SPERM RELEASE FROM THE OVIDUCT RESERVOIR

BY

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DISSERTATION

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ABSTRACT

Sperm undergo capacitation while traveling through the female reproductive tract, a process including several biochemical and physiological events before fertilization. Also prior to fertilization, sperm bind to the epithelial cells of the oviduct isthmus to form a sperm reservoir. This sperm reservoir regulates sperm capacitation, reduces polyspermy and prolongs fertile lifespan. It has been proposed that oviduct glycans interact with sperm, which leads to the formation of sperm reservoir. Our lab also has previously demonstrated that porcine sperm bind to the oviductal glycans biantennary 6-sialylated N-acetyllactosamine (bi-SiaLN) oligosaccharide and a Lewis X trisaccharide (Le^X) to form the sperm reservoir. These glycans are also implicated in the regulation of sperm capacitation by regulating calcium influx and extending sperm life span. My objective was to identify how sperm bound to oviduct glycans are released from the reservoir to fertilize the oocyte. The results of this study indicate that soluble oviduct glycans do not alter sperm capacitation. Progesterone and the related steroid pregnenolone release 40-60% of sperm from oviduct cells and immobilized glycans. Progesterone release requires the ion channel CatSper and protein degradation by proteasomes. Finally, the development of sperm hyperactivation was sufficient to induce sperm release, but complete release also required the function of CatSper and proteasomes. My results are the first to show that hyperactivated motility is sufficient to induce sperm release from the oviduct reservoir and implicate progesterone as an important signal to sperm promoting their release and movement towards the egg.

ACKNOWLEDGEMENTS

First and foremost, I would like to acknowledge and thank my advisor and mentor, Dr. David J. Miller, for his mentorship, guidance, never ending patience and support throughout my graduate journey. Under his guidance I have, without a doubt, become a better learner, scientist, mentor and a person with a better temperament. I am extremely grateful to have had the opportunity to be trained under his supervision, where he lets his students learn at their own pace and approach.

I would also like to thank my committee members for their support and advice. My lab mates, both current and former also deserve recognition for their support and friendship. A special thanks to my friend, Roop whose support is always by my side to offer me with vital sustenance during grad school.

Many thanks to my family, another integral part of my journey through graduate school. My parents and siblings have provided endless love, support, and encouragement. My siblings look up to me as a role model and thanks God I made it through.

Lastly, I would like to thank my spouse Arslan, over the last decade has steadfastly stood by my side and supported me through life. He has always given me full autonomy over my decisions and also supported me through them. I cannot thank him enough for all the love, support, and tolerance. He has always facilitated me on the path of becoming a “strong independent women”, which is my life’s moto. I am also extremely thankful for our daughter Ayza who is a fairly new addition to our lives. She has reminded me to enjoy and appreciate life at another level. She has made me happier than I ever thought possible and has pushed me to work even harder so that she can be very proud of her mother.

I Dedicate this thesis to my grandfather "Muhammad Sharif", who strongly believed in women empowerment and higher education. Without his support I would never have pursued a doctoral degree.

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CHAPTER 1

LITERATURE REVIEW

1.1. INTRODUCTION

After sperm deposition at mating, sperm are moved towards the oviduct where they are transiently retained to form a reservoir. Sperm are eventually released and move to the ampulla to fertilize oocytes (Hunter, 2004; Kadirvel et al., 2012; Hunter et al., 1981). Storage in the isthmus prolongs sperm lifespan and reduces polyspermy (Hunter, 1973; Holt and Fazeli, 2010; Gist and Jones, 1987). It is known that formation of this reservoir is due to sperm interaction with specific glycans on the epithelial cells in the isthmus (Miller, 2015; Kadirvel et al., 2012; Machado et al., 2014; Suarez, 2001). These oviduct glycans have been identified using a novel glycan array approach (Kadirvel et al., 2012; Machado et al., 2014). This approach has shown that porcine sperm preferentially binds to either of two abundant glycan motifs, a biantennary 6-sialylated *N*-acetyllactosamine (bi-SiaLN) oligosaccharide and a Lewis X trisaccharide (Le^X) to form the reservoir (Kadirvel et al., 2012; Machado et al., 2014).

The general model for this dissertation is that sperm are released from oviduct glycans in the sperm reservoir in response to progesterone. I propose that oviduct glycans regulate sperm capacitation, and that release requires progesterone signaling through CatSper and hyperactivation, and that proteasomal sperm protein degradation is necessary. (Figure 1.1)

1.2. RATIONALE AND SIGNIFICANCE

Improper or failed fertilization are amongst the primary causes of infertility and subfertility (Hargreave, 2000; Hasson and Stone, 2009; Yanagimachi, 2008; O'Flynn O'Brien et al., 2010). The factors behind infertility include gamete age and asynchrony between them, sperm storage problems and other complications leading to low reproductive success (Georgiou et al., 2007; Hawk, 1983; Tienthai et al., 2004; Nissen et al., 1997).

It is important to gather more knowledge about sperm storage and release in swine for two main reasons:

1-Appropriate synchronization between AI, the release of sperm from the reservoir and ovulation

Predicting when females will ovulate in necessary for deciding when to perform artificial insemination. In swine, estrus is of variable length and because of this determination of ovulation and the appropriate time of insemination is difficult. The most common solution is to inseminate at the beginning of estrus and every 24 hr until estrus concludes, which is a laborious procedure (Nissen et al., 1997).

2-Premature sperm capacitation due to use of cryopreserved semen

Capacitation is the final maturation step, which enables sperm to fertilize the oocyte (Austin, 1952; de Lamirande et al., 1997; Chang, 1984). But premature capacitation appears to reduce the ability of sperm to be retained in the oviduct reservoir (Dobrinski et al., 1995; Bailey et al., 2008). Sperm cryopreservation is done routinely in cattle but is much less common in

swine. Cryopreservation is desirable to be able to store sperm for long periods to allow transportation with negligible effects on fertility. But porcine sperm have reduced fertility following cryopreservation, perhaps due to reduced retention in the sperm reservoir (Dobrinski et al., 1995; Bailey et al., 2008). My research may lead to improved reservoir formation and, ultimately, the development of more successful porcine sperm cryopreservation methods.

1.3. BACKGROUND

Sperm capacitation includes multiple processes

Capacitation, the final maturation step of sperm in the female reproductive tract is necessary for fertilization (de Lamirande et al., 1997). It includes a series of biochemical and physiological changes. The molecular basis of this process is unclear at numerous levels (Aitken and Nixon, 2013). Capacitation prepares the sperm for the acrosome reaction at the sperm head while preparing the tail for hyperactivation (Jin and Yang, 2017). Steroid hormones are extrinsic factors known to affect capacitation. Progesterone, present in the oviductal fluid, is a well-known inducer hyperactivation in sperm (Miller et al., 2016; Teves et al., 2006). It is also known that acrosomal exocytosis happens after the completion of capacitation while hyperactivation occurs either spontaneously or in a time-dependent manner during the capacitation process (Chang and Suarez, 2010; Vicente-Carrillo et al., 2017). Several cellular pathways are interlinked and work synergistically to help sperm fertilize the oocyte. These include the cyclic adenosine monophosphate dependent protein kinase A pathway (cAMP-PKA), endocannabinoid pathway, extracellular-regulated kinase (ERK) pathway, protein tyrosine

phosphorylation, protein Ser/Thr phosphorylation, sperm proteasomes and proteases (Jin and Yang, 2017; Miller et al., 2016).

Sperm oviduct reservoir

After insemination, artificial or natural, the semen which is deposited in the uterus must move to reach the oviduct before the immune response in the uterus affects it (Bischof et al., 1994). During this journey, sperm passes through the uterus via uterine-tubal junction (UTJ) towards the isthmic region where the sperm binds the oviduct epithelial cells to form a sperm reservoir (Suarez and Pacey, 2006). Sperm binding to the reservoir is carbohydrate-mediated. Receptors for those specific carbohydrates are found on sperm apical surface, which binds to the carbohydrates (glycans) present on the oviduct epithelial cells (Benoff, 1997; DeMott, 2005; Suarez, 2001) **(Figure 1.2)**. In the oviduct, sperm go through their final maturation step when they come in contact with the oviductal fluid and the epithelium (Nixon et al., 2007). The environment in the female reproductive tract induces multiple changes in sperm function, membrane structure and motility, encompassing the process of capacitation (Hawk, 1983; Gadella, 2008). Formation of the sperm reservoir in the oviduct delays these changes. This reservoir may delay capacitation, but in turn prolong sperm lifespan, decrease sperm motility and prevent polyspermy (Rodriguez-Martinez, 2007; Miller, 2015).

The sperm reservoir is essential for proper and well-timed fertilization to take place in the ampulla (Tienthai et al., 2004; Rodríguez-Martínez et al., 2005). The sperm are released from this reservoir during the pre-ovulatory period in a sequential manner as the process of capacitation completes (Kadirvel et al., 2012; Ho and Suarez, 2001). How this attachment

between the oviduct cells and sperm is broken is still uncertain. It has been suggested that the release of sperm from the reservoir happens due to programmed changes in sperm during capacitation leading to hyperactivation (Chang and Suarez, 2010; Hunter and Gadea, 2014). It could also be due to alterations happening in oviduct, which can encompass oviduct epithelial cell surface modification, changes in oviductal fluid and steroid concentration due to the presence of oocyte and follicular fluid at ovulation (Roldan et al., 1994; Uhler et al., 2016; Nahed et al., 2016).

As mentioned in the rationale, knowledge about the formation of oviduct sperm reservoir and release of sperm in a timely manner is significant for advancement in improving AI in the animal industry, particularly in swine.

Oviduct glycans

There is considerable evidence present that in several species, sperm binding to the reservoir in oviduct is mediated by glycans present on oviduct epithelial cell (Miller, 2015; Kadirvel et al., 2012; Machado et al., 2014; Kuo et al., 2009; DeMott, 2005; Lefebvre et al., 1997). The glycocalyx, a complex structure made up of glycoprotein and glycolipids on the surface of the cell membrane is primarily made up of sugars is responsible for the regulation of cell-cell adhesion, interaction, recognition, and oviduct sperm binding (Brandley and Schnaar, 1986; Kuo et al., 2009; Vjugina, 2008; Kumaresan et al., 2012). The specific identity of the molecules that mediate sperm oviduct binding varies from species to species. The identity of these glycans, their location, abundance and candidate receptors are under investigation and little information is available for most species. It is important to identify the precise identity of

the glycans and their candidate receptors to completely elucidate the role of glycans in the formation of the sperm reservoir.

There is evidence that the sperm reservoir forms in the lower oviduct of the female, known as the isthmus (DeMott, 2005). Our lab screened 377 glycans (sugars) to identify the glycans which are responsible for the sperm reservoir formation in porcine oviduct using a glycan array (Kadirvel et al., 2012; Miller, 2015; Machado et al., 2014). Uncapacitated sperm bound to glycans were assessed to identify that glycans that bound sperm. The glycan array screening for specific ligands indicated that a significant number of **uncapacitated boar sperm bound to biantennary 6-sialylated *N*-acetyllactosamine** (bi-SiaLN; Neu5Ac□2-6Galβ1-4GlcNAcβ1-2Man□1-3(Neu5Ac□2-6Galβ1-4GlcNAcβ1-2Man□1-6)Manβ1-4GlcNAcβ1-4GlcNAc), **and dimers/trimers of Lewis X trisaccharide** (Le^x; Galβ1-4(Fuc□1-3)GlcNAc) (Kadirvel et al., 2012) **(Figure 1.3 and 1.4)**. When these glycans or their putative receptors were blocked it reduced the sperm binding to the oviduct cells by 60%. These data showed that both bi-SiaLN and Le^x are necessary to retain porcine sperm and form the sperm reservoir (Miller, 2015; Kadirvel et al., 2012; Machado et al., 2014) **(Figure 1.4)**.

Moreover, the release of sperm from the reservoir has been proposed to occur after sperm are capacitated. Our lab has tested whether capacitated sperm retained an affinity for glycans. Capacitated sperm were incubated with fluorescein-labeled 6-sialylated lactosamine biantennary glycan (Flu-bi-SiaLN). About 40% of sperm bound Flu-bi-SiaLN, much less than the number of uncapacitated sperm that bound branched sialylated lactosamine **(Figure. 1.5, D–F, and J)**. Because capacitation does not occur synchronously, likely, not all sperm in the sample that was prepared under conditions that promote capacitation were capacitated. Controls using

fluoresceinated lactosamine (Flu-LN) bound to less than 5% of sperm **(Figure. 1.5J)**. Therefore, during capacitation, most sperm lost their ability to initiate binding to bi-SiaLN (Kadirvel et al., 2012). Preliminary investigation has also shown that binding to these oviduct glycans prolongs sperm lifespan and delays sperm capacitation **(Figure 1.6)**.

Candidate glycan receptors in porcine sperm

The glycans on the oviduct epithelial cell surface are not cell-permeable and thus require a receptor for communication with the sperm for the formation of the reservoir. Glycans are recognized by lectin-like proteins which have a carbohydrate recognizing domain and recognize specific sugar residues by fitting them in their binding pockets (Brandley and Schnaar, 1986; Benoff, 1997). There is evidence that spermadhesin (AQN1), which originates from accessory gland secretion, is a glycan-binding protein but AQN1 does not bind bi-SiaLN and Le^x motifs (Ekhlasi-Hundrieser et al., 2005). Moreover, the epididymal porcine sperm not exposed to AQN1 can still bind oviduct cells suggesting there are other molecules involved in sperm binding to the oviduct (Ekhlasi-Hundrieser et al., 2005; Petrunkina et al., 2001). Our lab identified candidate glycan receptors using affinity chromatography and reverse-phase HPLC, which led to the characterization of eluted proteins by SDS-PAGE and glycan blots. Three proteins which were identified using mass spectrometry were MFGE8 (SP47, SED1 or lactadherin), ADAM5 (A Disintegrin and Metalloproteinase Domain 5) and PKDREJ (Polycystic Kidney Disease (Homolog) to Receptor for Egg Jelly in Sea Urchin). MFGE8 was found to be receptor protein for Le^x while PKDREJ was found to be a receptor for both Le^x and bi-SiaLN. Our

lab has proposed that these proteins play a significant role in sperm progression, retention, and formation of the sperm reservoir (Silva et al., 2014, 2017).

Sperm release from oviduct cells

The mechanism behind the release of sperm from oviduct cells is unclear. It has been suggested that progesterone from the ovulated follicle and cumulus-oocyte complex (COC), is able to cause Ca^{2+} influx in the sperm which leads to changes in motility pattern, leading the sperm into hyperactivated motility (Ho and Suarez, 2001; Pacey et al., 1995; Suarez and Ho, 2003). Our lab has proposed that capacitation and hyperactivation jointly help the sperm free itself from the oviduct reservoir. The initial investigation of this model (**Figure 1.1)** has confirmed that the progesterone in physiological concentrations can cause an increase in intracellular Ca^{2+} via a non-genomic endocannabinoid pathway. CatSper is the principal Ca^{2+} channel in the sperm, which is regulated by progesterone (Niederberger, 2017). Also, a recent in-silico study showed via knock-out calculations that CatSper is the main generator of Ca^{2+} oscillations in sperm flagella and that other Ca^{2+} channels, if present, just have a complementary role (Espinal-Enríquez et al., 2017). A preliminary experiment supported this mechanism. Blocking CatSper channels with a T-type blocker prevented the progesterone-induced release of sperm from oviduct cells (Machado et al., 2019, submitted) (**Figure 1.7 and 1.8)**. Moreover, a preliminary experiment also showed that only longer incubation times under capacitating conditions did not cause the release of sperm from oviduct glycans, which indicates that multiple factors are involved in sperm release from the reservoir (**Figure 1.9)**.

Protein degradation in sperm during capacitation and fertilization

Proteasomes are barrel-shaped protein complexes which degrade unnecessary or damaged intracellular proteins by proteolysis (**Figure 1.1)** (Matsumura and Aketa, 1991). Proteasomes are part of a mechanism by which cells regulate the abundance of particular proteins and degrade misfolded proteins. A small protein called ubiquitin tags proteins for degradation via proteasomes (Sawada et al., 2002). Like somatic cells, sperm have protease complexes or proteasomes that possess multi-enzymatic properties of trypsin-like, chymotrypsin-like and peptide-hydrolyzing activities (Morales et al., 2003; Tipler, 1997; Wojcik et al., 2000). The 20S core subunit of proteasome in boar sperm has been found to associate with several proteins that are candidate receptors for oviduct glycans and are implicated in sperm capacitation and sperm-zona binding **(Figure 1.10, Left)** (Raymond et al., 2009; Miles et al., 2013; Liberda et al., 2006). The ubiquitin-proteasome system is essential for both intracellular and extracellular protein degradation in sperm (**Figure 1.10, Right)** (Sawada et al., 2002).

Sperm hyperactive motility

To fertilize the oocyte, sperm need to be able to move through the female reproductive tract towards the ampulla. Two distinctive types of motility have been exhibited by sperm that are very well characterized: active (or progressive) and hyperactive motility (**Figure 1.11)** (Martin-Hidalgo et al., 2018). In the uterus, inseminated sperm show active motility described by straight-lined paths. As sperm reach the final maturation state (capacitation) in the oviduct, they exhibit hyperactive motility, which is described by a large asymmetrical beating of the

sperm tail with less progressive movement that results in a circular or helical path (Yanagimachi, 1969). This hyperactive motility is proposed to allow spermatozoa to release sperm from the oviductal mucosa and allow them to move through the oviductal mucus towards oocyte (Suarez and Osman, 1987; Smith and Yanagimachi, 1989; Suarez et al., 1992; Katz et al., 1989).

1.4. PRELIMINARY CONCLUSIONS

The current model for the sperm reservoir formation in porcine oviduct has provided some insight into how it is formed but to understand how the sperm are released to move on and fertilize the oocyte is a critical question. Use of a novel glycan screening approach has shown that uncapacitated porcine sperm bind preferentially to glycans containing bi-SiaLN and Le^x motifs. The candidate receptors for these sugars are MFGE8, PKDREJ, and ADAM5. These candidates are present on the sperm head. Our lab's initial work has determined the identity of oviduct glycans and identified candidate receptors on sperm that form the reservoir. In this dissertation, I have investigated how the sperm are released from accumulation in the lower isthmus region and the factors involved in this release. I have studied whether the release is pre-programmed into sperm or is regulated by progesterone coming from mature follicles or COC in the oviduct during ovulation. This significant clarification in the sperm release mechanism from oviduct reservoir may lead to novel techniques to improve AI methods and more success with cryopreserved porcine sperm.

1.5. FIGURES

FIGURE 1.1

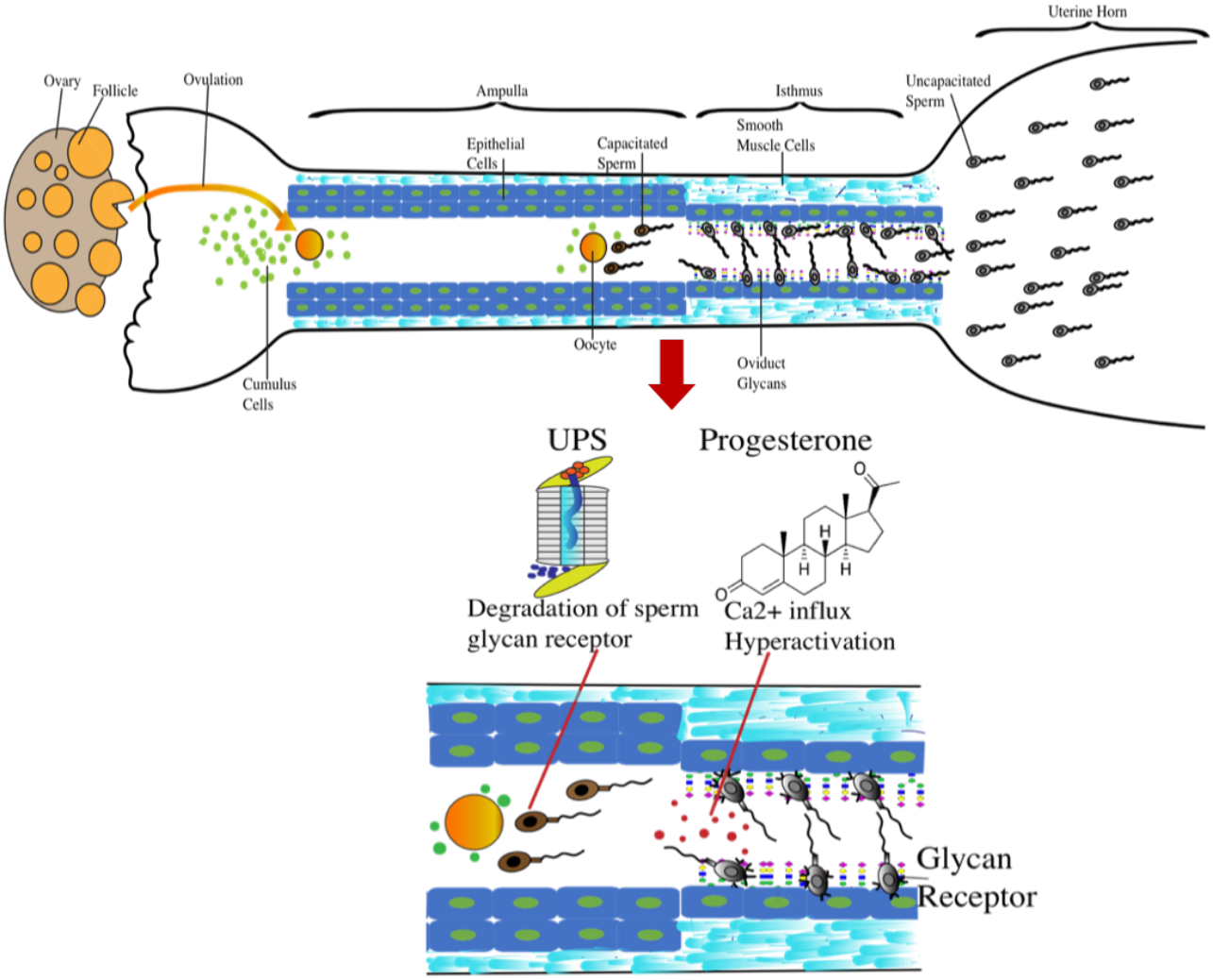

Figure 1.1 Overall model: Schematic representation of overall objectives.

Sperm form a reservoir in the isthmic region of the oviduct that maintains sperm viability and suppresses motility. Sperm are released to move to the ampulla to fertilize oocytes. The ubiquitin-proteasome system (UPS) is responsible for proteolysis of >75% of intracellular eukaryotic proteins and may also degrade extracellular candidate glycan receptors. Progesterone

Figure 1.1

(P4) is responsible for calcium (Ca^{2+}) influx and hyperactivity. A combination of proteasomal degradation and hyperactivated motility is hypothesized to be responsible for the release of sperm from sperm oviduct reservoir.

FIGURE 1.2

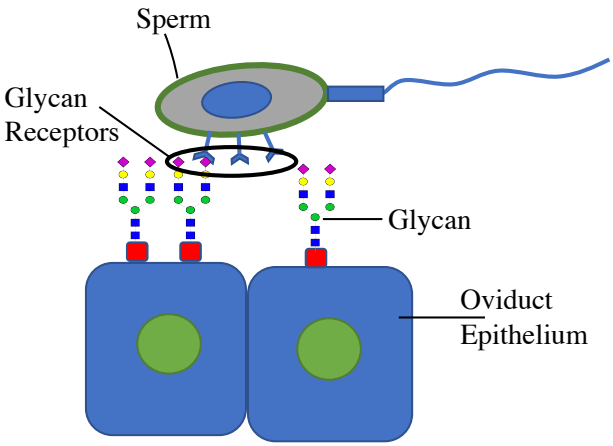

Figure 1.2. Sperm binding to oviduct epithelial cells.

