of Control Cells Over a 180 min incubation.

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Chapter III

In Vitro Fertilization of Bovine Oocytes with Capacitated Stallion Spermatozoa

Introduction

The limited availability of equine oocytes for research has forced researchers to develop heterologous oocyte binding assays (Alm et al., 2001; Sinowatz et al., 2003; Coutinho da Silva et al., 2004; Mugnier et al., 2009). In fact, stallion spermatozoa can bind to, acrosome react, and fuse with heterologous oocytes (Sinowatz et al., 2003; Mugnier et al., 2009). The use of zona intact bovine oocytes permits evaluation of the ability of stallion sperm to bind to the zona pellucida and undergo the acrosome reaction (Brackett et al., 1982; Sinowatz et al., 2003). In addition, stallion sperm exhibit reduced capacity to bind and undergo the acrosome reaction of equine oocytes compared to heterologous oocytes (Mugnier et al. 2009). These studies were designed to address *in vitro* stallion sperm capacitation difficulties and optimize stallion sperm capacitation so that they can fertilize oocytes *in vitro.*

The ZP is a significant barrier to stallion sperm fertilizing capacity and limits equine IVF success, since equine sperm are capable of penetrating zona-free equine oocytes but not zona-intact oocytes (Mugnier et al., 2009). This potential problem in the equine IVF system (passage of a sperm through the ZP) can be circumvented

by partially disrupting of the ZP by mechanical or chemical techniques (Choi et al., 1994; Mugnier et al., 2009) or bypassing capacitation and the ZP altogether in the case of intracytoplasmic sperm injection (ICSI; García-Roselló et al., 2009). Because IVF could be more readily used than ICSI, this study focused on determining where the lesion(s) in equine IVF exist with hopes to develop a greater understanding of the lack of success in equine IVF.

Materials and Methods

Preparation of Medium

Media utilized in the *in vitro* maturation, fertilization, and embryo culture were those described by De La Torre et al. (2006). Sperm were capacitated in MW as previously described (McPartlin et al., 2008).

Oocyte Preparation and Maturation

Bovine ovaries were obtained at a local abattoir and transported to the laboratory in 37 °C saline (0.15M NaCl) within 2 h of collection. Ovaries were rinsed in saline and follicles of approximately 2.5-8 mm in diameter were aspirated using an 18 gauge needle attached to a 50-100 mm Hg vacuum source. The follicular fluid was examined using a stereomicroscope and compact cumulus oocytes were recovered and washed in H-CDM-M (De La Torre et al., 2006). The oocytes (50 oocytes/well) were placed into four well maturation dishes; each well contained 1 mL equilibrated IVM (De La Torre et al., 2006) and the oocytes matured for 28 h (5%CO_2 in air; 38.5°C).

In vitro Fertilization

Sperm were prepared for capacitation as previously described. Briefly, ejaculates were diluted 1:1 (v:v) with MW ± 5 mM Procaine and centrifuged through 30% Percoll® (400 x g; 5.5 min). The resultant pellet was suspended to 50 x 10^6 spermatozoa/ml in MW. The samples were incubated (5%CO_2; 38.5°C; 20 min) with PC12 (40 μm), MBC (1 μm), or left untreated. A subsample of spermatozoa from the 40 μM PC12 treatment were killed by snap freezing/thawing three times and served as a parthenogenetic control.

During capacitation of spermatozoa (20 min), mature oocytes were placed in drops of FCDM-M (85 μL; De La Torre et al., 2006) under mineral oil (7.5 ml). Following sperm capacitation, oocytes were fertilized with 250,000 spermatozoa from each treatment (5 μL), and the gametes were co-incubated for 18 h (5%CO_2; 38.5°C). Potential zygotes were rinsed through drops of HCDM-1 (De La Torre et al., 2006) and transferred to 0.5 mL wells of CDM-1 (De La Torre et al., 2006), and the presumptive zygotes were incubated for 30 h at 38.5°C in an atmosphere of 5%CO_2, 5%O_2 and 90%N_2. Following incubation, the zygotes were placed into 500-μL microcentrifuge tubes, shaken for 1 min using a vortex mixer to remove cumulus and loosely bound sperm, and visually assessed for cell cleavage. Fertilization was defined as the presence of multiple evenly sized blastomeres.