Sperm bound via receptors for the glycan structures present on oviduct epithelial cells.

FIGURE 1.3

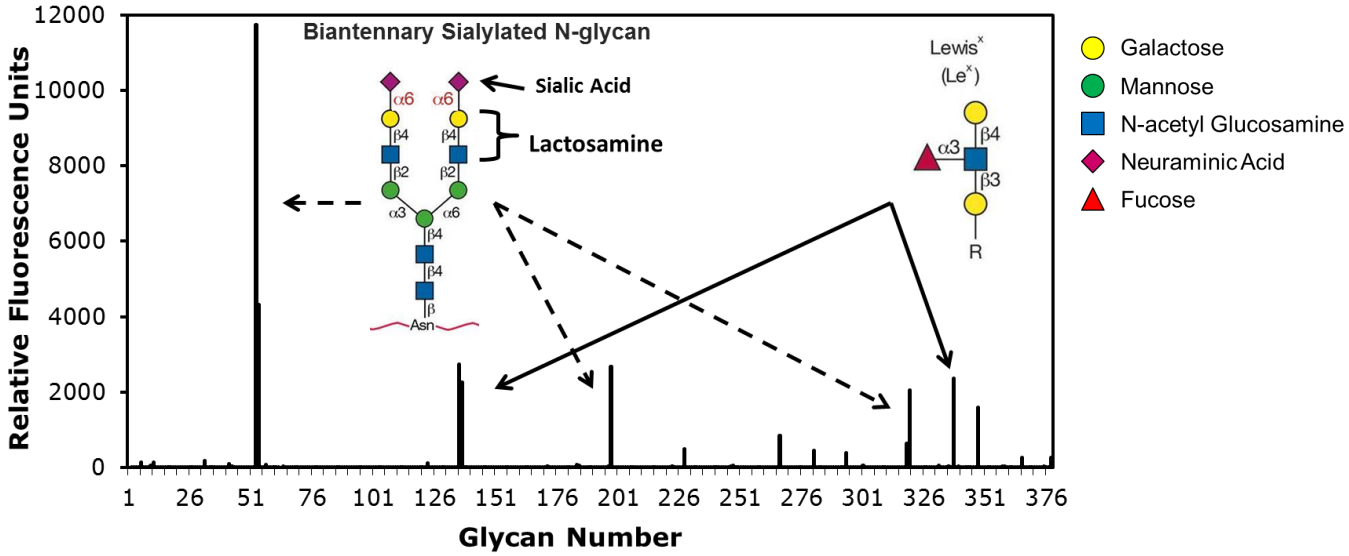

Figure 1.3. Uncapacitated boar sperm bind to specific bi-SiaLN and Le^X moieties.

Sperm was stained with the fluorochrome Syto-16 and allowed to bind to a glycan array containing 377 numbered sugars. Peaks of RFU (Relative Fluorescence Units) represent fluorescence due to the total number of sperm bound to spots containing a specific glycan. Arrows represent fluorescence peaks of sperm bound to bi-SiaLN (dashed arrows) and Le^X (solid arrows), n=3. (Kadirvel et al., 2012).

FIGURE 1.4

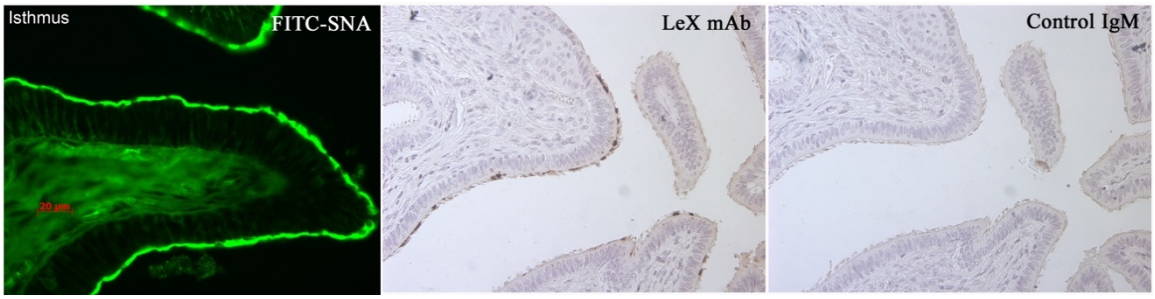

Figure 1.4. Localization of Le^x structures to the luminal epithelium of regions of the oviduct. **Left**, Oviduct tissue from the ampulla and isthmus stained with fluoresceinated Simbucus nigra agglutinin lectin (SNA). bi-SiaLN was detected on the epithelium of the oviduct. **Middle**, Fixed oviduct tissue stained with monoclonal antibody to Le^X. Staining was detected in the epithelium of the isthmus. **Right**, Minimal staining of isthmus adjacent section with normal IgM, n=3. (Kadirvel et al., 2012)**.**

FIGURE 1.5

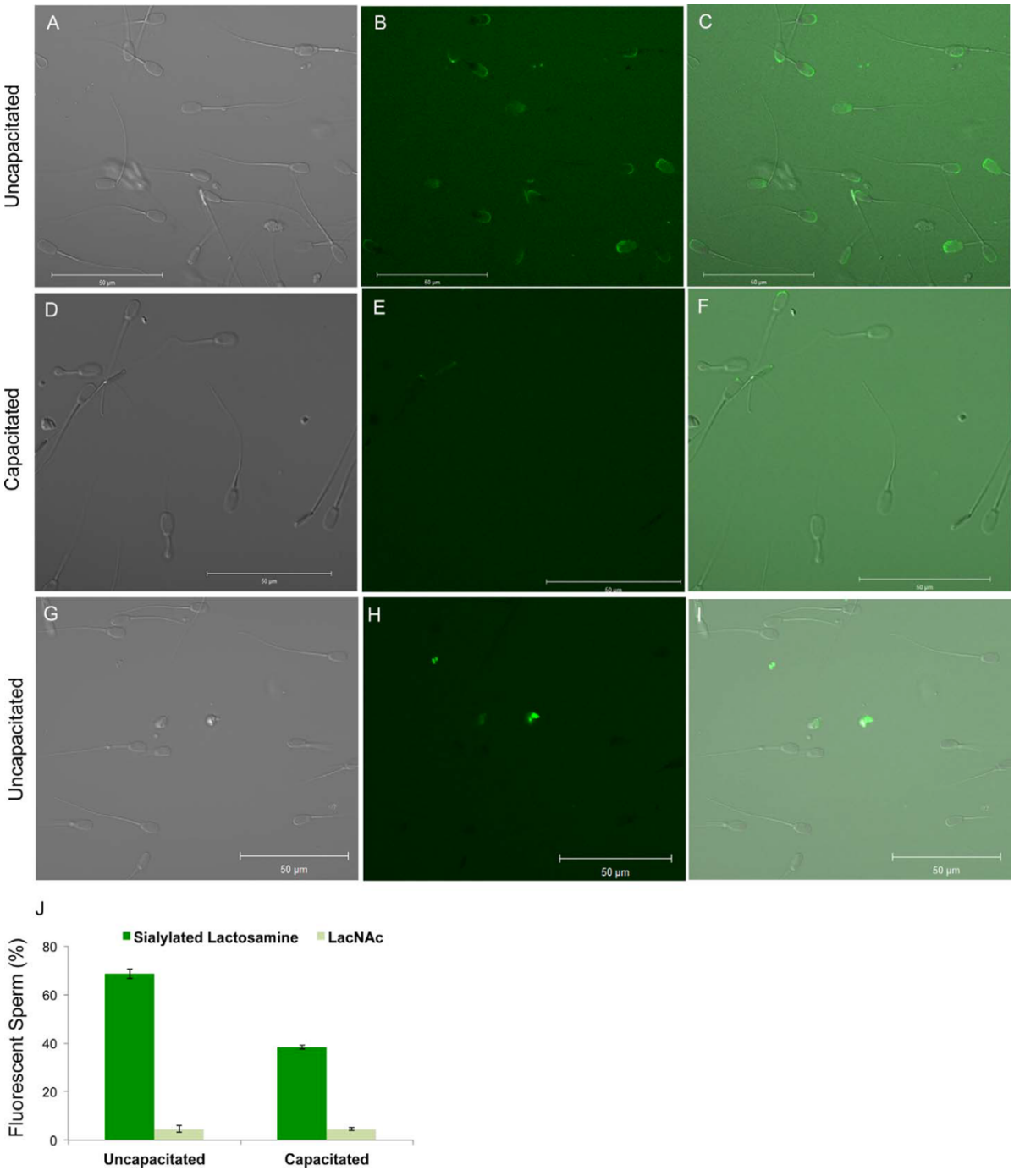
A
B
C
D
E
F
G
H
I
Uncapacitated
Capacitated
Uncapacitated
50 µm
J
Sialylated Lactosamine
LacNAc
Fluorescent Sperm (%)
80
60
40
20
0
Uncapacitated
Capacitated

Figure. 1.5. The 6-sialylated biantennary glycans bound to the sperm head, but binding was reduced after capacitation.

Sperm were allowed to bind soluble oviduct glycans in capacitating dmTALP (C) and non-capacitating dmTALP (NC). Incubated with fluorescein-labeled, (Flu-bi-SiaLN) or fluorescein-labeled lactosamine (Flu-LN). The photomicrographs show representative fields of differential interference contrast (DIC), fluorescence, and merged images. DIC, fluorescence, and merged images of non-capacitated sperm incubated with Flu-bi-SiaLN are in A–C, respectively. DIC, fluorescence, and merged images of capacitated sperm (C) incubated with Flu-bi-SiaLN are in D–F, respectively. DIC, fluorescence, and merged images of non-capacitated sperm incubated with the Flu-LN are in G–I, respectively. The percentage of either non-capacitated sperm or capacitated sperm that bound Flu-bi-SiaLN or the Flu-LN is shown in J. n=3. (Kadirvel et al., 2012).

FIGURE 1.6

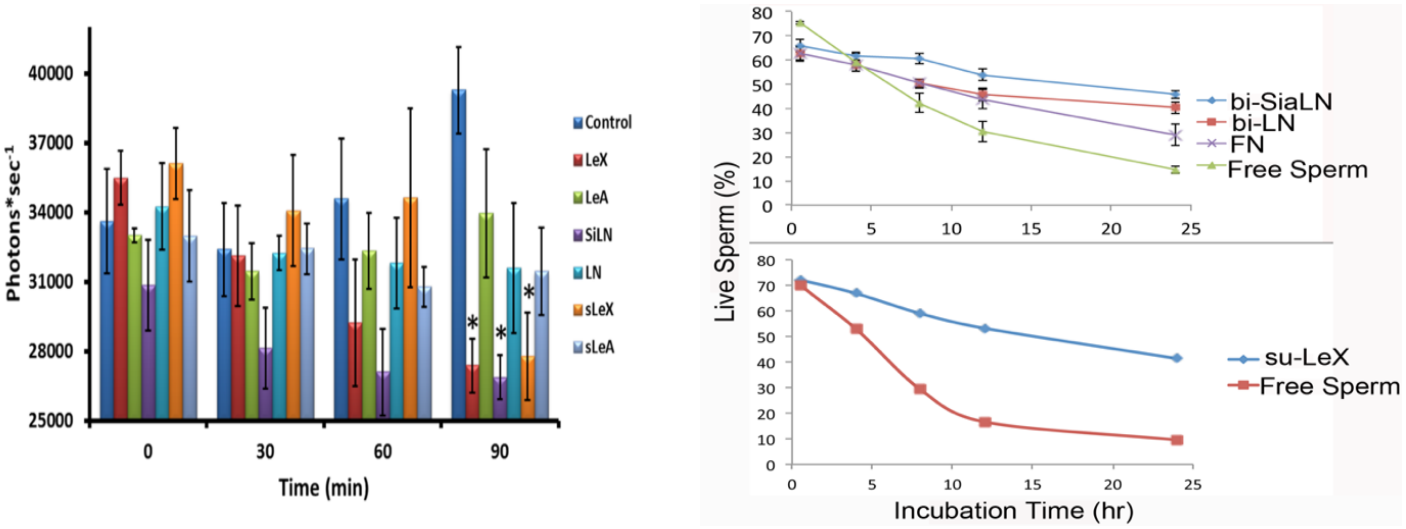

Figure 1.6. Binding to oviduct glycans suppresses an increase in intracellular Ca^{2+} during capacitation and prolongs porcine sperm lifespan.

Left, Fluo-4 loaded sperm were incubated with glycans in dmTALP and control conditions at 39°C. Spectrofluorometric readings were taken 0, 30, 60, and 90 min. Statistical differences ($p < 0.05$) relative to control are represented by an asterisk. **Right**, Biotinylated glycans were bound to streptavidin beads and then incubated with uncapacitated porcine sperm in dmTALP and control conditions. Aliquots were removed various incubation times, stained with SYBR14 and propidium iodide to label live and dead sperm. Sperm bound to glycans had a longer lifespan compared to control conditions, n=3. (Machado et al., 2019, submitted).

FIGURE 1.7

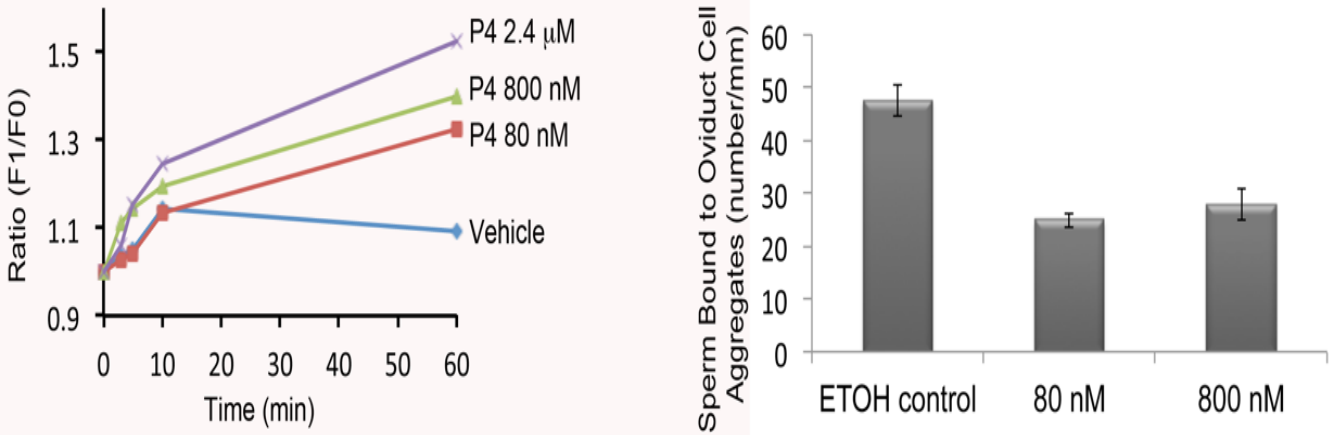

Figure 1.7. Progesterone increases intracellular free Ca^{2+} in sperm and release of sperm from oviduct cell aggregates.

Left, Fluo-4 loaded sperm were incubated with several concentrations of progesterone in dmTALP and control conditions at 39°C. Ca^{2+} was detected using spectrophotometer at different time points. **Right**, sperm, were allowed to bind oviduct cell aggregates for 15 min and progesterone was added (80 and 800nM) along with control steroids. Sperm bound to aggregates were counted after 30 min of incubation with steroids, n=3. (Machado et al., 2019, submitted).

FIGURE 1.8

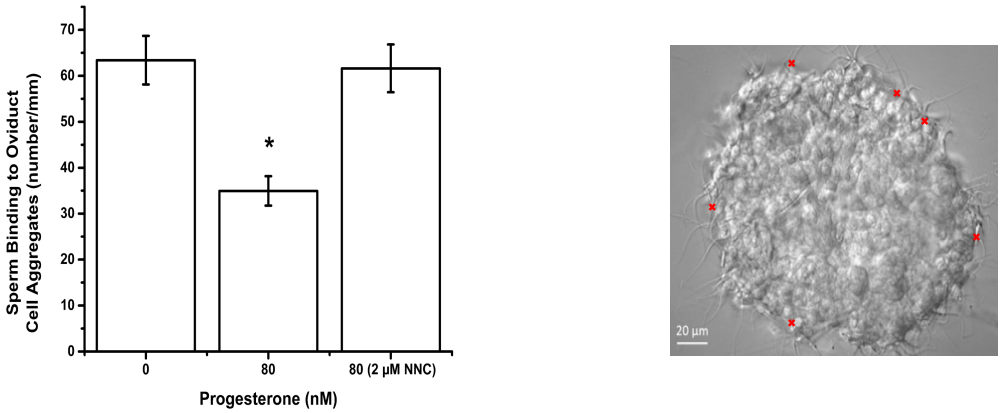

Figure 1.8. Inhibition of CatSper prevents sperm release from oviduct cells.

Left, Sperm were allowed to bind oviduct cell aggregates and then aggregates with bound sperm were either incubated with the CatSper T-type channel inhibitor NNC 55-0396 and progesterone (80 and 800nM). After 30 min, the number of sperm bound to the aggregates was counted. **Right**, A representative image of sperm (a few marked red) bound to oviduct cell aggregates, n=3. (Machado et al., 2019, submmited).

FIGURE 1.9

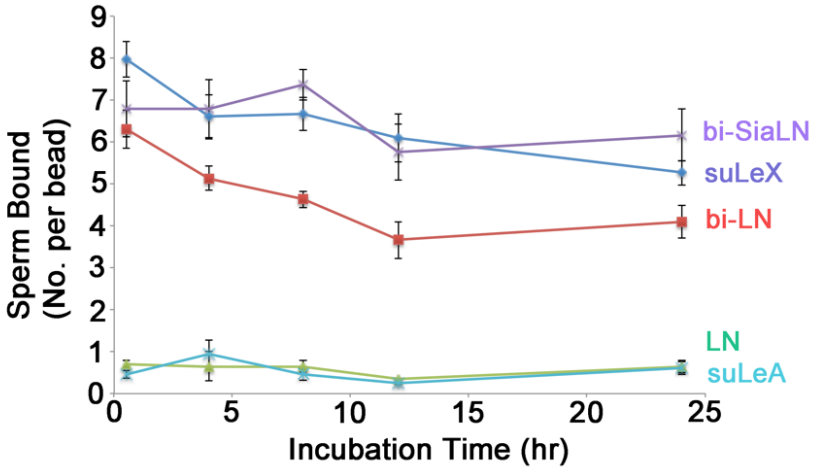

Figure 1.9. Sperm capacitation does not promote spontaneous sperm release from oviduct glycan-coated beads.

Sperm retention on oviduct glycan-coated beads was measured over 0, 4, 8, 12 and 24 hr. No significant spontaneous release was observed in sperm incubated with oviduct glycan-coated beads and controls, n=3. (Kadirvel Govindasamy, unpublished).

FIGURE 1.10

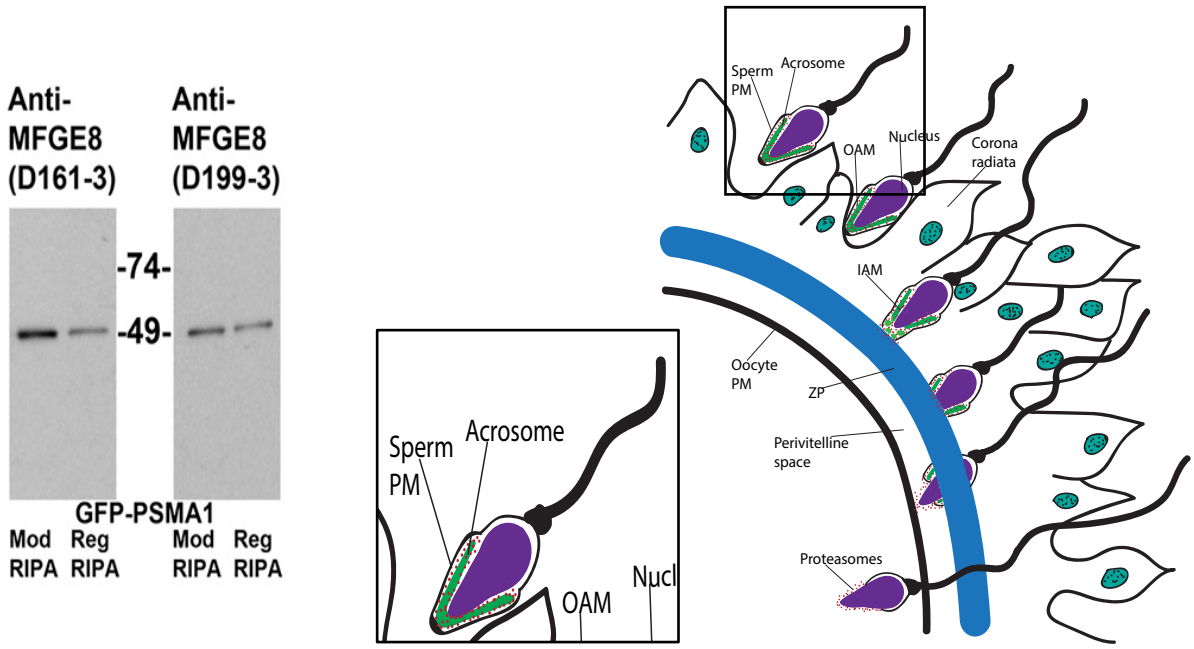

Figure 1.10. Protein degradation in sperm capacitation.

Left-Western blotting was used to confirm the co-immunoprecipitation of MFGE8 with sperm proteasomes from transgenic sperm extracts, using two different anti-MFGE8 antibodies, D161-3 and D199-3 (Miles et al., 2013). **Right-** In an intact acrosome (green), proteasomes (red dots, better visualized in the enlarged square) are bound to the inner (IAM) and outer (OAM) acrosomal membranes and dispersed throughout the acrosomal matrix. Upon sperm-zona binding, acrosomal membrane vesicles are coated with proteasomes. During sperm-zona penetration, a subpopulation of proteasomes remains associated with IAM.

FIGURE 1.11

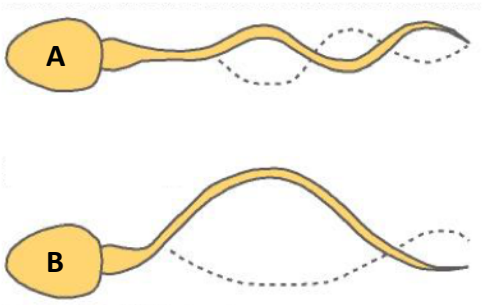

Figure 1.11. Types of sperm motility.

A- Active motility, sperm swim with small, but fast tail movements. Ineffective for fertilization.

B- Hyperactive motility, sperm tail movement is whip-like and at a higher amplitude. Effective for movement towards ampulla and fertilization.

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CHAPTER 2

AIM 1. EFFECTS OF THE OVIDUCT GLYCANS ON SPERM CAPACITATION

2.1. ABSTRACT

Before fertilization, sperm bind to the epithelial cells of the oviduct isthmus to form a reservoir that regulates sperm function, including viability and capacitation. The sperm reservoir may maintain reproductive efficiency in females in which semen deposition and ovulation may not be synchronized. We have demonstrated previously that porcine sperm bind specifically to either of two oviductal glycan motifs, biantennary 6-sialylated *N*-acetyllactosamine (bi-SiaLN) oligosaccharide and Lewis X trisaccharide (Le^X). These glycans increase sperm viability and regulate Ca^{2+} influx. Ca^{2+} Because the increase in intracellular Ca^{2+} was suppressed by oviduct glycans, we examined whether capacitation was altered by incubating soluble glycans with sperm during capacitation and assessed three different components of capacitation. First, the increase phosphorylation of a 32 kDa sperm protein, which is related to capacitation was assessed. Although tyrosine phosphorylation of this protein increased during capacitation, the phosphorylation increase was not affected by oviduct glycans. Second, the response of sperm after capacitation with or without oviduct glycans to addition of Ca^{2+} ionophore (A23187) was evaluated. We observed no suppression of the acrosome reaction in sperm bound to oviduct glycans. Because sperm hyperactivation is also associated with capacitation, we tested the effect of oviduct glycans on hyperactivation. No suppression of hyperactivation was detected. These results demonstrate that oviduct glycans containing Le^X and bi-SiaLN motifs can suppress the Ca^{2+} influx that occurs during

capacitation and extend porcine sperm lifespan but the glycans do not affect three components of capacitation.