Producing holes in the Zona Pellucida

Holes were made in bovine oocyte zona pellucida's (Figure 3.1), using a XYClone infrared laser + integrated 40X objective (Hamilton Thorne Biosciences;

Beverly, MA). This instrument uses a 1480λ nm beam (300 mW laser; single pulse; 800 microsecond pulse width) to create holes in the zona pellucida of *in vitro* matured bovine oocytes (Tadir and Douglas-Hamilton, 2007).

Experiment 1. Determine fertilization rates of in vitro matured bovine oocytes when inseminated with capacitated stallion sperm.

Spermatozoa, from each of seven treatments: 1) control (untreated); 2) 15 µm PC12; 3) 20 µm PC12; 4) 40 µm PC12; 5) 0.5 µm MBC; 6) 1 µm MBC; and 7) killed by freezing (parthenote of 15 µm PC12-treated sperm); were added to thirty bovine oocytes. Presumptive zygotes were visually assessed for cell cleavage following a 48 h co-incubation using a phase contrast microscope (Table 3.1).

Experiment 2. Determine if bypassing the ZP by laser disruption will increase fertilizing capacity of stallion sperm capacitated in vitro.

Thirty bovine oocytes per drop per replicate were collected matured as described above. Following maturation, the cumulus cells were removed using a five min incubation in hyaluronidase (200 IU in 1ml) and vigorous pipetting. The denuded oocytes were then placed into a petri dish (35 x 15 mm; BD Biosciences; Franklin Lakes, NJ) containing 4 ml HCDM-M (De La Torre et al., 2006). Each oocyte was then centered under the orange isothermal ring (140°C; Hamilton Thorne; XyClone Laser Ablator Operator's Manual, 2009) of the laser, and the laser activated in pulses until a hole was present in the zona using a phase contrast microscope (Tadir and Douglas-Hamilton, 2006). Following laser treatment, the oocytes were

rinsed in HCDM-M and added to 85 µL droplets of FCDM (De La Torre et al., 2006), and sperm were added as described above. Spermatozoa treated with 40 µM PC12 serving as a positive control, were added to zona-intact (untreated) oocytes. Zygotes were visually assessed for cell cleavage following a 48 h co-incubation, as described above.

Statistical Analysis

Data were subjected to an analysis of variance (ANOVA; SAS Institute Inc., 1985) and treatment means were separated by Student-Newman-Keuls (SNK) mean separation technique (SAS Institute Inc., 1985). Treatments were considered different if $P < 0.05$.

Results

Experiment 1

Sperm capacitated with 15µM PC12 induced significantly higher bovine oocyte cleavage rates than oocytes inseminated with sperm of other treatments ($p<0.05$; Table 3.1). Procaine did not affect cleavage rates and actually resulted in lower rates than sperm incubated in MW ($p<0.05$; Table 3.1).

Experiment 2

The zona intact bovine oocytes exhibited significantly higher cleavage rates than laser-treated bovine oocytes ($p<0.05$; Table 3.2). In addition, sperm

capacitated in 15 μM PC12 induced higher oocyte cleavage rates than noncapacitated sperm ($p<0.05$; Table3.2).

Discussion

Spermatozoal capacitation is a poorly understood process, and not being able to effectively capacitate stallion sperm *in vitro* may account for the poor results achieved for IVF. Recently, it was reported that stallion spermatozoa treated with 5mM procaine yielded encouraging fertilization rates (60.7%) when these sperm were added to equine oocytes *in vitro* (McPartlin et al., 2009). However, because equine oocytes are not readily available, experiments using heterologous oocytes to study the equine IVF system are necessary(Alm et al., 2001; Sinowatz et al., 2003; Coutinho da Silva et al., 2004; Mugnier et al., 2009).

Previously, we reported that stallion sperm treated with PC12 fertilized *in vitro* matured bovine oocytes (Spizziri et al., 2008). This study confirms that spermatozoa capacitated with PC12 (15μM) induced higher bovine oocyte cleavage rates than any other sperm treatment.