2.2. INTRODUCTION

After mating in mammals (Baillie et al., 1997; Holt, 2011; Hunter et al., 1980; Smith and Yanagimachi, 2004; Suarez, 2008; Tienthai et al., 2004; Troedsson et al., 1998) birds, reptiles and amphibians (Almeida-Santos et al., 2004; Das et al., 2008; Dias et al., 2008), sperm are stored by females in a reservoir. A functional sperm reservoir in the mammalian lower oviduct, known as the isthmus, regulates sperm function and extends cell viability, traits necessary for high fertility, given that semen deposition and ovulation are not always well synchronized (Belstra et al., 2004; Holt and Fazeli, 2010; Wagner et al., 2002). Upon semen deposition into the female tract, a sperm subpopulation is transported to the isthmus, where the sperm attaches to epithelial cells until unidentified cues trigger their gradual release towards the ampulla, the fertilization site (Hunter et al., 1999; Suarez, 2008). Sperm binding to epithelial cells regulates cell function by suppressing sperm motility and prolonging sperm lifespan (Rodríguez-Martínez et al., 2005; Rodriguez-Martinez, 2007). Sperm binding to the oviduct is mediated by oviduct carbohydrates (Suarez, 2008; Green et al., 2001; Talevi and Gualtieri, 2010; Topfer-Petersen et al., 2008) and the particular adhesion molecules involved in the formation of the sperm reservoir may be species-specific (Suarez, 2008; Topfer-Petersen et al., 2008; Cortés et al., 2004; Sostaric et al., 2004). The binding of lectin-like receptors on the sperm head to isthmic cell glycans regulates the succession of changes necessary for fertilization,

collectively known as sperm capacitation (Dobrinski et al., 1997; Fazeli et al., 1999; Holt and Fazeli, 2010).

During capacitation, plasma membrane potential and ion transport are altered (Hernández-González et al., 2006; Lishko et al., 2012; López-González et al., 2014; Nishigaki et al., 2014), protein phosphorylation is modified (Morgan et al., 2008; Visconti and Kopf, 1998), and there is an efflux of plasma membrane cholesterol (Shadan et al., 2004; Boerke et al., 2008; Gadella, 2008). Intracellular free Ca^{2+} is central to sperm function in preparation for fertilization (Lishko et al., 2012; Darszon and Hernández-Cruz, 2014; Publicover et al., 2007; Breitbart, 2002). Ca^{2+} influx is necessary for sperm to hyperactivate and ascend beyond the oviduct isthmus to fertilize eggs; failure results in infertility (Ho et al., 2009; Qi et al., 2007). Along with HCO_3^-, Ca^{2+} can activate in sperm a soluble adenylyl cyclase (sAC) (Hess et al., 2005). The product of sAC, cyclic AMP, activates protein kinase A leading to phosphorylation of a series of proteins (Visconti, 2009).

I hypothesized that sperm capacitation may be controlled or regulated by sperm attachment to oviduct glycans. I proposed this hypothesis because the glycans can regulate sperm intracellular Ca^{2+} (Machado, et al, submitted) but the outcome of that regulation not completely clear. Although there is considerable evidence that glycans function in cell adhesion (Taylor and Drickamer, 2007), it is not known if glycan-mediated adhesion regulates cell function. A glycan array screening of hundreds of specific glycans indicated that all glycans that bound porcine sperm with high affinity contained one of two motifs, either a biantennary 6-sialylated *N*-acetyllactosamine (bi-SiaLN) structure or a Lewis X trisaccharide (Le^X) (Kadirvel et al., 2012; Machado et al., 2014). Because these sugars are at least partially involved in sperm

binding to porcine isthmic epithelial cells, oviduct glycoproteins may regulate sperm capacitation, motility, and acrosome reaction.

2.3. MATERIALS AND METHODS

Collection and Processing of Sperm

For each replicate, semen collected from 3 to 5 mature boars (Prairie State Semen Supply, Champaign IL or PIC, Hendersonville, TN) Semen was extended, cooled to 17 °C, transported to the laboratory, and processed within 24 h. The extended semen was pooled and 3 mL were washed through a Percoll cushion containing 4 mL of dmTALP (2.1 mM $CaCl_2$, 3.1 mM KCl, 1.5 mM $MgCl_2$, 100 mM NaCl, 0.29 mM KH_2PO_4, 0.36% lactic acid, 26 mM $NaHCO_3$, 0.6% BSA, 1 mM pyruvic acid, 20 mM HEPES pH 7.3, sterile filtered), 0.6 mL of 10X HBS (1.3 M NaCl, 40 mM KCL, 10 mM $CaCl_2$, 5 mM $MgCl_2$, 140 mM fructose, 5% BSA, sterile filtered), and 5.4 mL of Percoll for 10 min at 800 x g. The supernatant was discarded, and the resulting pellet was re-suspended in 14 mL of dmTALP and centrifuged for 3 min at 600 x g. The supernatant was discarded again, and the resulting pellet was re-suspended in 1 mL of dmTALP. Sperm concentration was estimated by hemocytometer and only samples with greater than 75-80% motile sperm were used for experiments.

Assessing capacitation by sperm protein tyrosine phosphorylation

Changes in protein tyrosine phosphorylation of sperm during capacitation were assessed using SDS-PAGE and immunoblotting. Sperm were incubated with soluble oviduct glycans aliquots were collected at 0, 2 and 4 h, and lysed under reducing conditions for SDS-

PAGE and immunoblotted with a monoclonal antibody (4G10, EMD-Millipore) to detect phosphotyrosine on proteins. Sperm after processing were adjusted to 5 x 10^6 cells for each treatment and incubated in NC-TALP (2.1 mM $CaCl_2$, 3.1 mM KCl, 1.5 mM $MgCl_2$, 100 mM NaCl, 0.29 mM KH_2PO_4, 0.36% lactic acid, 0.6% polyvinyl alcohol, 1 mM pyruvic acid, 35 mM HEPES, [pH 7.3], sterile filtered), (negative control for tyrosine phosphorylation), dmTALP (positive control for tyrosine phosphorylation), and dmTALP containing 40 µg/mL of either bi-SiaLN, $suLe^X$, Le^X, LN, $suLe^A$ or Le^A. The sperm were centrifuged at 13,000 x g for 5 min at 4 °C. The supernatant was discarded, and the pellet was re-suspended in ice-cold Nonidet-P40 Lysis Buffer (150 mM NaCl, 50 mM Tris, pH 8.0, and 1 % NP-40) containing 0.2 µM sodium orthovanadate, in addition to a protease inhibitor cocktail containing AEBSF, bestatin, E-64, pepstatin A, phosphoramidon, and leupeptin (Sigma-Aldrich Co., St. Louis, MO). After homogenization by repeated pipetting, the samples were boiled for 5 min and centrifuged at 13,000 x g for 5 min at 4 °C. The resulting supernatant was transferred to a fresh micro-centrifuge tube containing 5% β-mercaptoethanol (final concentration) and boiled 5 min. Aliquots containing 5 x 10^6 sperm were diluted in 5X loading buffer (4% SDS, 20% glycerol, 0.1% bromophenol blue, 0.125 mM Tris HCl, pH 6.8), and loaded into a 4-20% gradient gel (Thermo Fisher Scientific Inc., Waltham, MA). After electrophoresis, proteins were transferred to a nitrocellulose membrane. The membranes were blocked with 5% BSA. Phosphotyrosine antibody was used at 1:1000 dilution in TBST (Tris-buffered saline, 0.1% Tween 20) with 5% BSA. Membranes were washed in TBST and incubated with a polyclonal anti-mouse IgG conjugated to HRP (BD Pharmingen, San Jose, CA) diluted 1:2000. After washing, the membranes were incubated with a chemiluminescent peroxidase substrate (Thermo Fisher

Scientific Inc., Waltham, MA). Chemiluminescent signals were documented using an ImageQuant LAS 4000 (GE Healthcare Bio-Sciences, Pittsburgh, PA). Three biological replicates were done for each treatment and time point.

Evaluating Sperm Motility and Hyperactivation

Motility assessment of sperm bound to soluble glycans (bi-SiaLN, $suLe^X$, Le^X, LN or $suLe^A$) was done using the Hamilton Thorne Semen Analysis CASA system (IVOS, Version 12.3K, Build 003). Sperm were incubated with 40 µg of soluble glycans at 39 °C in normal dmTALP and NC-TALP. Motility of aliquots at 0, 2 and 4 h, was assessed. For each experimental condition, 5 random fields were evaluated for a minimum total of at least 100 cells in each replicate. Five biological replicates were done for each treatment and time point.

Assessing capacitation by induction of the acrosome reaction

Capacitation status in sperm incubated with soluble oviduct glycans (bi-SiaLN, $suLe^X$, Le^X, LN or $suLe^A$) was assessed by whether sperm can undergo the subsequent acrosome reaction induced by Ca^{2+} ionophore (A23187). 40 µg of soluble glycans were incubated with sperm at 39 °C in normal dmTALP and NC-TALP. Coomassie staining was done on aliquots at 0, 2 and 4 h to verify the acrosome status of the sperm. Sperm were fixed with 4% paraformaldehyde solution (110 mM Na2HPO4, 2.5 mM NaH2PO4, pH 7.4) for 10 min at 24°C. Sperm were centrifuged and washed twice using 1.5 ml of 100 mM ammonium acetate. The final sperm pellet was resuspended in 1 ml of 100 mM ammonium acetate, 50 µl of the sperm suspension was smeared on glass microscope slides. Sperm on the slides were incubated in Coomassie stain

(0.22% Coomassie Blue G-250, 50% methanol, 10% glacial acetic acid, 40% water) for 2 min. Slides were washed thoroughly using distilled water to remove excess stain. Slides were air-dried and coverslips were placed on slides. Stained sperm were examined under bright-field microscopy. Cells were classified as either acrosome intact or reacted. A minimum of 200 sperm were examined for each treatment in replicates. Three biological replicates were done for each treatment and time point.

Statistical Analysis

Differences among means were determined using a one-way analysis of variance run in SAS (SAS Institute, Inc, Cary, NC). The results are shown as means ± SEM and the means were considered to belong to distinct populations if $P < 0.05$ using Tukey's test for multiple comparisons.

2.4. RESULTS

Sperm protein tyrosine phosphorylation was used as an assessment of capacitation. Tyrosine phosphorylation of a 32 kDa protein in porcine sperm has been most closely linked with capacitation and addition of calcium in the incubating medium (Tardif et al., 2001, 2004). Phosphorylation of the 32 kDa protein increased as sperm were incubated for 4 hr in capacitating or non-capacitating medium. However, that increase was not affected by addition by either soluble suLe^X or bi-SiaLN or control glycans at 0, 2 (data not shown) or 4 hr. **(Figure 2.1).**

Intracellular free Ca^{2+} influences many sperm behaviors such as the development of hyperactivated motility, sperm protein tyrosine phosphorylation and the ability to respond to acrosome reaction inducers (Harrison and Gadella, 2005). But neither $suLe^X$ or bi-SiaLN affected sperm motility. CASA evaluation at 0 (data not shown), 2 (data not shown) or 4 hr **(Table 2.1)** showed no differences between sperm incubated with soluble $suLe^X$ or bi-SiaLN or control glycans that do not bind porcine sperm. Binding to glycans did not delay hyperactivation or affect any sperm motility kinematics.

The sperm acrosome reaction requires that sperm are first capacitated. I hypothesized that oviduct glycans would delay capacitation, resulting in a reduction in the ability of sperm to undergo an ionophore-induced acrosome reaction. There were significant differences ionophore-induced acrosome reaction between 0,2, 4 hr of capacitation compared to control incubation conditions, which acted as a positive control. However, even at 4 hr incubations in the presence of $suLe^X$ or bi-SiaLN, the ionophore-induced acrosome reaction in sperm was not affected when compared with control conditions. Sperm incubated with the soluble oviduct glycans showed no delay in acrosome reaction induced by either 5 μM or 10 μM of Ca^{2+} ionophore (A23187). Values were expressed as the percentage of the total sperm evaluated **(Figure. 2.2).**

2.5. DISCUSSION

Formation of a sperm reservoir *in vivo* requires sperm binding to oviductal cells of the lower oviduct or isthmus. Binding to oviductal cells lengthens sperm lifespan and suppresses Ca^{2+} influx (Machado et. al., 2019, submitted). Previously, the oviduct cell components that

mediated these functional outcomes on sperm were unknown. It was also not clear whether the sperm-oviduct adhesive molecules might have additional roles in regulating sperm function. The previous study (Machado et. al., 2019, submitted) showed that oviduct glycan motifs on the luminal epithelium of the isthmus, both bi-SiaLN and suLe^X, regulate sperm intracellular Ca^{2+} concentration and lengthen sperm lifespan. However, we found no evidence that binding to soluble oviduct glycans affects sperm capacitation or development of hyperactivated motility.

Binding to oviduct glycans may prevent premature capacitation. Once capacitated, unless the sperm is near an egg, its fate is death; fertility is maximized if capacitation is completed near the time a sperm encounters the egg (Rodríguez-Martínez et al., 2005; A.p. Harrison and Gadella, 2005; Harrison, 1996). But we did not observe any effect of oviduct glycans on protein tyrosine phosphorylation, the ability to undergo an induced acrosome reaction or the development of hyperactivated motility.

Previous studies have demonstrated that the addition of extracellular Ca^{2+} is required for increased tyrosine phosphorylation during capacitation; medium containing Ca2+ but lacking BSA and bicarbonate was still able to increase tyrosine phosphorylation in porcine sperm (Tardif et al., 2004). So, we anticipated that if the increase in intracellular Ca^{2+} were suppressed, capacitation would also be diminished. But none of the measures of capacitation we examined was affected by soluble oviduct glycans. One possible explanation is that the oviduct glycans must be immobilized to affect capacitation; however, a previous study showed that soluble glycans suppressed the increase in intracellular Ca^{2+} (Machado et. al., 2019, submitted). An alternative possibility is that the reason was no effect on capacitation is that

capacitation includes many different processes, some of which are not linked together, and those we examined were among those that were unchanged. Verifying the status of capacitation to this point has been difficult. The common capacitation assessments focus on one aspect of capacitation rather than the broader processes that encompasses capacitation. We hypothesized that capacitation would be delayed in sperm bound to soluble oviduct glycans but assessing the three indicators of capacitation, protein tyrosine phosphorylation, development of hyperactivated motility or induction of the acrosome reaction after incubation in capacitating conditions provided no evidence that capacitation was affected.

Direct membrane contact between spermatozoa and epithelial cells of the isthmus is necessary to regulate Ca^{2+} entry, capacitation, minimize oxidative damage and maintain sperm viability over extended periods (Dobrinski et al., 1997; Murray and Smith, 1997; Smith and Nothnick, 1997; Huang et al., 2013). The carbohydrate interactions studied in this work are at least partially responsible for sperm adhesion to the oviduct and indicate that bi-SiaLN and Le^X do not regulate measures of capacitation in their soluble form that was tested. It is possible that insoluble glycans may have a more noteworthy effect on sperm capacitation, as they are anchored to a bead akin to glycans anchored on oviduct epithelial cells. This possibility remains to be examined.

2.6. FIGURES AND TABLE

TABLE 2.1

Parameters	Treatments (4 hr of incubation)					
	C	**NC**	**suLe^X**	**bi-SiaLN**	**suLe^A**	**LN**
Motility %	41.2 ± 7.8	38.8 ± 7.4	41.8 ± 8.6	41.2 ± 8.7	47.6 ± 8.5	44 ± 8.6
Progressive cells %	21 ± 5.6	14.2 ± 3.2	16.4 ± 3.8	18.6 ± 4.9	22.8 ± 4.8	15.6 ± 3.5
Rapid cells %	29.6 ± 5.7	24.7 ± 4.5	26.6 ± 5.3	27.8 ± 6.5	33.2 ± 6.0	29.4 ± 6.6
(VAP) µm/sec	68.7 ± 3.3	57.0 ± 5.1	66.1 ± 6.3	69.0 ± 5.3	68.3 ± 1.8	65.2 ± 4.8
(VSL) µm/sec	40.6 ± 3.1	31.3 ± 2.9	36.5 ± 2.6	39.7 ± 3.5	41.4 ± 1.4	37.3 ± 3.9
(VCL) µm/sec	150.2 ± 6.7	128.8 ± 9.4	151.8 ± 3.2	155.1 ± 9.6	150.4 ± 3.2	145.8 ± 8.3
(ALH) µm	7.9 ± 0.18	7.5 ± 0.4	8.4 ± 0.3	8.5 ± 0.2	8 ± 0.27	7.6 ± 0.2
(BCF) Hz	37.6 ± 0.9	37.0 ± 1.7	38.8 ± 0.6	36.0 ± 0.8	37.1 ± 0.4	37.6 ± 0.8
Straightness %	56.6 ± 3.5	54.4 ± 1.9	53.8 ± 2.5	54.8 ± 2.3	57.8 ± 2.7	54.2 ± 2.8
Linearity %	27.8 ± 1.4	25 ± 0.5	24.8 ± 1.1	26 ± 1.6	31 ± 3.1	29.4 ± 3.1
Static cells %	50 ± 8.1	54.6 ± 8.3	46.4 ± 10.	49.6 ± 9.1	43.4 ± 8.2	47.4 ± 8.7

*Path"Velocity"(VAP)"µm/sec,"Progressive"Velocity"(VSL)"µm/sec,"Track"Speed"(VCL)"µm/sec, "Lateral"Amplitude"(ALH)"µm,"Beat"Frequency"(BCF)"Hz."

Table 2.1. Sperm motility parameters after 4 hr incubation with suLe^X, bi-SiaLN, and control conditions.

Motility parameters of sperm incubated with soluble oviduct glycans. Sperm incubated in capacitating dmTALP (C), NC-TALP (NC), C with suLe^X, C with bi-SiaLN, C with suLe^A and C with LN. Results are means and SEM from 3-5 experiments.

FIGURE 2.1

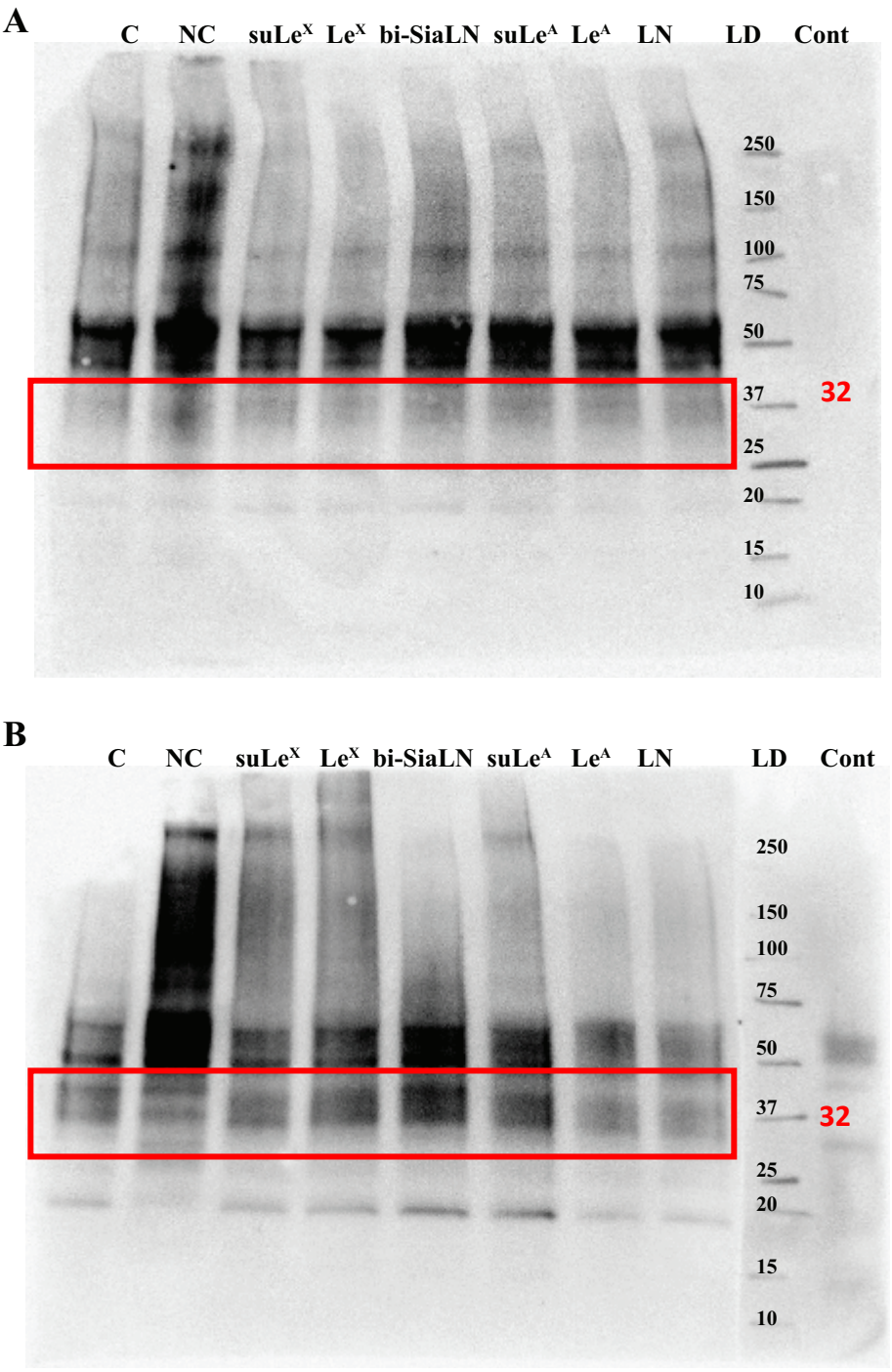
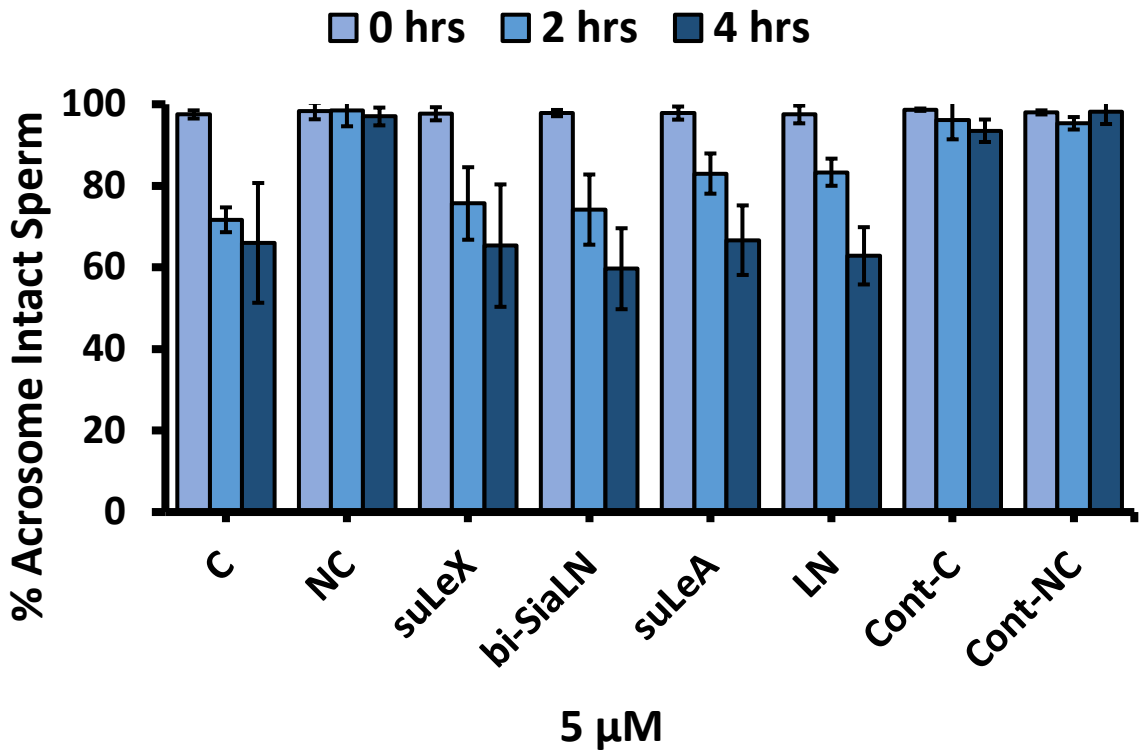

A
C
NC
suLe^X
Le^X
bi-SiaLN
suLe^A
Le^A
LN
LD
Cont
250
150
100
75
50
37
25
20
15
10
32
B
C
NC
suLe^X
Le^X
bi-SiaLN
suLe^A
Le^A
LN
LD
Cont
250
150
100
75
50
37
25
20
15
10
32

Figure 2.1. Sperm protein tyrosine phosphorylation increased during capacitation but was not affected by oviduct glycans.

Immunoblotting with a monoclonal phosphotyrosine antibody (4G10) to detect specific phosphotyrosine-containing proteins showed no differences in the sperm incubated with soluble oviduct glycans compared to controls at *A)* 0 hr or *B)* 4 hr of incubation. Capacitating dmTALP (C), non-capacitating dmTALP (NC), C with $suLe^X$ ($suLe^X$), C with bi-SiaLN (bi-SiaLN), C with $suLe^A$ ($suLe^A$), C with lactosamine disaccharide (LN), Ladder (LD) and negative control (Cont), n=3. The box shows the region containing the 32 kDa phosphoprotein, the phosphorylation of which is related to capacitation.

FIGURE 2.2


0 hrs
2 hrs
4 hrs
% Acrosome Intact Sperm
100
80
60
40
20
0
C
NC
suLeX
bi-SiaLN
suLeA
LN
Cont-C
Cont-NC
5 µM

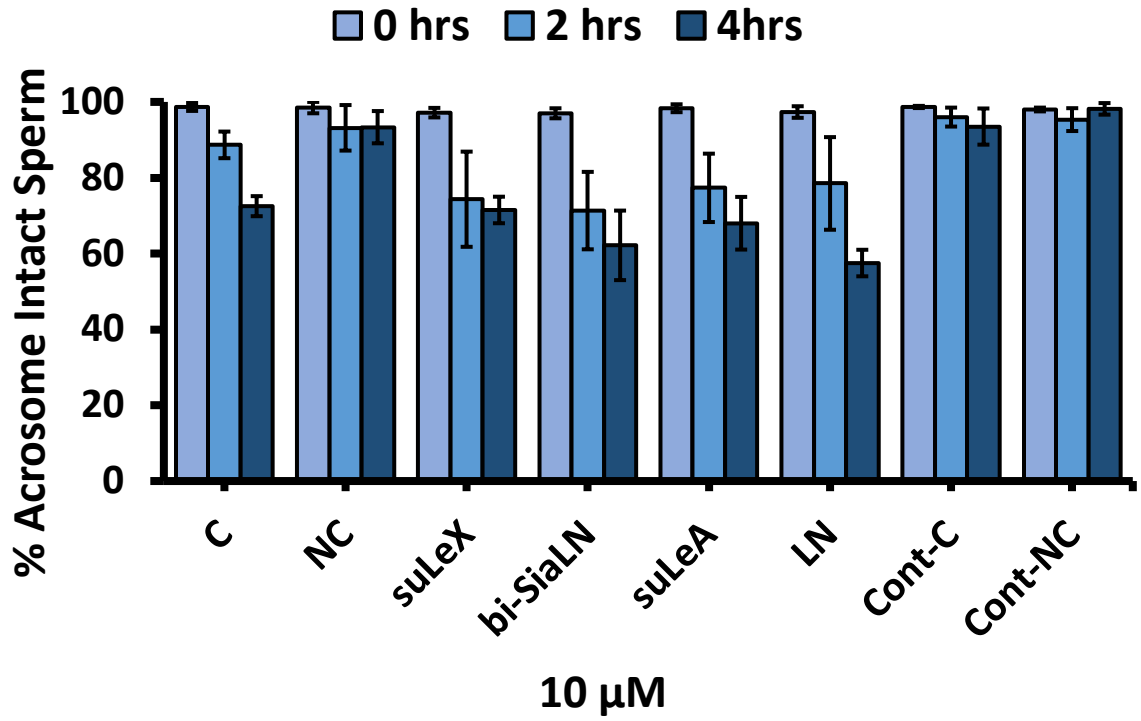

0 hrs
2 hrs
4hrs
% Acrosome Intact Sperm
100
80
60
40
20
0
C
NC
suLeX
bi-SiaLN
suLeA
LN
Cont-C
Cont-NC
10 µM

Figure 2.2. Soluble oviduct glycans do not affect capacitation, as assessed by the ionophore-induced acrosome reaction.

The graph shows the percentage of A23187-induced acrosome-reacted sperm after pre-incubation with soluble oviduct glycans suLe^X and bi-SiaLN for 0, 2 and 4 hr and addition of A23187. **A,** 5 μM concentration of A23187. **B,** 10 μM concentration of A23187. The acrosome reaction induced by either concentration of ionophore was not affected by oviduct glycans. Sperm incubated in dmTALP (C), NC-TALP (NC), C with suLe^X, C with bi-SiaLN, C with suLe^A, C with LN, all with A23187, C with A23187 (Cont-C) and NC without A23187 (Cont-NC). These results are means and SEM from 3-5 experiments.

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CHAPTER 3

AIM 2. PROGESTERONE INDUCES SPERM RELEASE FROM THE OVIDUCT GLYCANS IN A PROTEASOME-DEPENDENT MANNER.

3.1. ABSTRACT

In mammals, the oviduct retains sperm forming a reservoir from which the fertilizing sperm is released. However, the mechanisms underlying sperm release are unclear. Herein, I tested if steroids can release sperm in the oviduct reservoir and if the ubiquitin-proteasomal system is necessary for sperm release. Sperm were allowed to bind to oviduct cells or immobilized oviduct glycans, either 6-sialylated branched glycan (bi-SiaLN) or a 3-O-sulfated Lewis X trisaccharide (suLe^X) and then challenged with steroids in the presence or absence of proteasome inhibitors. Progesterone induced sperm release from oviduct cells and immobilized glycans in a steroid-specific manner; pregnenolone and 17α-OH-progesterone did not affect sperm release. Ca^{2+} influx into sperm through CatSper ion channels is associated with capacitation and development of hyperactivated motility. NNC 055-0396, a CatSper blocker, inhibited the progesterone-induced sperm release from oviduct cells or immobilized glycans. One mechanism for sperm release is that glycan receptors on sperm are inactivated by proteasomal degradation. Inhibition of proteasomal degradation blocked sperm release from oviduct cell aggregates or immobilized oviduct glycans. In summary, progesterone induced sperm release that required both active CatSper channels and proteasomal degradation, suggesting that hyperactivation and proteolysis are a vital part of the mechanism by which sperm move from the oviduct reservoir to the site of fertilization.

3.2. INTRODUCTION

The fertilizing ability of sperm is sustained by their interaction with lower oviduct epithelial cells in the isthmus before sperm move to the isthmus to fertilize oocytes (Suarez, 2016; Pollard et al., 1991; Kadirvel et al., 2012). It has been observed in mammalians that fertility and motility are prolonged by sperm retention in the isthmus (Hunter, 1973; Suarez, 2008; Pollard et al., 1991). By retaining sperm and releasing a limited number for fertilization, the isthmus also helps prevent polyspermy (Hunter, 1973; Suarez, 2008; Pollard et al., 1991).

Little is known about how sperm release from the oviduct so that they can progress towards the ampulla to reach oocytes. It is not clear whether the release is activated by changes in the oviduct fluid, oviduct cells, or sperm. One hypothesis is that sperm detachment is due to a change in oviduct fluid components or volume (Ardon et al., 2016). The release signal may also be secreted from the cumulus-oocyte complex to act on sperm or the oviduct (Strünker et al., 2011). Finally, the release may be pre-programmed in sperm and activated as part of capacitation. In support of the role of capacitation, following capacitation, fewer sperm bind to oviductal cells or oviduct glycans (Petrunkina et al., 2001; Kadirvel et al., 2012; Machado et al., 2014). This indicates that glycan receptors become less functional after capacitation, perhaps due to membrane modifications and/or receptor degradation concurrent with capacitation.

Another outcome of capacitation is that sperm become hyperactivated. Sperm hyperactivation increases the force required to tether sperm (Curtis et al., 2012) and may thereby add tension to the adhesion holding sperm to oviduct cells. Progesterone (P4) has been implicated in sperm hyperactivation, particularly human sperm (Niederberger, 2017).

Progesterone stimulates Ca^{2+} entry in human sperm in a fast and non-genomic fashion (Blackmore et al., 1991; Falkenstein et al., 1999). Over the last decade, it has been demonstrated that progesterone stimulates Ca^{2+} influx by binding to a receptor that depletes an endocannabinoid, releasing inhibition of sperm Ca^{2+} channels known as CatSper channels (Lishko et al., 2011; Strünker et al., 2011; Miller et al., 2016; Lishko et al., 2012; Chang and Suarez, 2011). CatSper channels are localized in the principal piece of the sperm flagellum and are essential for development of human and mouse sperm hyperactivation and male fertility (Lishko et al., 2011; Strünker et al., 2011; Alasmari et al., 2013; Lishko et al., 2012; Barratt, 2011; Lishko and Mannowetz, 2018; Chung et al., 2014; Smith et al., 2013; Carlson et al., 2009; Ren et al., 2001). Although there is evidence that CatSper channel subunit genes are expressed in the porcine testis and localized in porcine sperm (Vicente-Carrillo et al., 2017; Song et al., 2011) much less is known of progesterone and CatSper function in species other humans and mice.

Another hypothesis for sperm release from the oviduct reservoir is that putative glycan receptors on sperm are degraded via the ubiquitin-proteasome system (UPS) (Miles et al., 2013), allowing sperm detachment. Unidentified glycan receptors bind to oviduct glycans in the isthmus retaining sperm in the sperm reservoir (Topfer-Petersen et al., 2008; Kadirvel et al., 2012; Machado et al., 2014). Spermatozoa from many animals contain proteasomes and the UPS has been implicated in many cellular processes including fertilization (Pizarro et al., 2004; Morales et al., 2003; Miles et al., 2013; Sutovsky, 2011, 2003; Yokota and Sawada, 2007). The UPS is responsible for proteolysis of >75% of intracellular proteins but may also have a role in extracellular protein degradation in sperm (Zigo et al., 2018; Kerns et al., 2016). Previous

studies have demonstrated that a subunit of the 20S proteasomal core co-precipitates with several proteins that have been implicated as candidate receptors for oviduct glycans (Miles et al., 2013). It is possible that these candidate receptors may be degraded by the UPS to promote sperm release from oviduct glycans.

The hypotheses of this study were that progesterone induces sperm release by activating CatSper and that UPS protein degradation was necessary for sperm release from the oviduct reservoir **(Figure 3.1).**

3.3. MATERIALS AND METHODS

Collection and processing of sperm

For each replicate, semen collected from 3 to 5 mature boars (Prairie State Semen Supply, Champaign IL or PIC, Hendersonville, TN). Semen was extended, cooled to 17 °C, transported to the laboratory, and processed within 24 hr. The extended semen was pooled and 3 mL were washed through a Percoll cushion containing 4 mL of dmTALP (2.1 mM $CaCl_2$, 3.1 mM KCl, 1.5 mM $MgCl_2$, 100 mM NaCl, 0.29 mM KH_2PO_4, 0.36% lactic acid, 26 mM $NaHCO_3$, 0.6% BSA, 1 mM pyruvic acid, 20 mM HEPES pH 7.3, sterile filtered), 0.6 mL of 10X HBS (1.3 M NaCl, 40 mM KCL, 10 mM $CaCl_2$, 5 mM $MgCl_2$, 140 mM fructose, 5% BSA, sterile filtered), and 5.4 mL of Percoll for 10 min at 800 x g. The supernatant was discarded, and the resulting pellet was re-suspended in 14 mL of dmTALP and centrifuged for 3 min at 600 x g. The supernatant was discarded again, and the resulting pellet was re-suspended in 1 mL of dmTALP.

Sperm concentration was estimated by hemocytometer and only samples with greater than 75% motile sperm were used for experiments.

Sperm binding to glycan coupled to beads

Glycan-coated streptavidin-Sepharose High-Performance beads (GE Healthcare Bio-Sciences, Pittsburgh, PA) with an average diameter of 34 µm were used to test the ability of porcine sperm binding to glycans (bi-SiaLN and Le^X). To link glycans to beads, approximately 60 µg of glycans (Bovin et al., 1993), covalently attached to a biotinylated polyacrylamide core, were incubated with 20 µL of streptavidin-Sepharose beads for 90 min at room temperature. Each 30-kDa molecule of polyacrylamide had 20% glycan and 5% biotin, by molarity.

Beads incubated with glycans were washed twice in dmTALP and re-suspended in 100 µl of dmTALP. Once the glycan-coupled beads were ready for use, a 50 µL-droplet containing 1.5 x 10^6 sperm/mL was prepared to receive 1 µL of glycan-coated beads. Sperm and beads were co-incubated at 45 min at 39°C, 0% CO_2. After that, the appropriate amount of progesterone (P4; 80 nM or 800 nM), pregnenolone (P5; 80 nM or 800 nM), or 17∝ hydroxy-progesterone (17∝OHP: 80 nM or 800 nM) was added. After incubation for 30 min at 39 °C for each treatment, 25 beads were randomly selected and the total number of bound sperm was enumerated in three replicates. Sperm that were self-agglutinated were not included in the counts. At least three biological replicates were done for each treatment. The experiments were documented using AxioVision or ZEN software (Zeiss, Thornwood, NY).

Sperm Release from Glycan Coupled Beads and inhibition of CatSper

To test the function of CatSper channels during the progesterone-mediated sperm release, sperm were allowed to bind to glycan coated bead as stated earlier. 2 µM of T channel blocker (NNC 055-0396) was added to the sperm bound to glycan coated beads 15 min prior to progesterone addition. For each treatment, 25 beads were randomly selected and the total number of bound sperm was enumerated in three replicates. At least three biological replicates were done for each treatment. The experiment was documented using a Zeiss Axioskop (Zeiss, Thornwood, NY).

Acrosome reaction induction in non-capacitated sperm by progesterone

To determine if the acrosome reaction is induced by progesterone in the non-capacitated sperm, sperm were incubated in dmTALP with progesterone and control steroids at 39°C with for 30 min. Coomassie Blue staining (Larson and Miller, 1999) was done to verify the acrosome status of the sperm. At least 200 sperm were analyzed in 3 replicates.

Collection of Oviduct Epithelial Cells

Oviducts were provided by Rantoul Foods. For each experiment, the isthmus of 15-20 oviducts were collected from pre- and post-pubertal females and transported in PBS in a sterile 50 mL conical tube on ice. After 2 hr on ice, the oviducts were processed at the lab. The isthmus was trimmed, and the edge of a microscope slide was used to apply pressure to the outside of the oviduct to strip sheets of oviduct epithelial cells from the isthmus. Epithelial sheets in PBS were transferred to a 15 ml conical tube and centrifuged at 100 x g for 1 min. After removing

the supernatant, the cells were disaggregated by passage through a 1 ml pipette tip 10 times. After bringing the volume to 15 mL with PBS, the suspension was centrifuged again. The partially disaggregated cells in the pellet were passed through a 22-gauge needle ten times. After adjusting the volume to 12 mL with dmTALP, the cells were divided evenly into three 100-mm tissue culture dishes. Cells were allowed to re-aggregate for 90 min at 39°C. Spherical aggregates that were 100-150 µm in diameter were selected for experiments.

Sperm Binding to and Release from Oviduct Epithelial Cells

Spherical oviduct cell aggregates were selected and washed twice in 100 µL drops of fresh dmTALP. A Stripper Pipette (MidAtlantic Diagnostics, Inc., Mount Laurel, NJ) with a 250 µm internal diameter tip was used to collect oviduct epithelial cell aggregates and wash them. Sperm at a final concentration of 5 x 10^6 cells/mL were added to 50 µL droplets (total volume) containing oviduct cell aggregates. Sperm and oviduct cell aggregates were pre-incubated at 39°C for 15 min to allow sperm binding. Proteasome inhibitor cocktail, 10 µM was added at 39°C for 15 min. Then, 80 nM of progesterone and 17α-OH-progesterone for 30 min at 39°C or vehicle control. After incubation, free and loosely attached sperm were removed by washing with 30 µl of dmTALP. Aggregates were transferred onto a microscope slide in a volume of 3 µL. Each droplet with 10 aggregate-sperm complexes was considered an experimental unit for statistical analysis. Images were captured using a Zeiss Axioskop and AxioCam digital camera (Carl Zeiss, Thornwood, NY). The number of sperm bound to the periphery of each aggregate was enumerated and the circumference of the aggregate calculated using Zen (Carl Zeiss, Thornwood, NY). The number of sperm bound per mm circumference was calculated for each

aggregate. The average number of sperm bound to the aggregates counted in each droplet was used for statistical analysis.

Effects of NNC 55–0396 and Methoxy Arachidonyl Fluorophosphonate on Sperm Motility

The motility of sperm incubated with 80 nM progesterone, progesterone and CatSper inhibitor NNC 55-0396 (2μM), 2μM NNC 55 0396 alone, progesterone and the progesterone non-genomic receptor blocker, methoxy arachidonyl fluorophosphonate (2 μM), and methoxy arachidonyl fluorophosphonate (2 μM) alone was assessed using a Hamilton Thorne Semen Analysis CASA system (IVOS, Version 12.3K, Build 003), (Hamilton Thorne, Beverly, MA, USA). Sperm were incubated with the treatments at 39°C in dmTALP for 30 min. For each experimental condition, 5 random fields were evaluated for a minimum total of 100 cells (in each field) in 5 replicates.

Necessity of the UPS in release of sperm bound to oviduct glycans coated beads

To test for the function of protein degradation by proteasomes in the release of sperm from oviduct sperm reservoir, sperm were allowed to bind to beads coated with suLe^{X}, bi-SiaLN and related control glycans as described above. Three different treatment sets were tested: (i) without proteasomal inhibitors; (ii) with proteasomal inhibitors (purchased from Enzo Life Sciences), (100 μM MG132 in dmTALP (475.63 g/mol in 0.5% DMSO), (100 μM MG132 and clasto-Lactacystin beta-Lactone (CLBL) in dmTALP (475.63 g/mol and 213.2 g/mol in 2.5% DMSO), (10 μM MG132, CLBL and Epoxomicin in (475.63, 21.3.2 and 554.7 g/mol in 0.25% DMSO), aliquoted and stored at -80°C for working concentration of 5 μl for 100 μM and 0.5 μl

for 10 μM inhibitor combinations per 50 μl treatment droplet ; and (iii) vehicle control, 0.5% DMSO. Three different combinations of proteasome inhibitors, 1. 100 μM MG132, 2. 100 μM MG132 and CLBL and 3. 10 μM MG132, CLBL, and EPOX were added to the bead-bound sperm. After 15 min of incubation with the proteasome inhibitors, 80 or 800 nM of steroids (progesterone, 17-OHP or vehicle control) were added to sperm bound to beads. Thirty min later, the sperm bound to glycan-coated beads were counted.

Necessity of the UPS in release of sperm bound to oviduct aggregates

Sperm were allowed to bind to oviduct cell aggregates as described above. A combination of 3 proteasome inhibitors (10 μM each of MG132, CLBL and Epoxomicin in dmTALP (475.63, 21.3.2 and 554.7 g/mol in 0.25% DMSO) was added to the aggregate-bound sperm. After 15 min of incubation with the proteasome inhibitors, 80 nM of progesterone, 17-OHP or vehicle control was be added to the aggregates. Thirty min later, the number of sperm bound per mm circumference was calculated for each aggregate

Statistical Analysis

Differences among means were determined using a one-way analysis of variance run in SAS (v. 9.1 SAS Institute, Inc, Cary, NC). The results are shown as means ± SEM and the means were considered to belong to distinct populations if $P < 0.05$ using Tukey's test for multiple comparisons.

3.4. RESULTS

Progesterone activated the release of sperm from oviduct glycans

I used an in vitro assay to test the ability of progesterone and related steroid hormones to release porcine sperm from oviduct glycans. Sperm were allowed to bind beads that had suLe^X attached to them and release was assessed. Following progesterone addition, about 65% fewer sperm were bound to immobilized suLe^X compared to 17-αOHP (80 and 800 nM) **(Figure 3.2)**. P5 at only the higher concentration (800 nM) promoted sperm release; fewer sperm remained bound to immobilized suLe^X than after addition of 17-αOHP. These data indicated that progesterone was the most effective of the steroids tested to release sperm.

One potential mechanism by which progesterone could cause sperm release is by inducing the acrosome reaction, which happens when sperm are capacitated prior to progesterone exposure (Roldan et al., 1994). The acrosome reaction would result in a loss of the plasma membrane which contains glycan receptors. In the current experiments, sperm that were incubated with immobilized glycans were not capacitated. Nevertheless, to be certain that the acrosome reaction was not the reason for sperm release, sperm were incubated under conditions that allowed them to bind immobilized beads and then progesterone was added. Progesterone did not increase the frequency of acrosome reaction compared to controls **(Figure 3.3).** Hence the release of sperm from oviduct glycans in the presence of progesterone is not due to induction of the acrosome reaction in sperm.

CatSper is required for progesterone induced release of sperm bound to the oviduct glycans

The T-type channel blocker (NCC 55-0396) was used to inhibit CatSper and test the function of this cation channel in progesterone induced sperm release from beads coated with either suLe^X or bi-SiaLN. About 50% of sperm bound to either immobilized suLe^X or bi-SiaLN was released by 80 nM progesterone **(Figure 3.4)**. When sperm were incubated with NNC 055-0396 (2 μM), nearly all sperm release was abrogated. Addition of progesterone also induced the release of about 50% of sperm from immobilized fibronectin, demonstrating that progesterone induced release was not limited to sperm adhering to glycans and CatSper was required for progesterone induced sperm release from suLe^X, bi-SiaLN and fibronectin.

Effects of NNC 55–0396 and Methoxy Arachidonyl Fluorophosphonate (MAFP) on Sperm Motility

Inhibitory compounds like NNC 55-0396 that reduce sperm release from immobilized glycans could do so by a possible toxic effect on sperm (Vicente-Carrillo et al., 2017; Carrillo, 2016), reducing the number of sperm that are motile, since it is likely that motility is important for sperm release (Talevi and Gualtieri, 2010; Strünker et al., 2011). To test this, the motility of free sperm exposed to progesterone, NNC, and MAFP, an inhibitor of the non-genomic progesterone receptor, was assessed using CASA. There were no differences in any of the sperm motility characteristics during the 30 min required for sperm release. **(Table 3.1)** Therefore, sperm release was not blocked due to an effect of NNC and MAFP on sperm motility. This demonstrates that porcine sperm release from isthmic epithelial cells was promoted by progesterone using a mechanism that requires functional CatSper channels and that inhibitors

did not have toxic effects on sperm, reducing sperm motility. We also did not detect changes in hyperactivation, as determined by CASA, in response to progesterone. But it is challenging for CASA to discern traits in porcine sperm that are associated with hyperactivation in other species (i.e. changes in BCF, ALH, straightness, and linearity) because of the very asymmetrical full-type hyperactivated motility that porcine sperm display (Kojima et al., 2015; Arai et al., 2019).