Previously, McPartlin et al. (2008) reported that sperm treated with 5mM procaine supported sperm changes consistent with capacitated sperm and that these sperm induced substantially higher equine oocyte cleavage rates than sperm that were not treated with procaine (60.7% vs. 0%, respectively). It is not known why our data contradict these results, but addition of procaine to stallion sperm inhibited PC12-treated sperm from fertilizing bovine oocytes. One possibility for this discrepancy may be that subtle differences exist between the equine and bovine

oocytes. Although bovine oocytes have been utilized in the past for studying various aspects of equine IVF (Sinowatz et al., 2003; Coutinho da Silva et al., 2004; Spizziri et al., 2008), they may be sufficiently different from equine oocytes that very different aspects of sperm capacitation, zona binding, acrosome reaction induction, zona penetration, and oocyte fertilization are different between the equine and bovine oocytes.

However, the ability of stallion spermatozoa to successfully penetrate zona-intact bovine oocytes indicates that the ligand systems of the equine and bovine zona pellucida are at least partially conserved (Sinowatz et al.., 2003). Recent investigations of the structure of the equine zona pellucida indicate the mesh-like surrounding structure and pore sizes are different than those of porcine zona pellucida (Mugnier et al., 2009). In addition, the number of pores is significantly higher in *in vitro* matured equine oocytes than in in vivo matured oocytes(Mugnier et al., 2009). Also, the diameter of the pores was larger after *in vitro* maturation but did not change during in vivo maturation (Mugnier et al., 2009). These differences also may alter the localization of zona glycoproteins, which could also impact fertilization rates. Due to the differences between porcine and equine ZP and subsequent fertilization, it is possible that such oocyte architectural differences exist between bovine and equine oocytes. The altered ZP architecture and glycoprotein localization could account for our results with bovine oocytes.

Several studies report fertilization rates of essentially 0% for equine oocytes fertilized with *in vitro* capacitated stallion sperm(McPartlin et al., 2009; Alm et al., 2001; Dell'Aquila et al, 1997; Dell'Aquila et al., 1996; Zhang et al., 1990). The

highest fertilization rates reported (65%; Choi et al. 1994) were obtained with partial or complete removal of the equine zona pellucida. Therefore, the ZP appears to pose a significant barrier to successful equine IVF (McPartlin et al. 2009).

In order to circumvent the potential difficulty imposed by the zona pellucida, we tested whether a mechanical disruption of the zona pellucida (making a hole in the ZP) would promote higher sperm fertilization rates. However, sperm did not fertilize oocytes with holes drilled in their ZP's more efficiently than zona-intact oocytes. One potential reason putting a hole in the ZP did not improve fertilization is that drilling holes using the laser produces high temperatures in the medium that may be detrimental to the developing oocyte. Our treatment groups were comprised of large groups of oocytes that may have resulted in damage to the oocytes due to lengthy exposure to high temperatures.

In addition to the possible deleterious effects of high temperature, our laser technique may have caused considerable damage to the oocyte's cytoplasm. Anzai et al. (2006) found that incubating mouse oocytes in 0.5 M sucrose caused significant cytoplasm shrinkage, thereby creating a larger perivitelline space so as to prevent damage to the oocyte cytoplasm. Hiraoka et al. (2008) also utilized sucrose (0.2 mol/l) to create a larger space around the ZP of human blastocyst while decreasing potential damage to the embryo. A larger perivitelline space would allow a safer area to apply the laser pulse and prevent cytoplasmic damage.

Another difference between our study and others that utilized the laser for creating a hole in the ZP is the laser power. In our study, the laser was calibrated to apply 300 mW power for creating a zona pellucida hole. Others have reported

positive fertilization results using mouse and human oocytes when 165-200mW and 180mW, respectively were used to produce holes in the ZP (Anzai et al., 2006; Hammoud et al., 2010). Therefore, a lower power may be safer for creating holes in the ZP.

Tinney et al. (2005) examined multiple combinations of laser parameters for creating ZP holes in mouse blastocysts and determined that halving the laser intensity and doubling the laser pulse was more beneficial than a single, more intense pulse. Another possibility for the decreased results is the number of ZP holes. Our study utilized the creation of a single hole in the zona pellucida. Results are conflicting in the success of "zona thinning", the practice of creating several consecutive small holes versus a total dissection used in our study (Mantoudis et al., 2001; Tinney et al., 2005). Tinney et al. (2005) found that zona thinning decreased mouse oocyte hatching. Conversely, Mantoudis et al. (2001) obtained greater results when zona thinning was used for human embryos. The discrepancies may be attributed to a thicker human ZP, indicating a proportionally sized hole may be as beneficial (Tinney et al., 2005). Taken together, the laser parameters needed to effectively modify the ZP need further investigation for successful stallion sperm penetration of the ZP.

Since PC12-treated stallion spermatozoa fertilized the highest percentages of bovine oocytes, treating sperm with PC12 may be effective in providing consistent fertilization rates of oocytes as well. Even though there may have been a potential problem associated with the mechanical zona disruption technique used in this study, results using zona intact oocytes appear promising to efficiently capacitate

stallion sperm for equine IVF. However, additional work needs to address whether circumventing the zona pellucida barrier is necessary to improve fertilization rates of *in vitro* capacitated stallion sperm.

Conclusion

Treating stallion spermatozoa with PC12 (15μM) to "capacitate" sperm *in vitro* resulted in the highest cleavage rates for bovine oocytes. Incubating sperm in a capacitation medium containing 5mM procaine (McPartlin et al., 2009) did not improve bovine oocyte cleavage rates. In order to bypass the potential barrier of the zona pellucida, we used mechanical disruption of the bovine zona pellucida. However, cleavage results following mechanical disruption were low, which may be due to the laser's inducing significant damage to the oocyte as well as the ZP.

Tables

Table 3.1. Cleavage rates (%) for bovine oocytes (~30 oocytes/replicate) fertilized with stallion sperm treated with PC12 or MBC in the absence or presence of 5 mM procaine(n=8).

Treatment	Control	Procaine	SEM
Control	9[a]	11	4
15 μM PC12	25[c]	6*	3
20 μM PC12	20[bc]	6*	2
40 μM PC12	13[ab]	6*	2
0.5 μM MBC	10[a]	10	3
1 μM MBC	8[a]	11	2
Parthenote	4[a]	4	5

[a,b,c] denote column differences. p<0.05.
*** indicates mean is different from control.**

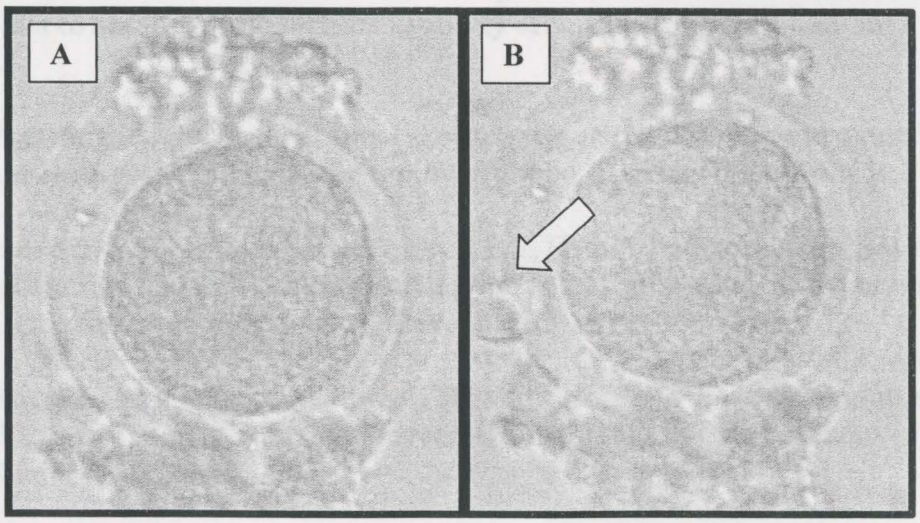
Table 3.2. Cleavage rate for zona intact oocytes and laser treated oocytes when bovine oocytes (~30 oocytes/replicate) were fertilized with control or 15 μM PC12 treated spermatozoa (n=4).

Treatment	Zona Intact	Lasered	SEM
Control	9	3*	2
15 μM PC12	30[a]	4*	2

[a] denotes column differences.
*** indicates mean is different from zona intact at p<0.05.**

Figure

Figure 3.1. Zona intact *in vitro* matured bovine oocyte prior to laser treatment (A). Zona intact *in vitro* matured bovine oocyte following laser treatment (B). Arrow indicates site of laser disruption.