Protein degradation by proteasomes is necessary for the release of sperm bound to oviduct glycans and oviduct cell aggregates

To determine if proteasome activity was necessary for sperm release from oviduct glycans induced by progesterone, three different types of cell permeable proteasome inhibitors were tested. First, MG132 which is reversible and inactivates 20S and 26S catalytic sites of proteasomes. Second, clasto-Lactacystin beta-Lactone (CLBL) which is irreversible and inactivates all catalytic binding sites of proteasomes. Third, epoxomicin which is also irreversible and modifies four subunits of 20S proteasomes.

Three proteasome inhibitors preparations were tested, 100 µM MG132, 100 µM CLBL and 100 µM MG132 and finally the combination of 10 µM MG132, 10 µM CLBL and 10 µM epoxomicin. Sperm release was promoted by 80 nM and 800 nM progesterone. Both progesterone concentrations stimulated the release of a similar percentage of sperm **(Figure 3.5).** MG132 inhibited the release of 71 % of sperm. The combination of MG132 and CLBL inhibited release of 89 % of sperm. A lower concentration 10 µ M of all 3 inhibitors was the most potent and inhibited the release of 98 % of sperm. To be certain that proteasomal activity

was also required for release of sperm from oviduct cells, the cocktail of 3 proteasomal inhibitors was tested for its ability to block progesterone -induced sperm release from oviduct cell aggregates. The inhibitor cocktail blocked release of just under 50% of sperm from oviduct cell aggregates **(Figure 3.6)**. Although it was less effective at inhibiting sperm release from cells than glycobeads, proteasomal activity was necessary for the maximum release of sperm from oviduct cells. The overall conclusion was that protein degradation by proteasomes plays a role in sperm release induced by progesterone.

3.5. DISCUSSION

This study investigated the importance of progesterone, CatSper and the UPS in the release of porcine sperm from the oviduct sperm reservoir. I have demonstrated that progesterone stimulates the detachment of porcine sperm from immobilized oviduct glycans and oviduct cell aggregates *in vitro.* Blocking CatSper channels inhibited this progesterone induced sperm release which shows that the release most likely occurs due to an influx of Ca^{2+} through CatSper channels. These findings indicate that when intracellular free Ca^{2+}, a central regulator of sperm function, is increased by the addition of progesterone, it promotes the sperm release from the oviduct cells or glycans.

Previous studies suggest that progesterone was involved in sperm detachment from the isthmus *in vivo,* although the target of progesterone was unclear, either sperm or oviduct cells (Hunter, 1973; Bureau et al., 2002; Jin et al., 2007). There is also evidence that progesterone promotes the release of avian sperm from the storage tubules in the uterovaginal junction (Ito et al., 2011) and from bull sperm from oviduct epithelial cells (Romero-Aguirregomezcorta et

al., 2019). Results herein are the first support for the hypothesis that progesterone acts directly on porcine sperm to release them from oviduct epithelial cells. The progesterone biosynthetic precursor pregnenolone caused some release at 800 nM concentration whereas progesterone's downstream derivative 17α-OH-progesterone, did not cause sperm release. The effect progesterone had on sperm release was as expected. I did not expect pregnenolone (800 nM) to stimulate release but the results with pregnenolone were in accordance with the recent study which showed that pregnenolone sulfate, a 3β-sulfate of P5, activates the sperm Ca^{2+} channel (CatSper) similar to progesterone and possibility binds to the same sperm receptor as progesterone (Brenker et al., 2018; Mannowetz et al., 2017). Previous studies also demonstrated that estradiol had no effect on sperm binding to oviductal vesicles *in vitro* (Bureau et al., 2002). Our results are consistent with the findings of an *in vivo* study that reported high rates of polyspermic fertilization, presumably by causing a massive release of oviduct-bound sperm, when the oviduct hormonal milieu was modified by injecting progesterone in the isthmic subserosa of gilts in the pre-ovulatory period (Hunter, 1973). Although progesterone concentrations are very high during the pre-ovulatory period, especially near the oviduct (Eiler and Nalbandov, 1977), how this steroid reaches sperm is unclear. But if produced in adequate amounts, progesterone could modify the oviductal environment and elicit changes in sperm behavior, including release from the isthmic epithelium (Coy et al., 2012).

Although there are many reports of the effect of progesterone on human sperm (Seshagiri, 2001; Calogero et al., 2000; Meizel and Turner, 1991), reports of the effects of progesterone on porcine sperm are very limited. The mechanism by which progesterone

increases intracellular Ca^{2+} through CatSper is best described in human sperm (Lishko et al., 2011). Moreover, increases in intracellular Ca^{2+} concentrations are commonly associated with a variety of changes that occur during capacitation (Darszon and Hernández-Cruz, 2014; Tateno et al., 2013; Tulsiani and Abou-Haila, 2004). A report using human sperm found that low progesterone concentrations (10 nM to 100 nM) affected sperm motility and protein tyrosine phosphorylation and higher concentrations (1-10 µM) activated MAP kinase signaling and phosphorylation of P90RSK (Sagare-Patil et al., 2012).

The observation that progesterone induced release of sperm suggests that progesterone alone is sufficient for release of a significant number of sperm. It is unclear if progesterone-induced hyperactivation is the reason that progesterone induces sperm release or if there is some other mechanism. My results also suggest that oviduct fluid flow or oviduct peristaltic contractions are not necessary for sperm release because release occurred in isolated epithelial cells or immobilized bead-bound glycans in droplets that have minimal fluid flow.

It is highly unlikely that NNC 55-0396, a T-type calcium channel inhibitor, prevented sperm release by acting on oviduct cells rather than sperm. The NNC compound blocked the release of sperm from beads (no oviduct cells present) as effectively as the release from oviduct cells. My data support the model that release of porcine sperm from oviduct isthmic cells is activated by progesterone and requires CatSper channels, which are only present in sperm. Increasing progesterone concentrations in the sperm reservoir might be one of the signals that accompanies ovulation and facilitates the release of sperm from the oviduct epithelium so that

they can be free to fertilize oocytes. These are the first data showing that progesterone is sufficient to release porcine sperm from oviduct epithelial cells.

The second part of this study demonstrates the importance of the UPS in progesterone-induced sperm release from the oviduct reservoir. Inhibition of proteasomes hindered the partial release of sperm bound to glycan coated beads and oviduct cell aggregates **(Figure 3.5, 3.6).** Greater inhibition of proteasomal activity using a cocktail of three inhibitors was more effective at blocking sperm release. Previous results showed that there is a wide variety of proteins tagged with ubiquitin for degradation, as determined by precipitation with a proteasomal subunit (Miles et al., 2013). Moreover, it has also been shown that proteasomal inhibitors protect sperm acrosomal surface-associated proteins from degradation including one of the glycan receptor protein (MFGE8) (Zimmerman et al., 2011). Proteasomal inhibitors may block release by preventing degradation of candidate glycan receptors on sperm. This implies that receptor proteolysis contributes to sperm release from the reservoir in the isthmus.

An alternative role for the UPS is a more indirect one. The activity of the ubiquitin-activating enzyme E1, which is the principal enzyme for the initiation of protein ubiquitination, is required for correct remodeling of acrosomal membranes and sperm capacitation (Sutovsky et al., 2019; Zigo et al., 2018; Kerns et al., 2016; Yi et al., 2012). As glycan receptors are expected to be components of the sperm plasma membrane, it is reasonable that their function may be modulated as an outcome of membrane remodeling. The exact nature of the remodeling that could affect membrane receptor function is unclear.

In summary, proteasome inhibitors play a key role in sperm release from the oviduct reservoir and partially inhibit the progesterone induced sperm release. The proteins that are

the target of proteasomes and the steps in fertilization in which they are ubiquitinated are the questions that need to be resolved to completely understand how sperm proteasomes are enabling sperm to release from oviduct glycans in the reservoir.

3.6. FIGURES AND TABLE

TABLE 3.1

Treatments	P4	P4-NNC	NNC	P4-MAFP	MAFP
Progressive %	35±2.2	31.1±0.7	28.9±0.7	36.4±4.0	32.1±6.0
Motility %	61.6±3.6	58.3±8.5	59.7±8.1	68.8±2.1	62.2±1.1
Rapid %	36.2±8.1	38.8±4.3	37.7±10.1	50.6±5.4	42.3±7.0
VAP µm/sec	70.2±8.9	75.0±14.1	69.5±13.8	81.9±7.1	75.5±13.4
VSL µm/sec	46.6±7.0	51.9±14.3	43.2±7.7	52.6±6.9	49.2±11.2
VCL µm/sec	151.4±9.8	154.6±19.2	149.3±20.8	163.5±17.7	155.8±21.1
ALH µm	8.6±0.9	8.1±0.5	8.4±1.0	8.9±0.7	8.5±0.6
BCF Hz	39.2±1.7	37.9±1.8	36.8±2.1	37.4±2.8	37.4±1.7
Straightness %	60.4±5.7	64.2±8.0	58.9±2.0	61.2±3.8	61.4±2.2
Linearity %	29.4±5.0	33.1±7.0	28.8±2.1	31.0±1.5	31.0±2.3
Elongation %	38.4±2.0	42.4±5.0	39.3±2.1	38.8±3.0	40.2±1.9
Area µm²	18.4±2.6	17.0±3.1	17.4±5.1	16.0±3.2	16.8±3.0

*Path"Velocity"(VAP)"µm/sec,"Progressive"Velocity"(VSL)"µm/sec,"Track"Speed"(VCL)"µm/sec, "Lateral"Amplitude"(ALH)"µm,"Beat"Frequency"(BCF)"Hz."

Table 3.1. Effects of NNC 55–0396 and Methoxy Arachidonyl Fluorophosphonate on Sperm Motility.

Motility parameters of sperm incubated with progesterone (P4), P4 and CatSper inhibitor NNC 55–0396 (P4-NNC), NNC, P4 and P4 receptor blocker MAFP (P4-MAFP), and MAFP alone. Results are means and standard deviations. n=5.

FIGURE 3.1

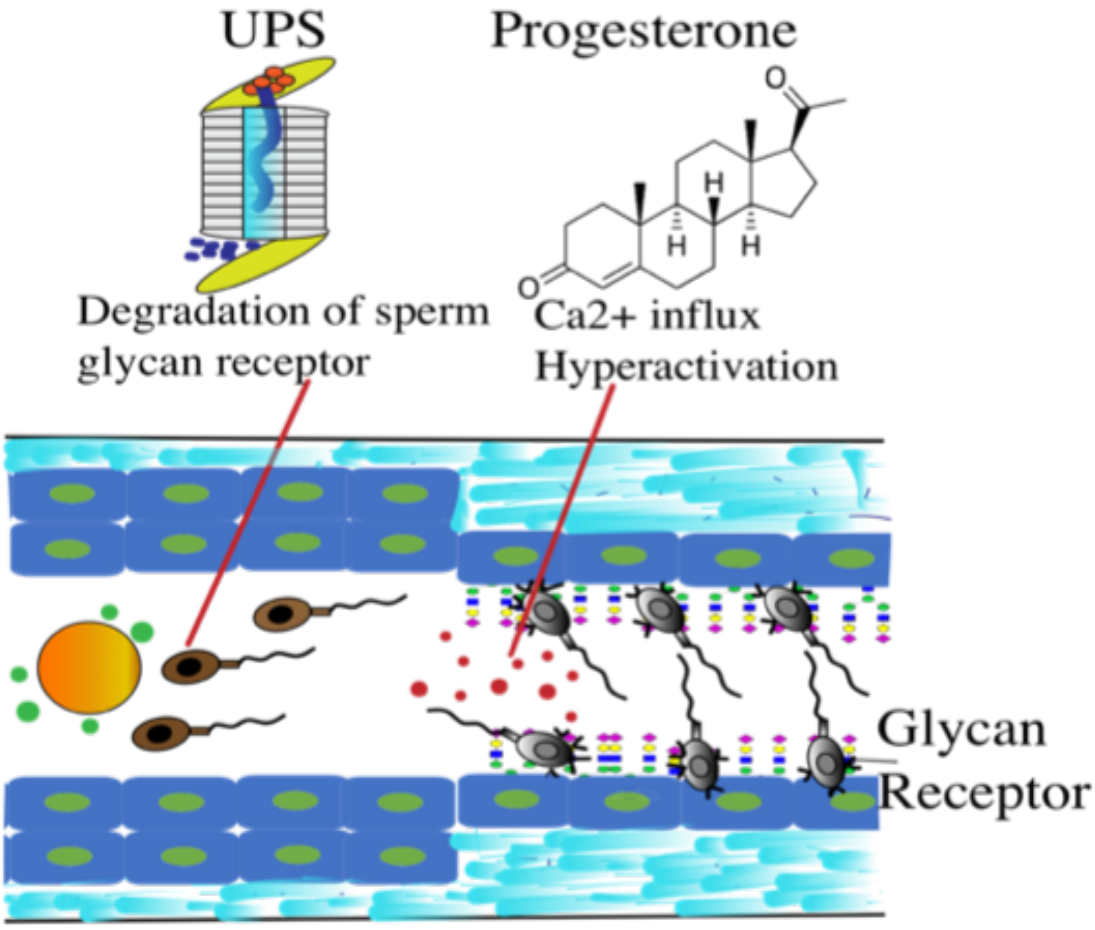


Figure 3.1. Potential mechanisms that control sperm release from the oviduct reservoir.

Left panel.

Sperm receptors could be degraded by the ubiquitin-proteasome system (UPS), perhaps in response to progesterone, causing sperm release. **Right panel**. Progesterone could induce sperm release by binding to a non-genomic receptor, activating CatSper, causing a Ca^{2+} influx and sperm hyperactivation.

FIGURE 3.2

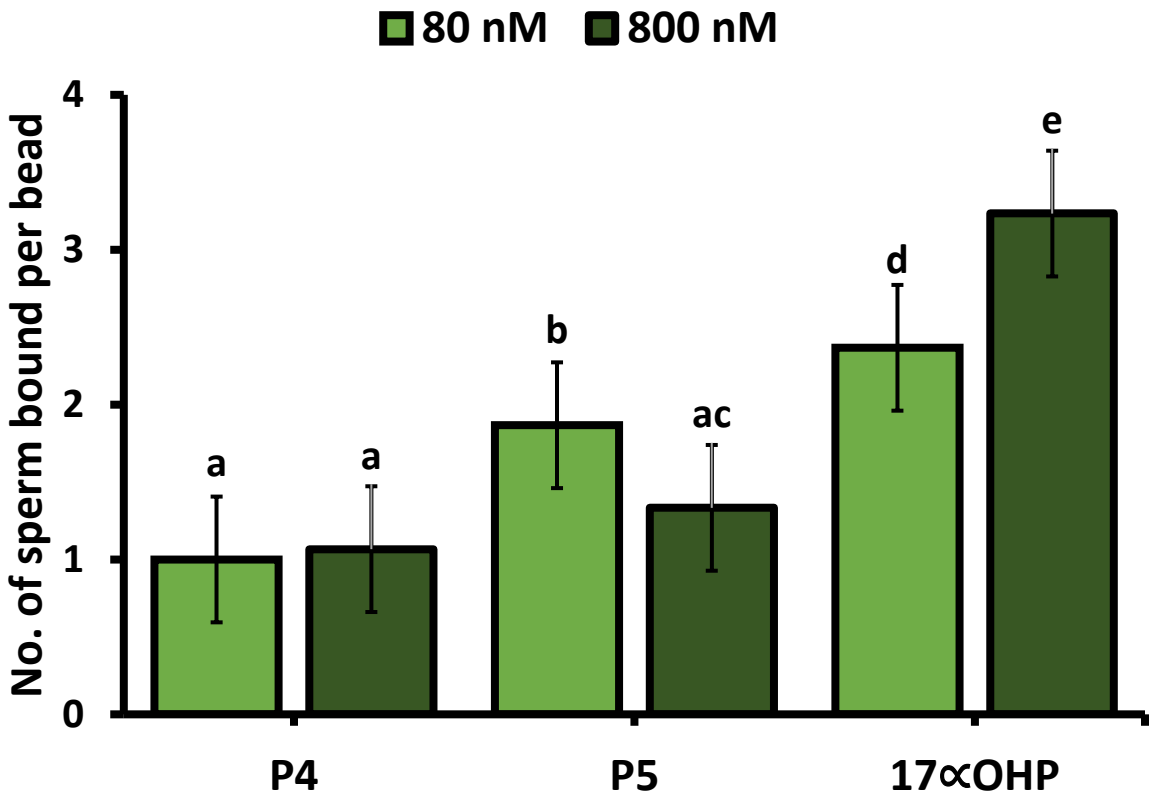


Figure 3.2. Progesterone induces sperm release from oviduct glycans.

The number of sperm bound per suLe^X coated bead after addition of steroid (either progesterone, P4; pregnenolone, P5 or 17 ∝-hydroxyprogesterone, 17 ∝OHP) at 80 or 800 nM. P4 (80 and 800 nM) and P5 (800 nM) induced sperm release when compared with 17-αOHP (80 and 800 nM). An asterisk indicates a significant difference from control, n=3.

FIGURE 3.3

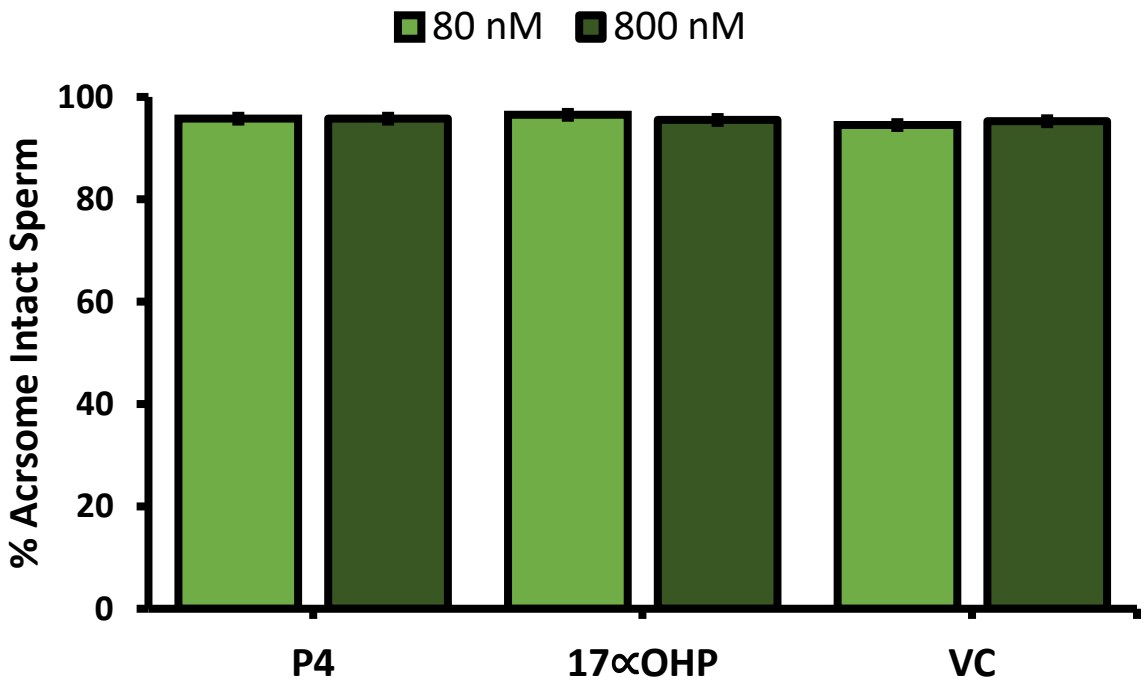


Figure 3.3. Progesterone does not induce acrosome reaction in sperm that are not capacitated.

Acrosome status was assessed in non-capacitated sperm 30 min after incubation with 80 nM progesterone or 17αOHP or vehicle control (VC). Over 90% of the sperm had intact acrosomes under the conditions used in the sperm release assays. (n=3).

FIGURE 3.4

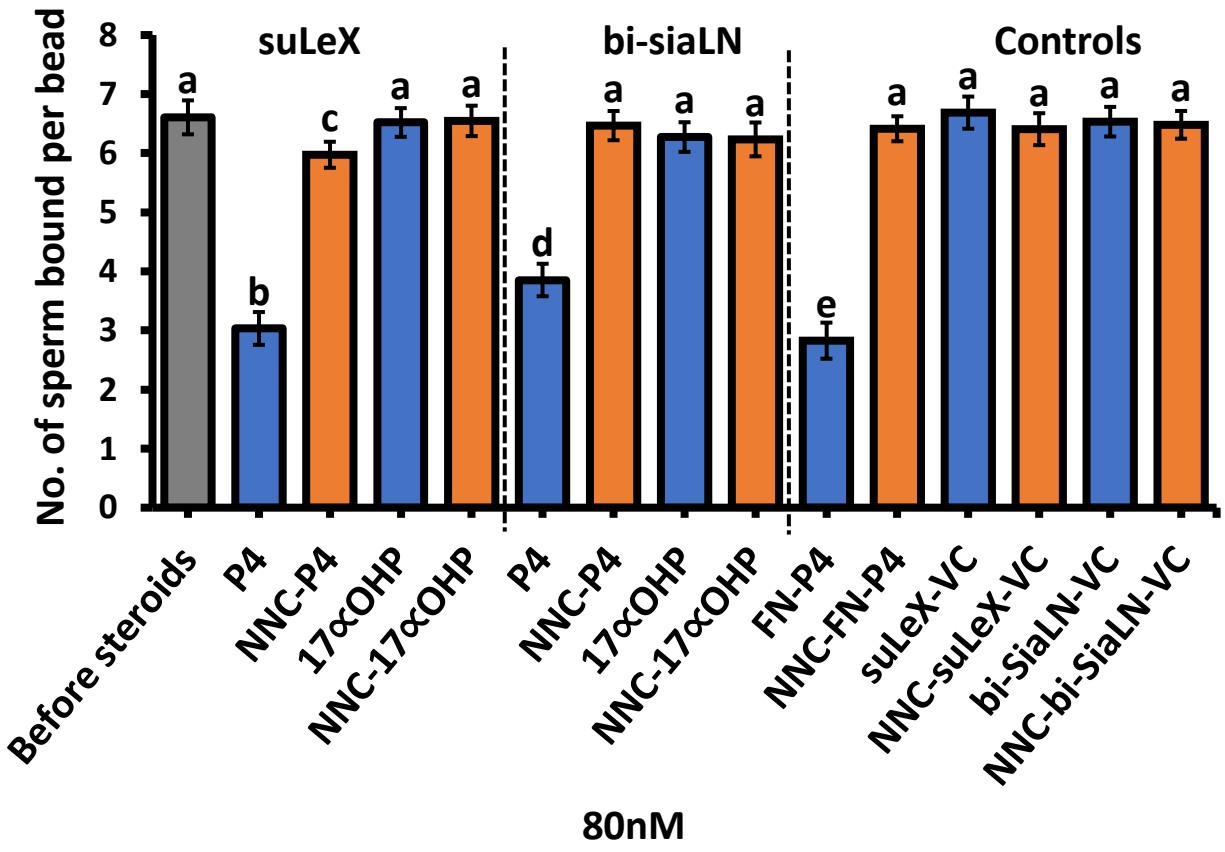
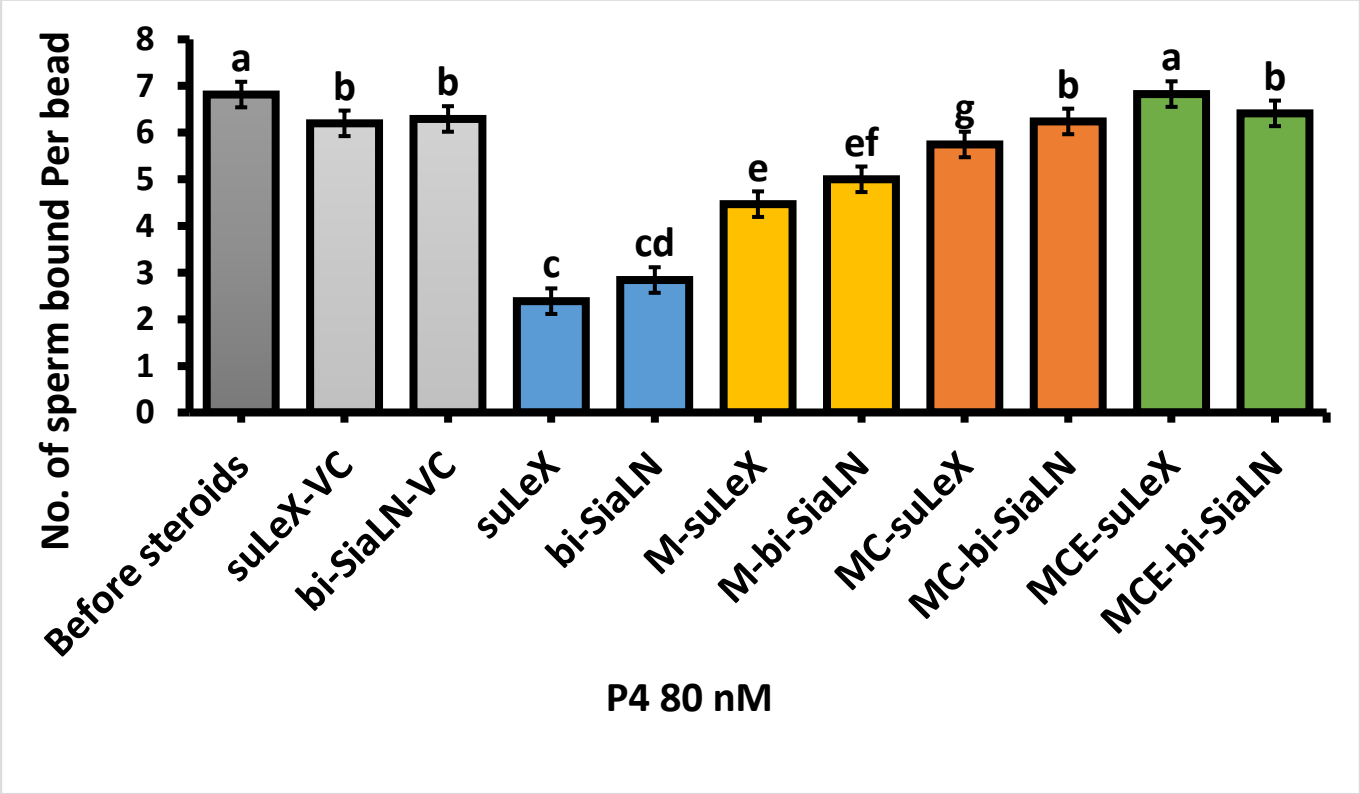


Figure 3.4. CatSper is required for the progesterone induced sperm release.

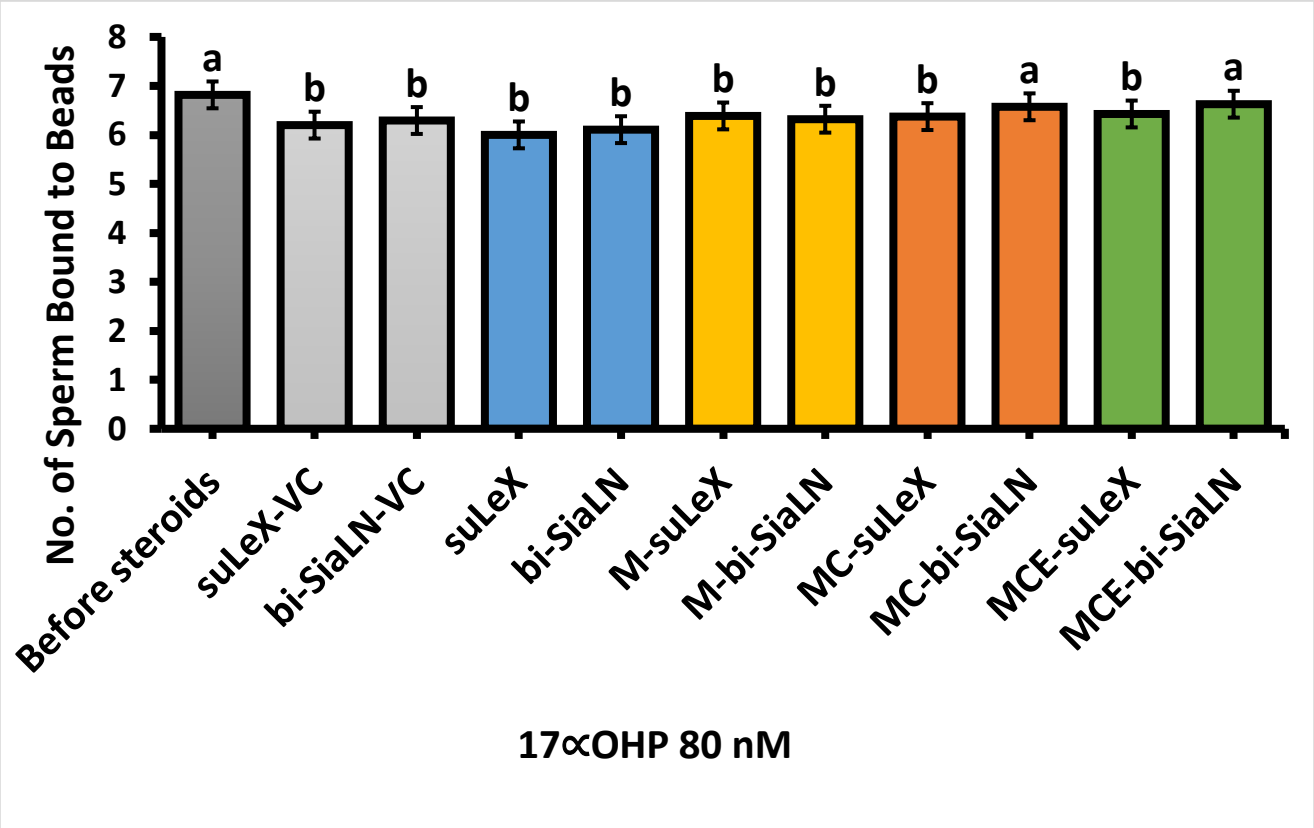
The number of sperm bound to oviduct glycan coated beads (immobilized $suLe^X$ or bi-SiaLN) before and after incubation with progesterone and 17∝OHP. The CatSper T- type channel blocker NCC 55-0396 was added prior to progesterone addition to test the function of CatSper. NNC blocked the progesterone-induced release of sperm from $suLe^X$- and bi-SiaLN-beads as well as immobilized fibronectin (FN). An asterisk denotes a significant difference from samples before progesterone addition, n=3.

FIGURE 3.5.A



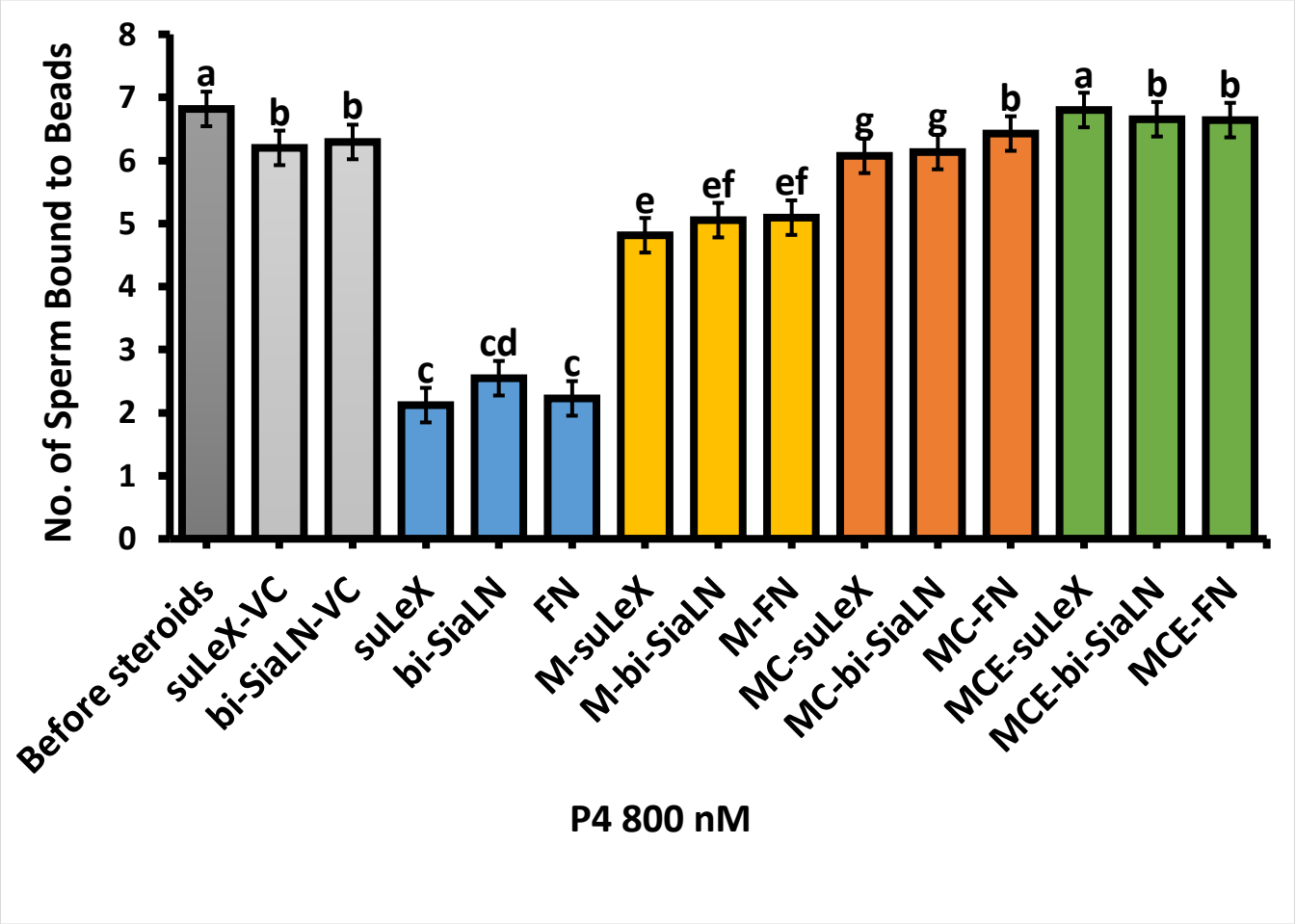
VC= vehicle control	M= MG132, 100 µM
suLeX= sulfated Lewis X	MC= MG132 +CLBL, 100 µM
bi-SiaLN= bi-sialylated lactosamine	MCE= MG132+CLBL+EPOX, 10 µM

FIGURE 3.5.B



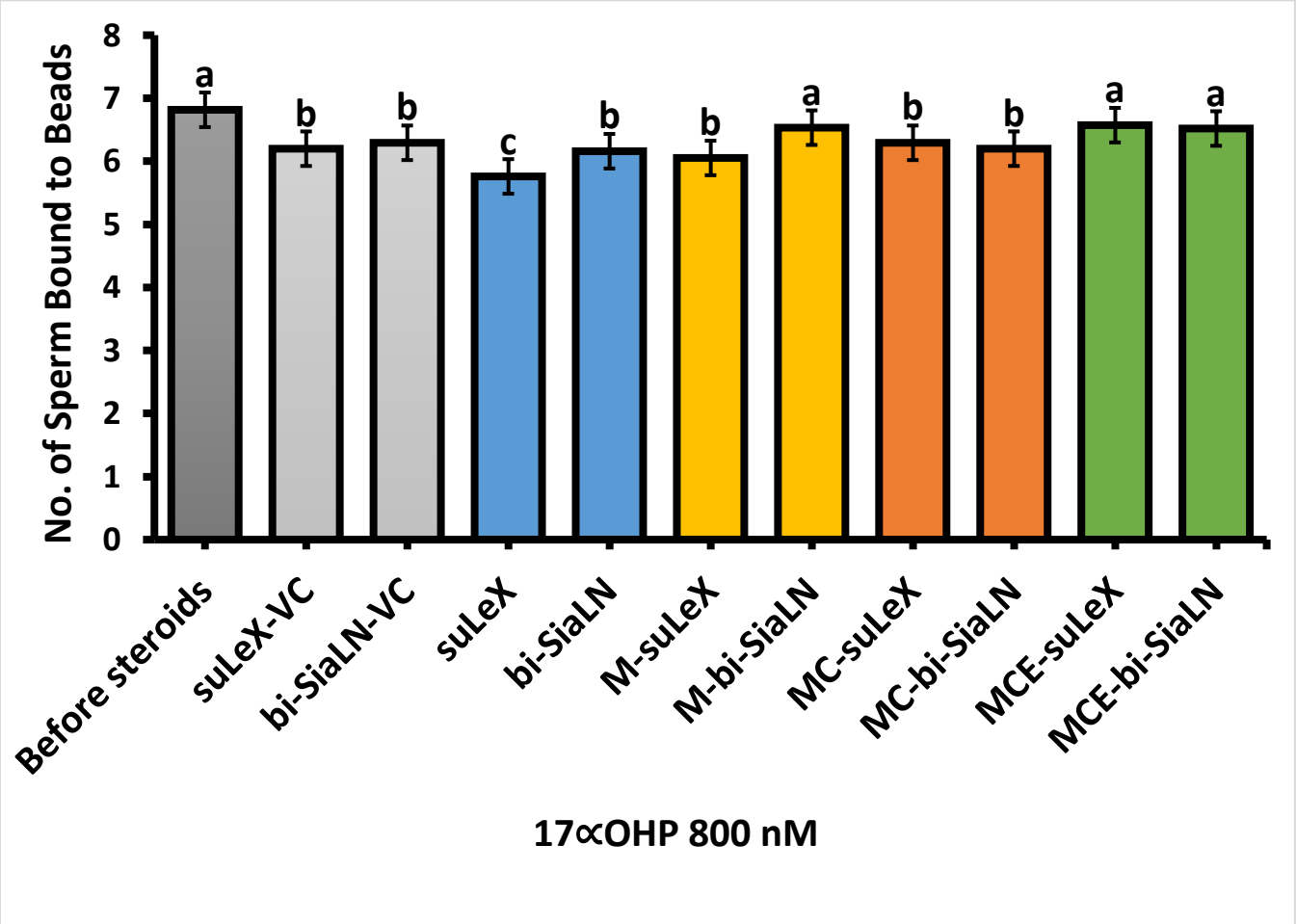
VC= vehicle control	M= MG132, 100 µM
suLeX= sulfated Lewis X	MC= MG132 +CLBL, 100 µM
bi-SiaLN= bi-sialylated lactosamine	MCE= MG132+CLBL+EPOX, 10 µM

FIGURE 3.5.C



VC= vehicle control	M= MG132, 100 µM
suLeX= sulfated Lewis X	MC= MG132 +CLBL, 100 µM
bi-SiaLN= bi-sialylated lactosamine	MCE= MG132+CLBL+EPOX, 10 µM

FIGURE 3.5.D



VC= vehicle control	M= MG132, 100 µM
suLeX= sulfated Lewis X	MC= MG132 +CLBL, 100 µM
bi-SiaLN= bi-sialylated lactosamine	MCE= MG132+CLBL+EPOX, 10 µM

FIGURE 3.5.E

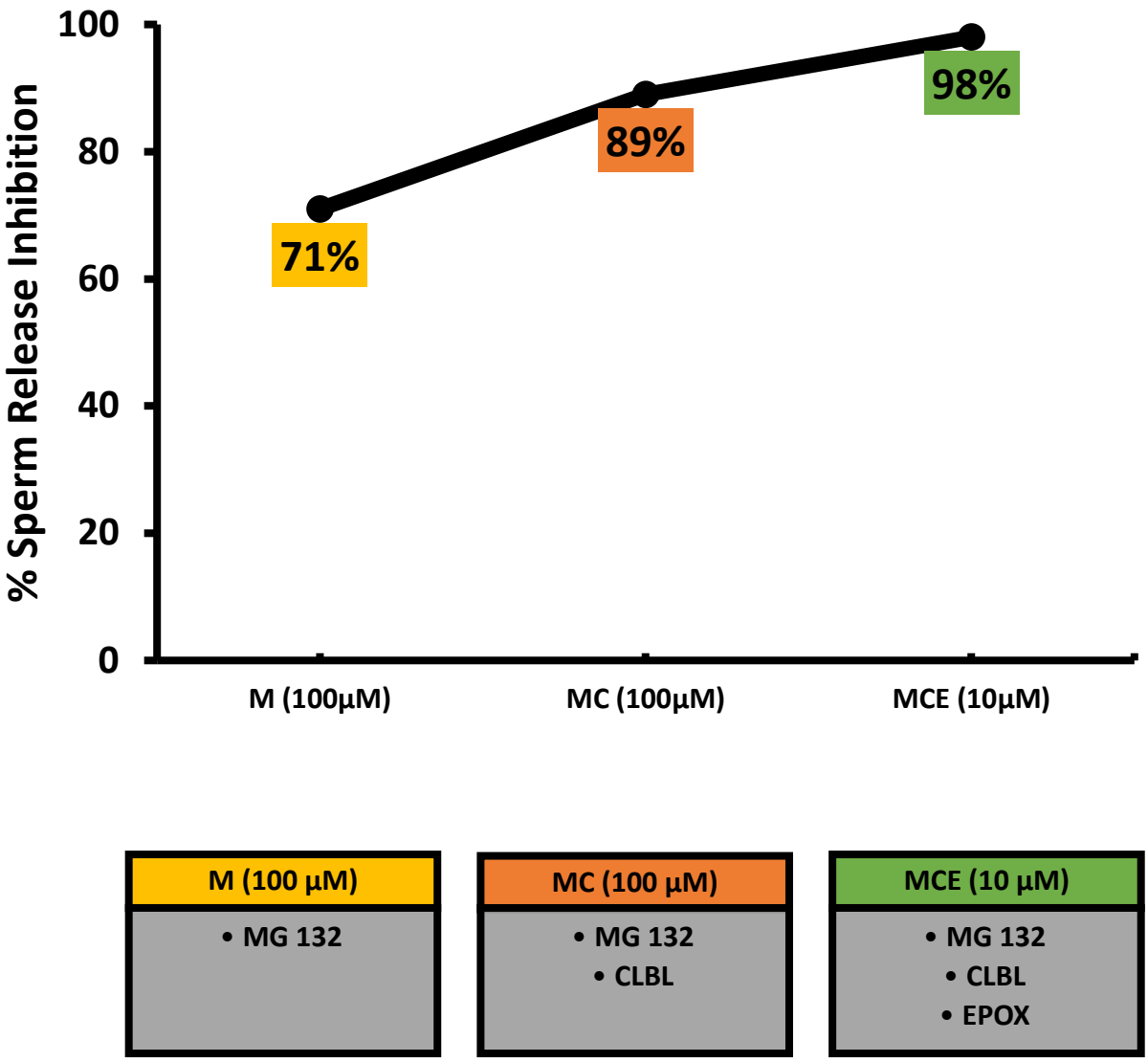

100
80
60
40
20
0
% Sperm Release Inhibition
71%
89%
98%
M (100µM)
MC (100µM)
MCE (10µM)
M (100 µM)
• MG 132
MC (100 µM)
• MG 132
• CLBL
MCE (10 µM)
• MG 132
• CLBL
• EPOX

Figure 3.5. Proteasome inhibitors block progestogen-induced sperm release from oviduct glycans.

Sperm were allowed to bind immobilized oviduct glycans (suLe^X or bi-SiaLN) for 30 min and sperm release was induced by progesterone. Proteasomal activity was inhibited using three different combinations of proteasome inhibitors M (100 μM MG132), MC (100 μM MG132 and CLBL), and MCE (10 μM MG132, CLBL, and EPOX) **A.** Proteasomal activity is necessary for progesterone-induced sperm release from either oviduct glycan (80 nM). More effective inhibition of proteasomal activity inhibited the release of more sperm. **B.** 17∝OHP (80 nM) had no effect on sperm release from oviduct glycans, **C.** Proteasome activity was necessary for sperm release from either oviduct glycan induced by a higher concentration of progesterone (800 nM), **D.** 17∝OHP (800 nM) had no effect on sperm release from oviduct glycans, **E.** Percentage of sperm release inhibition with the three different combinations of proteasome inhibitors (M, MC, and MCE). Addition of all 3 inhibitors was most effective at blocking sperm release (98%) compared to M (71%) and MC (89%), n=3.

FIGURE 3.6

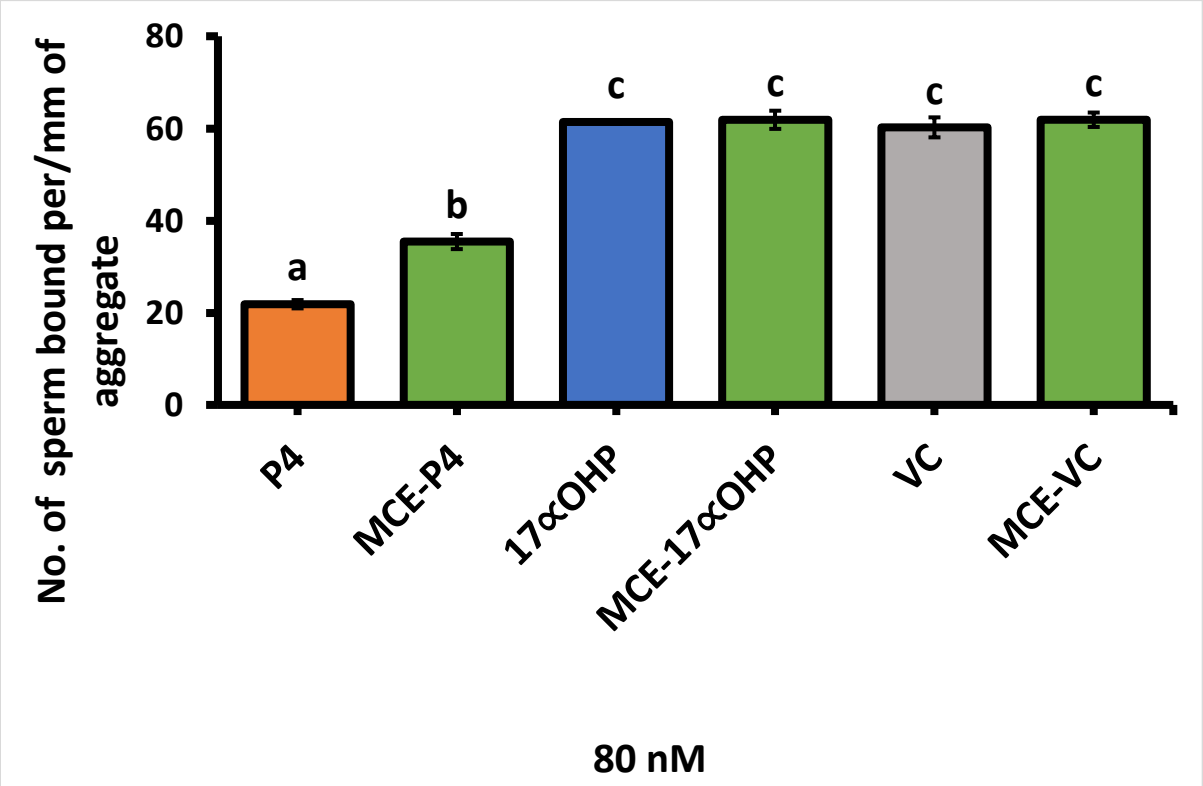


Figure 3.6. Proteasome activity is necessary for sperm release from oviduct cell aggregates. Sperm were allowed to bind to oviduct cell aggregates and release was induced by progesterone. Inhibition of release by the triple cocktail of proteasome inhibitors (MCE) was assessed. The release of more than 50% was blocked in the presence of MCE, n=3.