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Chapter IV

SUMMARY

Conclusions

A lack of understanding of and ability to induce capacitation in equine sperm may be a limiting factor in equine *in vitro* fertilization success. Sperm modifications that occur during capacitation include plasma membrane cholesterol efflux, increased intracellular bicarbonate and calcium concentrations, protein phosphorylation, acquisition of hyperactive motility, and actin polymerization (Jha et al., 2002; Brener et al., 2003; Breitbart et al., 2005). Together, these modifications allow the spermatozoa to gain hyperactive motility, bind to the zona pellucida, undergo the acrosome reaction, and initiate gamete fusion (Barbonetti et al, 2008). Unfortunately, a definitive test does not exist to assess sperm capacitation. Therefore, our research is limited to studying each of the steps in capacitation independent of each other. In addition, the limited availability of equine oocytes for research purposes has led to the development of heterologous binding assays to assess sperm fertilizing capacity.

In the present study, we confirmed that stallion sperm treated with methyl-β-cyclodextrins (MBC; Visconti et al., 1999; Purdy and Graham, 2004) lose cholesterol, a prerequisite of sperm capacitation. As we hypothesized, treating sperm with dilauroylphosphatidylcholine (PC12) did not decrease cholesterol levels

as PC12 utilizes an alternative method to disrupt plasma membranes. We also confirmed that treating sperm with cholesterol-loaded-cyclodextrin (CLC; Moore et al., 2005) increased membrane cholesterol levels.

Protein tyrosine phosphorylation, another aspect of sperm capacitation, is typically analyzed using western blot analysis. In the present study, we determined how treating stallion sperm with PC12, MBC, and calcium ionophore A23187 affected protein tyrosine phosphorylation. Neither MBC nor A23187 induced significant protein tyrosine phosphorylation. However, PC12 treatment induced protein tyrosine phosphorylation, similar to the amount of protein tyrosine phosphorylation that occurred in control sperm that were capacitated over an extended incubation in capacitating medium.

We then attempted to adapt the protein tyrosine phosphorylation assay to flow cytometery. However, we were unable to obtain consistent results using flow cytometery. This analysis appears to be very delicate in terms of optimizing fixation and permeabilization protocols (Krutzik and Nolan, 2003). Flow cytometric analysis is promising as a method for more accurate and complete analysis. However, additional research is necessary to optimize this procedure.

Hyperactive motility, acquired during capacitation, is usually assessed with computer assisted sperm analysis (CASA; Mortimer and Maxwell, 1999; Cancel et al., 2000; Suarez, 2008). Motility parameters are species dependent (Mortimer and Mortimer, 1990; Suarez et al.. 1992; Baumber and Meyers, 2006) and have recently been defined in the horse (McPartlin et al., 2009). In this study, we used a more sensitive method of analysis, a robust algorithm, termed Minimum Bounding Square

Ratio (MBSR). MBSR analysis is unique in that it can accurately detect motility patterns and classify sperm as being hyperactive, transitional, or progressive. Only MBSR analysis was able to confirm that adding procaine to stallion sperm induced hyperactive motility. This study indicated that typical CASA parameters are not the best method of assessing hyperactive motility and that the MBSR is more accurate in assessing hyperactive motility.

Our research confirmed that PC12 and MBC can induce the acrosome reaction in stallion sperm. However, procaine treatment, while inducing hyperactive sperm motility did not induce acrosome reactions, consistent with other studies (McPartlin et al., 2009).

Due to the limited number of equine oocytes that are available, we utilized *in vitro* matured bovine oocytes for IVF experiments. Stallion sperm, challenged with PC12 induced cleavage of bovine oocytes. However, when we disrupted the bovine zona pellucida to bypass the barrier of the ZP, fertility rates were significantly lower than zona-intact oocytes indicating the need for additional study to effectively disrupt the zona pellucida without damaging the oocyte.

In conclusion, these studies indicate that several methods to capacitate stallion sperm *in vitro* are concordant with several of the steps involved in sperm capacitation. In addition, treated sperm do fertilize *in vitro* matured bovine oocytes, indicating that at least some sperm are sufficiently capacitated to fertilize oocytes. However, additional research is still needed to address the complexity of steps involved in fertilization that potentially involves both stallion sperm capacitation and overcoming the barrier of the zona pellucida.

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