3.7. REFERENCES

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CHAPTER 4

AIM 3. SPERM HYPERACTIVATION IS SUFFICIENT TO RELEASE PORCINE SPERM FROM OVIDUCT GLYCANS

4.1. ABSTRACT

Sperm hyperactivation is critical for fertilization. It is known to be essential for penetration of the zona pellucida, but it may also enable the release of sperm from the oviduct reservoir and propel sperm through mucus in the oviduct. Hyperactivated motility of the sperm is characterized by high amplitude and asymmetrical flagellar beating. The main trigger for this change in motility is known to be Ca^{2+} influx. I tested if induction of full-type asymmetrical motility was sufficient to release sperm from oviduct reservoir. I induced hyperactivation in sperm using four different compounds, a cell-permeable cAMP analog (cBiMPS), CatSper activators (4-AP and procaine) and an endogenous steroid (progesterone). Motility changes were characterized using CASA and high-speed microscopy imaging. All agents induced sperm hyperactivation to varying degrees; procaine and 4-AP were most effective, as determined by CASA, which measures head movement and analysis of tail waveform. Each was then added to sperm bound to immobilized oviduct glycans and each induced sperm release. Procaine and 4-AP induced release of the highest number of sperm. Maximum sperm release required CatSper activation and fully active proteasomes. In summary, inducing sperm hyperactivation was sufficient for sperm release and release required CatSper channels and proteasome activity.

4.2. INTRODUCTION

At ejaculation, sperm develop activated motility by dilution in accessory gland secretions (Li et al., 2015; Morisawa and Yoshida, 2005). And as sperm develop the ability to fertilize oocytes, known as capacitation, they acquire a different type of activated motility, known as hyperactivated motility (Buffone et al., 2014; Harayama, 2018; Freitas et al., 2016; Chang, 1984; Visconti, 2009). Hyperactivated sperm have two distinct types of movements. Initially, an "asymmetrical" motility develops that is characterized by the beating of the middle and principal pieces of sperm more towards one side than the other and sometimes progressive movement in non-viscous medium (Kojima et al., 2015; Otsuka and Harayama, 2017). In more advanced sperm, an "extremely asymmetrical" pattern that is characterized by more exaggerated bending of the middle and principal pieces more towards one side of the sperm than the other and by the figure of eight-like movement or twisting movement in the non-viscous medium. This movement has also been termed full-type hyperactivation (Kojima et al., 2015; Otsuka and Harayama, 2017).

Development of hyperactivated motility requires the function of the major Ca^{2+} channels in sperm, known as CatSper channels (Lishko et al., 2012; Chung et al., 2014; Sun et al., 2017; Ren et al., 2001; Carlson et al., 2009). Mice or humans that lack CatSper subunits are infertile (Ho et al., 2009; Carlson et al., 2009) and mouse sperm deficient in CatSper are unable to hyperactivate (Carlson et al., 2003; Ho et al., 2009; Liu et al., 2007; Olson et al., 2011) . CatSper channels are only found in sperm and are activated by changes in pH or by progesterone in mouse or human sperm, respectively (Lishko et al., 2011; Sagare-Patil et al., 2012; Alasmari et al., 2013; Chang and Suarez, 2011; Strünker et al., 2011; Niederberger, 2017;

Chung et al., 2014; Lishko et al., 2012). The importance of CatSper emphasizes the central role that Ca^{2+} plays in sperm hyperactivation (Achikanu et al., 2018). But other signaling systems are also engaged to stimulate sperm hyperactivation including cAMP (Nolan et al., 2004; Buffone et al., 2012; Tateno et al., 2013; Alonso et al., 2017).

The development of hyperactivated motility occurs as sperm are capacitated in the oviduct. There are many reports that capacitated sperm are unable to bind to the oviduct epithelium and are thereby found in the lumen of the oviduct rather than retained by the epithelium (DeMott, 2005; Smith and Yanagimachi, 2004; Ardon et al., 2016; Fraser et al., 1995; DasGupta et al., 1993). Mouse sperm that lack the Ca^{2+} channel CatSper are unable to develop hyperactivated motility and fail to release from the sperm reservoir and move towards the upper oviduct (Ho et al., 2009). Based on these results, it has been proposed that the sperm reservoir in the isthmus is formed by adherence of non-capacitated sperm to the epithelium (Miller, 2015). A prominent hypothesis is that, as sperm are capacitated and hyperactivated, they are released from the reservoir to advance and fertilize oocytes. However, because capacitation and hyperactivation are usually coupled, it is not clear whether sperm release is due to capacitation and its associated membrane modifications (Leahy and Gadella, 2011; Sostaric et al., 2004) or specifically to hyperactivation.

The formation of a sperm reservoir in the isthmus is due, at least in part, to sperm retention by specific oviduct epithelial glycans. Porcine sperm bind glycans with either of two specific motifs, 3-O-sulfated Lewis X trisaccharide ($suLe^X$) and branched 6-sialylated lactosamine, SiaLN (Silva et al., 2017; Kadirvel et al., 2012; Miller, 2015; Machado et al., 2014). Capacitated sperm lose their ability to bind these two glycans (Kadirvel et al., 2012; Machado et

al., 2014) but how that loss is accomplished is uncertain. The loss or degradation of glycan receptors during capacitation is a possible cause of sperm release. Or alternatively, hyperactivation may provide the force to pull sperm away from their oviduct epithelial attachment sites (Ho and Suarez, 2001).

To distinguish if hyperactivation and not the other capacitation-associated changes can trigger sperm release, one can add to uncapacitated sperm several pharmacological agents that affect intracellular signaling systems and are sufficient to activate hyperactivated motility. For example, there are previous reports that procaine (which activates CatSper), 4-aminopyridine (which activates CatSper), and the cell-permeable cAMP analog Sp-5,6-dichloro-1-b-D-ribofuranosyl-benzimidazole-30,50-monophosphorothioate all induce full-type hyperactivation of porcine sperm (Kojima et al., 2015; Arai et al., 2019), which otherwise seldom undergo significant full-type hyperactivation in a capacitating medium, unlike mouse epididymal sperm (Mizuno et al., 2015; Arai et al., 2019; Otsuka and Harayama, 2017). Following the addition of these pharmacological agents, porcine sperm display a relatively high percentage of full-type hyperactivated motility before the time that sperm have normally completed capacitation (Kojima et al., 2015; Arai et al., 2019). This approach allows the separation of hyperactivation from other events in capacitation.

The purpose of this study is to determine if sperm hyperactivation was sufficient to promote sperm release from oviduct cells and immobilized glycans. I hypothesize that full-type hyperactivation in sperm is sufficient for sperm release from the reservoir. The role of sperm protein degradation by the proteasomal system during sperm hyperactivation and release was also investigated.

4.3. MATERIALS AND METHODS

Collection and processing of sperm

For each replicate, semen collected from 3 to 5 mature boars (Prairie State Semen Supply, Champaign IL or PIC, Hendersonville, TN). Semen was extended, cooled to 17 °C, transported to the laboratory, and processed within 24 hr. The extended semen was pooled and 3 mL were washed through a Percoll cushion containing 4 mL of dmTALP (2.1 mM $CaCl_2$, 3.1 mM KCl, 1.5 mM $MgCl_2$, 100 mM NaCl, 0.29 mM KH_2PO_4, 0.36% lactic acid, 26 mM $NaHCO_3$, 0.6% BSA, 1 mM pyruvic acid, 20 mM HEPES pH 7.3, sterile filtered), 0.6 mL of 10X HBS (1.3 M NaCl, 40 mM KCL, 10 mM $CaCl_2$, 5 mM $MgCl_2$, 140 mM fructose, 5% BSA, sterile filtered), and 5.4 mL of Percoll for 10 min at 800 x g. The supernatant was discarded, and the resulting pellet was re-suspended in 14 mL of dmTALP and centrifuged for 3 min at 600 x g. The supernatant was discarded again, and the resulting pellet was re-suspended in 1 mL of dmTALP Sperm concentration was estimated by hemocytometer and only samples with greater than 75-80% motile sperm were used for experiments.

Effects of hyperactivity-inducing compounds on sperm motility

The motility of sperm incubated with cBiMPS (50 and 100 μM), 4-AP (2 and 4 mM), procaine (2.5 and 5 mM), progesterone (80 nM) and vehicle control was assessed using a Hamilton Thorne Semen Analysis CASA system (IVOS, Version 12.3K, Build 003), (Hamilton Thorne, Beverly, MA, USA). Sperm were incubated with the treatments at 39°C in dmTALP for

30 min. For each experimental condition, 5 random fields were evaluated for a minimum total of 100 cells (in each field) in 5 replicates.

The treatments showing significant changes in CASA evaluation cBiMPS (100 μM), 4-AP (4 mM), procaine (5 mM), progesterone (80 nM) and vehicle control were used to evaluate sperm motility using high-speed imaging microscopy. After 30 min of incubation, as described, 20 μl of sperm droplet was placed on a slide with a coverslip on top and imaging was done using a motorized inverted microscope (Nikon Eclipse Ti). All images were taken using phase-contrast microscopy at 20X magnification. High-speed videos were recorded at 100 fps for 10 s each. The exposure was set to take fast time-lapses at less than 10 ms per frame.

Every tenth image (100 ms apart) was collected to create a minimum-intensity projection image (MinIP) using Nikon's NIS-Elements software. The algorithm uses all the data in a volume of interest to generate a single bidimensional image. This provided each sperm in the image to have localized pixels (darker values) in all the frames collected in 100 images over those 10s. The resulting images were quantified in two ways. First, sperm were placed in one of five groups (static, motile, progressive, asymmetrical and symmetrical) and data were graphed in the form of percentages. The groups were not mutually exclusive; that is, the same sperm could be placed into more than one group. For example, a single sperm could be motile, and its motility could be symmetrical. Second, sperm motility patterns were enumerated on the basis of geometric angles in six groups (0°), (1-90°), (91°-180°), (181°-359°), (360°) or not applicable (N/A).

Sperm release from glycan-coupled beads

Glycan-coated streptavidin-Sepharose High-Performance beads (GE Healthcare Bio-Sciences, Pittsburgh, PA) with an average diameter of 34 μm were used to test the ability of porcine sperm binding to glycans (bi-SiaLN and suLe^X). To link glycans to beads, approximately 60 μg of suLe^X and 60 μg of bi-SiaLN (Bovin et al., 1993) that were covalently attached to a biotinylated polyacrylamide core were, together, incubated with 20 μL of streptavidin-Sepharose beads for 90 min at room temperature. Each 30-kDa molecule of polyacrylamide had 20% glycan and 5% biotin, by molarity.

Beads incubated with glycans were washed twice in dmTALP and re-suspended in 100 μl of dmTALP. Once the glycan-coupled beads were ready for use, a 50 μL-droplet containing 1.5 x 10^6 sperm/mL was prepared to receive 1 μL of glycan-coated beads. Sperm and beads were co-incubated at 45 min at 39°C. After that, either cBiMPS (100 μM), 4-AP (4mM), procaine (5 mM), progesterone (80 nM) or vehicle control were added. After incubation for 30 min at 39 °C, for each treatment, 25 beads were randomly selected and the total number of bound sperm was enumerated in three replicates using a Zeiss Axioskop at 20X Sperm that were self-agglutinated were not included in the counts. At least three biological replicates were done for each treatment. The experiments were documented using AxioVision or ZEN software (Zeiss, Thornwood, NY).

Effect of proteasomal degradation, CatSper and progesterone receptor inhibitors on sperm release

To test the role of CatSper and progesterone, sperm were allowed to bind to glycan coated bead as stated earlier. A proteasome inhibitor cocktail (MG132, clasto-Lactacystin beta-Lactone (CLBL), and Epoxomicin (MCE,10 μM each)) or 2 μM of T channel blocker (NNC 055-0396) or 2 μM of methoxy arachidonyl fluorophosphonate (MAFP) was added to the sperm bound to glycan coated beads 15 min prior to treatments, cBiMPS (100 μM), 4-AP (4mM), procaine (5 mM), progesterone (80 nM) or vehicle control. Sperm were incubated with the treatments for 30 mins and imaging was done right after within 15 mins. For each treatment, 25 beads were randomly selected and the total number of bound sperm was enumerated in three replicates. At least three biological replicates were done for each treatment. The experiment was documented using a Zeiss Axioskop at 20X magnification (Zeiss, Thornwood, NY).

Statistical analysis

Differences among means were determined using a one-way analysis of variance run in SAS (v. 9.1 SAS Institute, Inc, Cary, NC). The results are shown as means ± SEM and the means were considered to belong to distinct populations if $P < 0.05$ using Tukey's test for multiple comparisons.

4.4. RESULTS

Effects of hyperactivity inducers on sperm motility

First, I confirmed that the pharmacological agents used, in fact, stimulated motility. Using CASA to track the sperm head, the motility of free sperm exposed to cBiMPS (50 and 100 μM), 4-AP (2 and 4 mM), procaine (2.5 and 5 mM), progesterone (80 nM) and vehicle control was assessed. There were significant differences in sperm motility characteristics within 30 min. Only the results from higher concentrations that affected sperm motility are shown **(Table 4.1)**. There were significant changes in beat-cross frequency (BCF), the amplitude of lateral head displacement (ALH), straightness and linearity in response to cBIMPS, 4-AP, procaine and progesterone, consistent with the asymmetrical full-type hyperactivated motility that porcine sperm display.

The motility patterns of free sperm exposed to cBiMPS (100 μM), 4-AP (4mM), procaine (5 mM), progesterone (80 nM) and vehicle control were also assessed using high-speed microscopy to observe the tail beat pattern and confirm the development of hyperactivated motility. Sperm tail motility patterns were divided on the basis of their motility (symmetric vs asymmetric). Progesterone, cBIMPS, 4-AP, and procaine all progesterone There were significant differences in sperm motility patterns compared to the control **(Figure 4.1)**. The highest percentage of sperm with asymmetric motility was seen in 4-AP treated cells. Procaine, cBIMPS and progesterone each induced a higher percentage of sperm to exhibit asymmetric tail beating than cells incubated with vehicle **(Figure 4.1)**. Many sperm heads were observed to adhere to the coverslip. In those cells, asymmetric tail beating induced rotation of the entire sperm in the XY plane. This rotation was most easily observed in the form of image-generated videos before

minimum-intensity projection image (MinIP) were generated. Great rotation was interpreted as a result of more asymmetrical tail beating. Sperm movement was categorized in 90° increments **(Figure 4.2)**. Procaine and 4-AP yielded the highest number of cells showing 360° and 180°-360° angles **(Figure 4.2).** Progesterone and cBIMPS also induced more sperm to show rotation in the XY plane than controls. Thus, all compounds tested induced highly asymmetrical beating of the sperm tail.

Hyperactivation is sufficient for sperm release from immobilized oviduct glycans

We used an in vitro assay to test the ability of hyperactivating compounds to release porcine sperm from oviduct glycans attached to beads. Beads that had both suLe^X and bi-SiaLN bound to the same beads were used as both are glycan motifs found in sperm-binding glycans (Machado et al., 2014; Kadirvel et al., 2012). Effects of cBiMPS (100 μM), 4-AP (4 mM), procaine (5 mM), progesterone (80 nM) and vehicle control were measured on sperm release from oviduct glycan-coated beads. All four compounds induced sperm release from immobilized oviduct glycans, as indicated by a lost in bound sperm after addition **(Figure 4.3).** Each released a number of sperm that was similar to the number released by P4. Therefore, inducing full-type hyperactivation was sufficient to activate sperm release from immobilized oviduct glycans.

Effect of inhibiting the non-genomic progesterone receptor, CatSper and proteasomes on sperm release

To understand the mechanism of sperm release, the effect of inhibitors of the non-genomic progesterone receptor, CatSper and proteasomes were examined. In human sperm, progesterone binds to a membrane receptor, abhydrolase domain-containing protein 2 (ABHD2), a serine hydrolase. An inhibitor of the non-genomic P4 receptor, methoxy arachidonyl fluorophosphonate (MAFP), was added to sperm bound to immobilized oviducgt glycans prior to addition of cBiMPS (100 μM), 4-AP (4mM), procaine (5 mM), and progesterone (80 nM). As expected, MAFP blocked release induced by progesterone **(Figure 4.4, A)**; however, it did not diminish sperm release in response to the hyperactivation inducers indicating their action was downstream of the non-genomic progesterone receptor **(Figure 4.4 A).**

Once progesterone binds to ABHD2, ABHD2 cleaves membrane 2 arachidonoylglycerol, releasing its inhibition of CatSper, allowing Ca^{2+} influx into sperm. The CatSper T channel inhibitor NNC 055-0396 was examined to determine if it would block induced sperm release. As expected, the NNC compound blocked release induced by progesterone **(Figure 4.4, B).** In contrast, blocking CatSper had a partial effect on release induced by the activator of the cAMP pathway, cBIMPS and by 4-AP and procaine, indicating that full release of sperm under these conditions required CatSper **(Figure 4.4, B).**

The possibility that 4-AP, cBIMPS, and procaine induced sperm release by activating protein degradation was also examined by inhibiting proteasomes using a cocktail of 3 proteasomal inhibitors, MG132, clasto-Lactacystin beta-Lactone (CLBL), and Epoxomicin each at 10 μM. Inhibiting protein degradation by proteasomes diminished but did not eliminate sperm

release induced by all three hyperactivity inducers **(Figure 4.4, C)**. These results suggest that for hyperactivation to fully release sperm, protein degradation by proteasomes is required.

4.5. DISCUSSION

Sperm hyperactivation is characterized by high-amplitude and asymmetrical flagellar bending (Kojima et al., 2015), is required to penetrate the oocyte zona pellucida (Harayama, 2013) and is also proposed to enable sperm escape from the oviduct isthmic reservoir because CatSper -/- sperm cannot ascend beyond the isthmus to fertilize oocytes (Ho et al., 2009). The importance of these two potential functions emphasizes why clarifying the precise role of hyperactivation is critical. As hyperactivation is linked to sperm capacitation, it has been challenging to distinguish the independent functions of each process. The discovery that several pharmacological agents could induce sperm hyperactivation in uncapacitated sperm allowed the evaluation of the effects of hyperactivation separately from the membrane modifying effects of sperm capacitation (Kojima et al., 2015; Arai et al., 2019).

In the present study, I characterized the ability four compounds, three different types of pharmacological agents (cBiMPS, 4-AP, and procaine) and an endogenous steroid (progesterone) that act on different signaling systems to induce full-type hyperactivated motility and to release sperm from oviduct glycans. Results showed that the tested compounds induced sperm hyperactivated motility prior to the time capacitation would be completed. Of the four inducers at the concentrations tested, 4-AP and procaine were the most potent inducers of hyperactivated motility. All four compounds also induced sperm release from oviduct glycans within 30 min. The fact that these compounds induce hyperactivation by

activating different intracellular signaling systems, CatSper/Ca^{2+}, and cAMP, but still induce some sperm release, suggests that the mechanism by which they induce release is by promoting hyperactivated motility rather than by some other phenomenon or off-target effect.

Inhibitors of ABHD2, the non-genomic progesterone receptor in human sperm and the ubiquitin-proteasomal system were tested for their effects on sperm release. As expected, MAFP, which inhibits ABHD2 (Miller et al., 2016; Niederberger, 2017), blocked release by progesterone but had a minimal effect on release triggered by the other hyperactivity inducers, indicating that they likely act downstream of ABHD2.

An inhibitor of CatSper, NNC 055-0396, did not affect sperm release due to activation of the cAMP pathway, suggesting that CatSper is not downstream of cAMP activation. This is in contrast to previous studies of mouse sperm that concluded CatSper was downstream of cAMP because no cAMP-induced Ca^{2+} influx was observed in CatSper-deficient sperm (Ren et al., 2001). However, blocking CatSper partially inhibited sperm release activated by 4-AP and procaine. Although 4-AP is said to be an activator of CatSper, the mechanism by which 4-AP activates CatSper is uncertain. It is known that millimolar (mM) concentrations of 4-AP can activate other Ca^{2+} channels directly (Wu et al., 2009). There is a previous report that 4-AP-induced human sperm penetration through viscous medium is inhibited by NNC (Alasmari et al., 2013), which is consistent with the NNC inhibition data reported here. Procaine is also believed to activate CatSper but there is evidence that procaine promotes partial hyperactivation of mouse sperm deficient in CatSper (Carlson et al., 2009). This indicates that there are targets of both 4-AP and procaine in sperm in addition to CatSper that can affect hyperactivation.

Previous studies have shown that the ubiquitin-proteasomal system was necessary for sperm release. Porcine sperm candidates for oviduct glycan receptors were co-precipitated with a 20S proteasomal subunit (Miles et al., 2013), suggesting their degradation might be involved with sperm release. Interestingly, inhibition of proteasomal protein degradation diminished sperm release from immobilized oviduct glycans. This indicates that hyperactivation appears to induce proteolysis of some membrane proteins, which may be important for sperm adhesion to the oviduct.

The trigger of sperm hyperactivation in vivo is uncertain. Progesterone is a strong candidate because it binds to a sperm membrane receptor, increases Ca^{2+} influx and affects sperm motility (Niederberger, 2017; Uhler et al., 2016; Strünker et al., 2011; Lishko et al., 2011; Sagare-Patil et al., 2012; Romero-Aguirregomezcorta et al., 2019; Calogero et al., 2000). The source of progesterone could be follicular fluid from the ovulated oocyte, production from the ovulated oocyte-cumulus complex or production from peri-ovulatory follicles and transport through counter-current mechanism (Hunter et al., 1983; Novak et al., 2003). There is some question that agents produced by the oocyte or cumulus could reach the isthmus at concentrations adequate to affect sperm (Chang and Suarez, 2010).

Assessment of sperm motility, sperm binding and release from immobilized glycans were performed in aqueous medium. However, it is important to consider that oviduct fluid is more viscous than typical aqueous media and that viscosity affects sperm tail beat patterns (Kirkman-Brown and Smith, 2011) and may thereby influence sperm release. It is not clear if hyperactivation may promote release under in vivo conditions.

Assessment of hyperactivated motility has most often been performed on video recordings of motility by manual analysis of patterns of individual sperm (Fujinoki, 2013; Marquez and Suarez, 2004) or by CASA. Manual analysis of sperm trajectories is relatively subjective. Automated methods of measurement such as CASA, although more objective, suffer from the problem that hyperactivity cannot be defined as a "one size fits all" (Bernecic et al., 2019) due to species-specific differences in the sperm flagellum and motility patterns (Kay and Robertson, 1998). Thus, CASA of porcine sperm when used to determine the percentage of hyperactivated sperm can sometimes yield unclear results (Daigneault et al., 2014). In these experiments, hyperactivation was observed by compression of a series of timed images into one image, which allowed observation of tail motion in a single image. The sperm head was frequently immobilized when tail beat patterns were assessed. In that situation, asymmetrical tail beating resulted in the sperm tail rotating around the head. The amount of rotation in the XY plane could be estimated fairly objectively, and the rotation was an indication of beat asymmetry and "full-type" hyperactivation. This approach proved very useful for more objective measurement of sperm hyperactivation.

In summary, results indicated that boar sperm "full-type" hyperactivation can be induced independent of capacitation by activation of the cAMP and CatSper/Ca^{2+} systems. Full-type hyperactivation was sufficient to induce sperm release from oviduct glycans. Full sperm release was dependent on CatSper and the function of the ubiquitin-proteasomal system, suggesting that both sperm membrane protein degradation and the additional force generated by hyperactivation have important roles in sperm release.

4.6. FIGURES AND TABLE

TABLE 4.1

Treatments	4-AP	cBIMPS	Procaine	P4	VC
Progressive %	40.8±4.0	40.6±4.5	39.4±5.8	35.0±2.2	26.6±2.7
Motility %	70.2±4.8	69.6±3.7	71.8±3.7	61.6±3.6	67.8±2.3
Rapid %	55.2±3.8	51.0±5.0	63.0±6.4	36.2±8.1	28.4±2.0
VAP □m/sec	80.26±6.1	82.3±8.2	98.78±14.8	70.22±8.9	49.2±6.0
VSL □m/sec	26.6±6.1	50.26±3.8	43.04±5.3	46.6±7.0	30.66±2.2
VCL □m/sec	180.84±28.8	156.26±33.8	228.76±43.6	151.44±9.8	110.28±13.3
ALH □m	11.28±0.9*	8.32±1.6*	9.76±0.8*	8.58±0.9*	7.44±1.6
BCF Hz	48.62±6.3*	39.48±6.7*	40.34±4.1*	39.24±1.7*	37.98±2.0
Straightness %	41.0±19.1	59.8±4.7	43.8±3.2	60.4±5.7	62.0±9.7
Linearity %	20.4±4.6	36.0±3.8	19.6±3.6	29.4±5.0	29.4±4.6
Elongation %	44.4±5.7	42.6±4.8	46.4±5.9	38.4±2.0	39.6±4.7
Area μm²	9.44±1.9	14.56±2.1	13.74±4.6	18.38±2.6	10.14±2.3

*Path"Velocity"(VAP)"μm/sec,"Progressive"Velocity"(VSL)"μm/sec,"Track"Speed"(VCL)"μm/sec, "Lateral"Amplitude"(ALH)"μm,"Beat"Frequency"(BCF)"Hz."

Table 4.1. Effects of hyperactivity inducing compounds on sperm motility measured by CASA.

Motility parameters of sperm incubated with cBiMPS (100 μM), 4-AP (4mM), procaine (5 mM), progesterone (80 nM) and vehicle control 30 min after addition. Results are means and standard deviations. (*) represent significant differences from vehicle control for ALH and BCF. n=5.

FIGURE 4.1.A

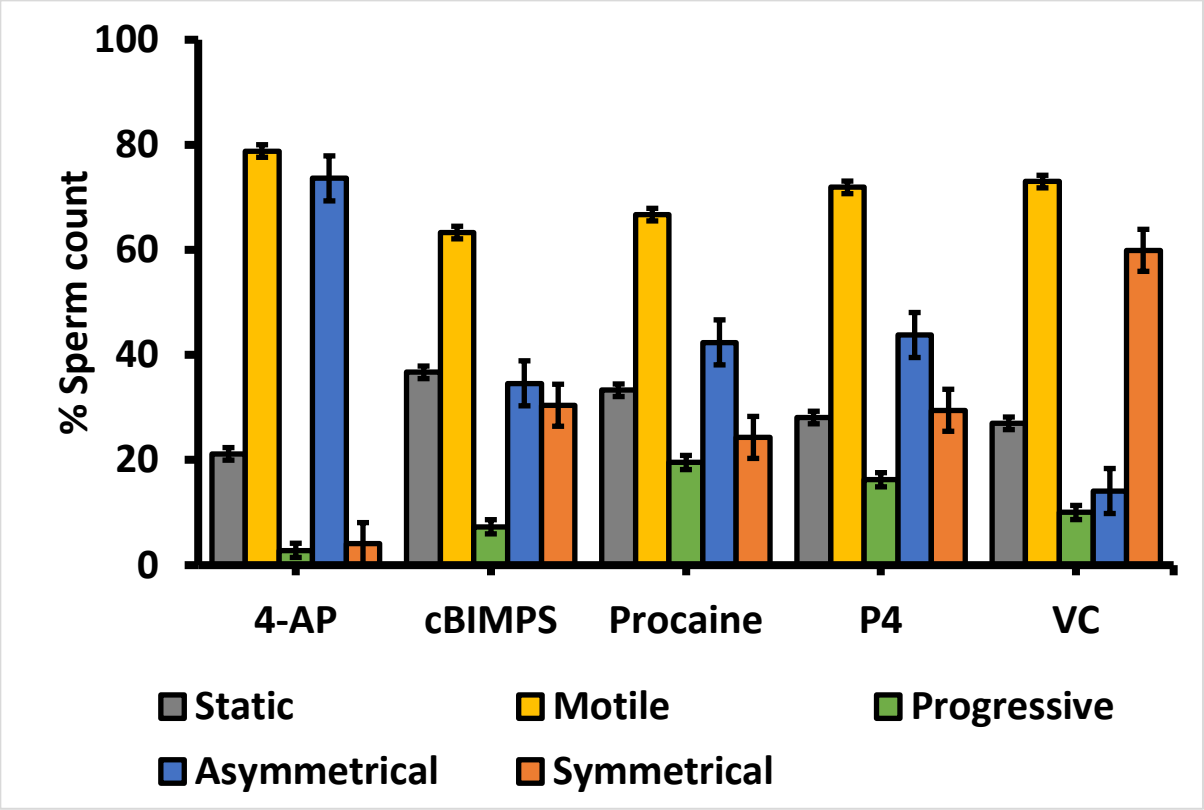

100
80
60
40
20
0
% Sperm count
4-AP
cBIMPS
Procaine
P4
VC
Static
Motile
Progressive
Asymmetrical
Symmetrical

FIGURE 4.1.B

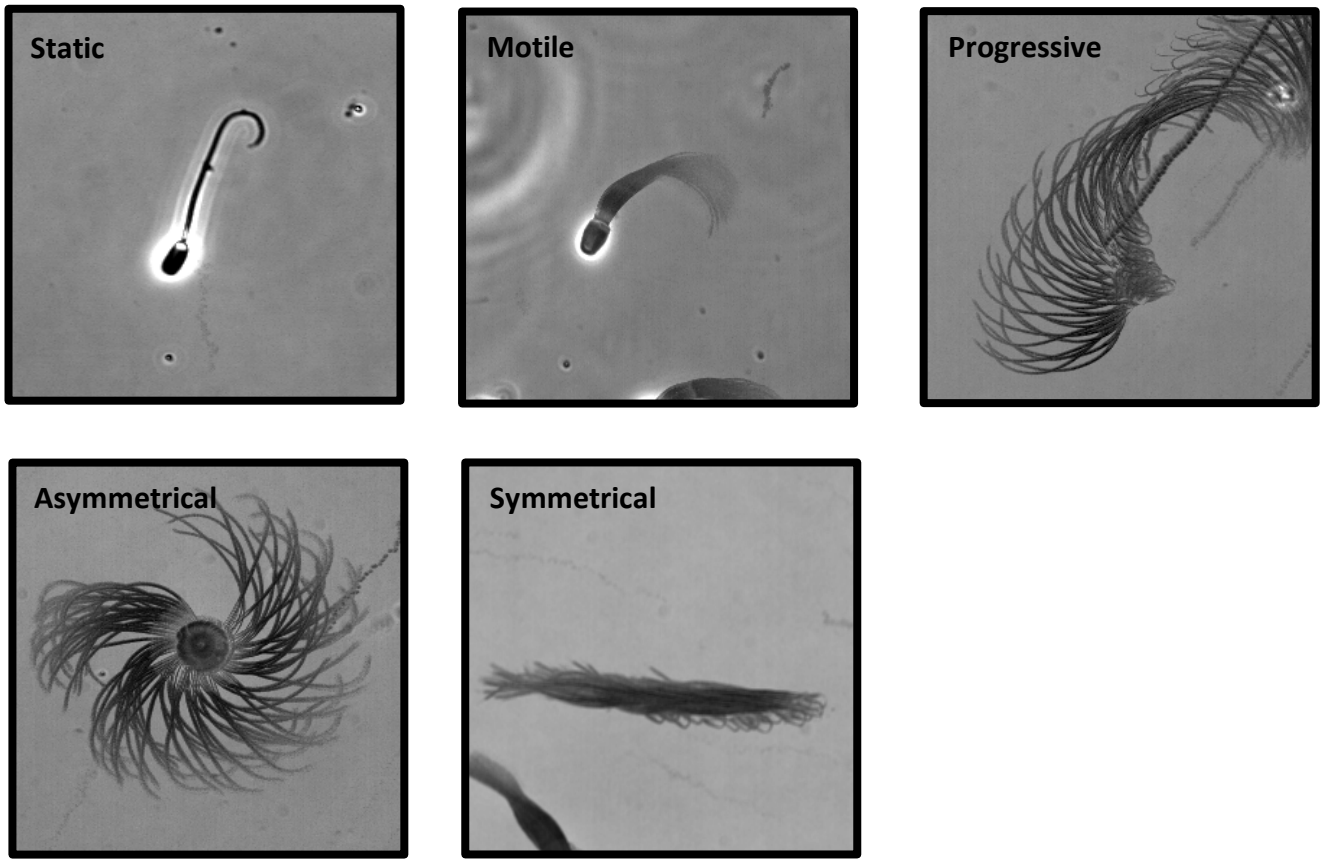
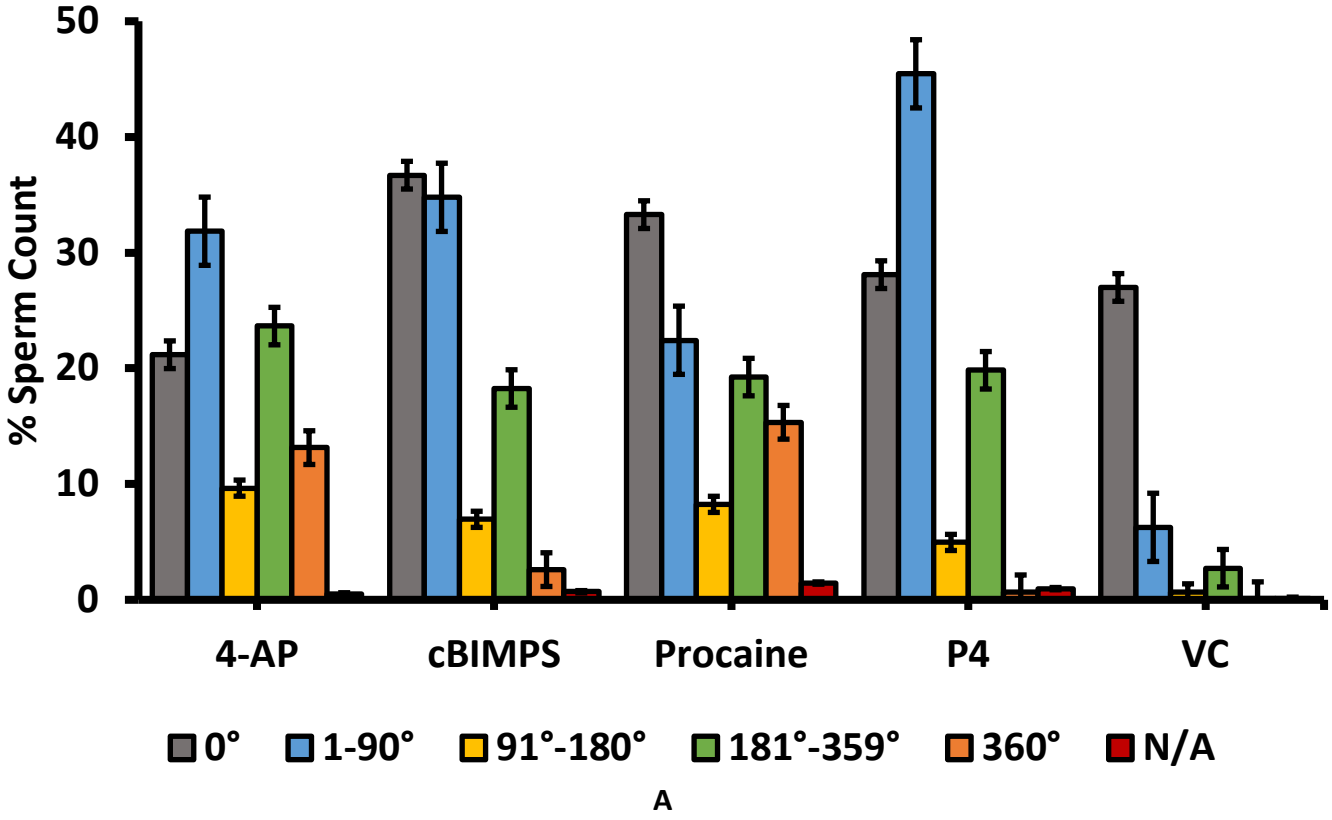


Figure 4.1. Effects of hyperactivity inducers on sperm motility patterns.

From the high-speed microscopic images, sperm were divided in five groups (static, motile, progressive, asymmetrical and symmetrical). The same sperm could be placed into more than one group (i.e. motile and symmetrical). Thus, the totals do not equal 100. Data are graphed as a percentage of total sperm. n=4. **B,** Examples of sperm with the specific motility pattern. n=4, for 350-500 sperm per replicate.

FIGURE 4.2.A


50
40
30
20
10
0
% Sperm Count
4-AP
cBIMPS
Procaine
P4
VC
0°
1-90°
91°-180°
181°-359°
360°
N/A

A

FIGURE 4.2.B

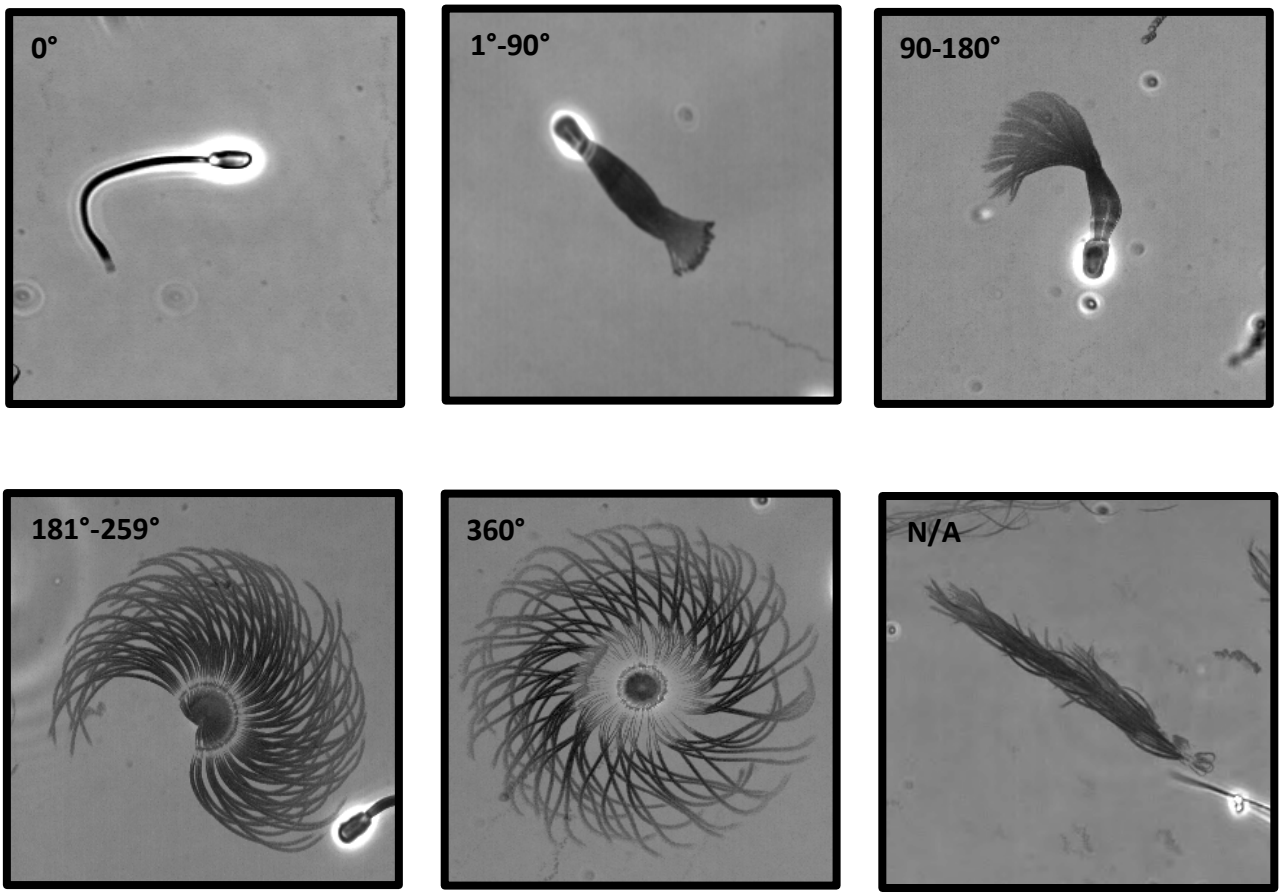


Figure 4.2. Effects of hyperactivity inducers on sperm asymmetry.

A, From the compressed image of high-speed video, sperm were divided into six groups of geometric angles (0°), (1-90°), (91°-180°), (181°-359°), (360°) and Not applicable (N/A). Data are graphed as a percentage of total sperm observed. n=4. **B,** Small subsets show the representative images of the geometric angles of sperm motility. n=4, for 350-500 sperm per replicate.

FIGURE 4.3

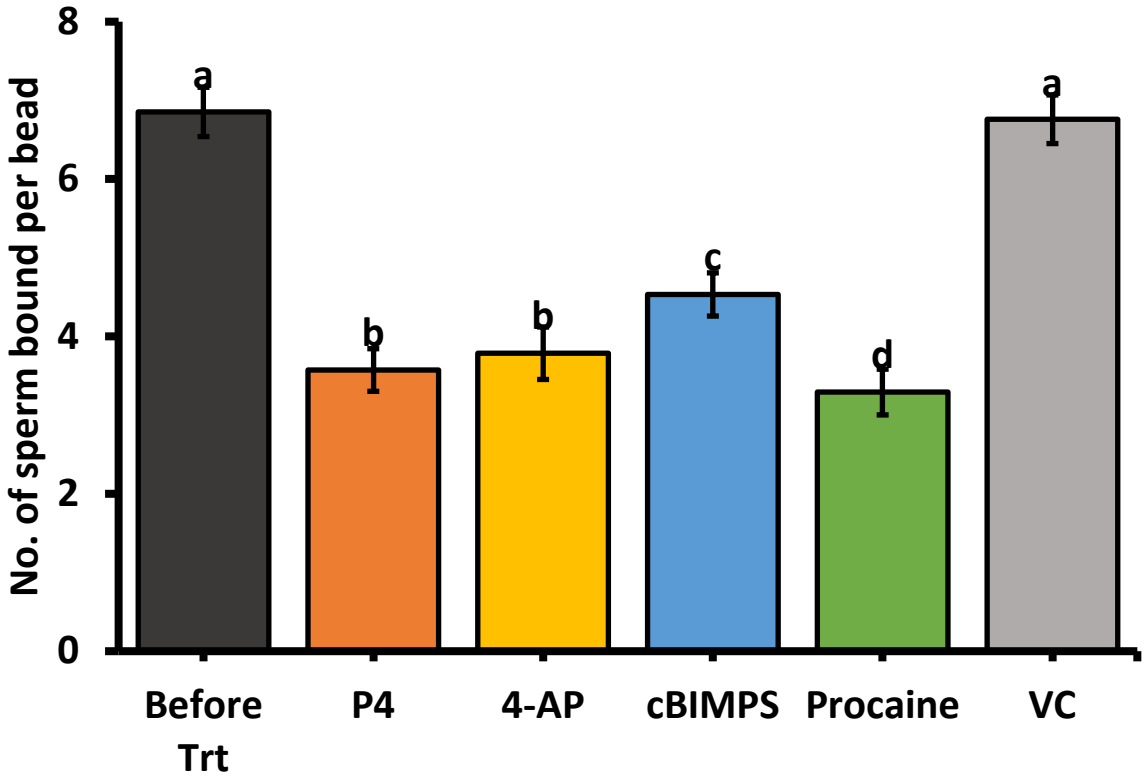


Figure 4.3. Hyperactivation is sufficient for sperm release from immobilized oviduct glycans. The number of sperm bound per glyco-bead prior to and following a 30 min incubation with cBiMPS (100 μM), 4-AP (4 mM), procaine (5 mM), progesterone (80 nM). All hyperactivity inducers promoted sperm release from immobilized oviduct glycans when compared with controls. Different letters indicate significant differences between treatments and controls, n=3.

FIGURE 4.4.A

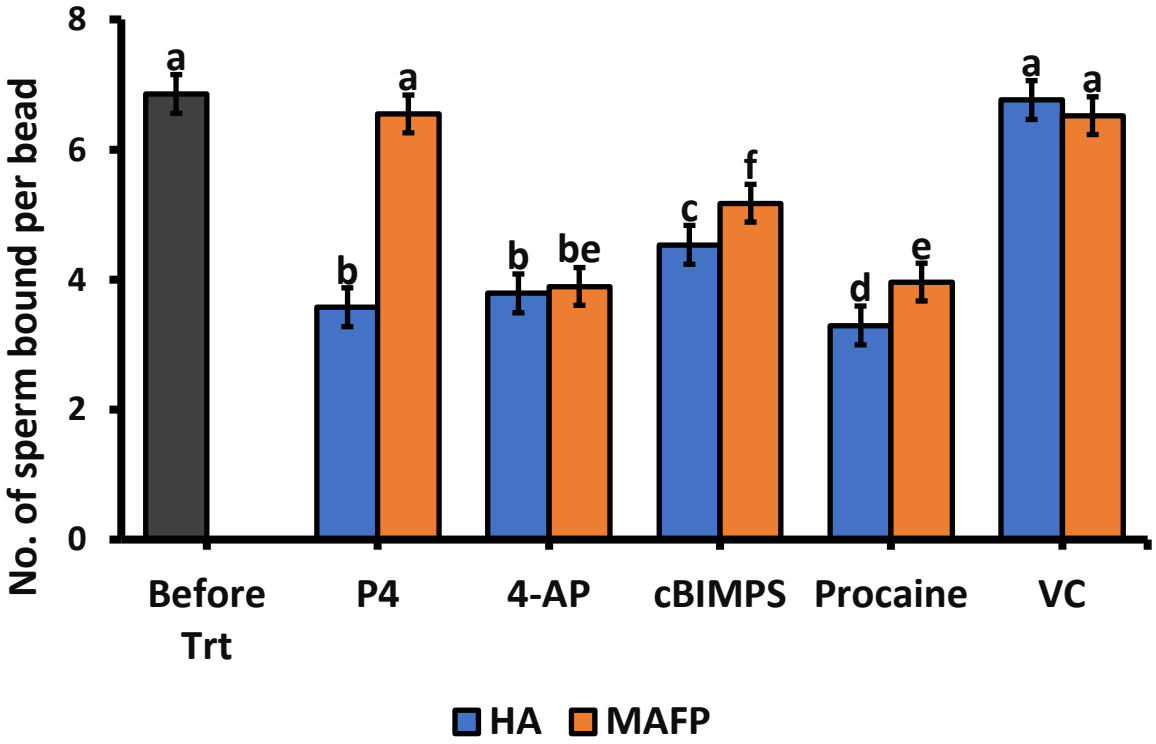

No. of sperm bound per bead
8
6
4
2
0
a
a
b
b
be
c
f
d
e
a
a
Before Trt
P4
4-AP
cBIMPS
Procaine
VC
HA
MAFP

FIGURE 4.4.B

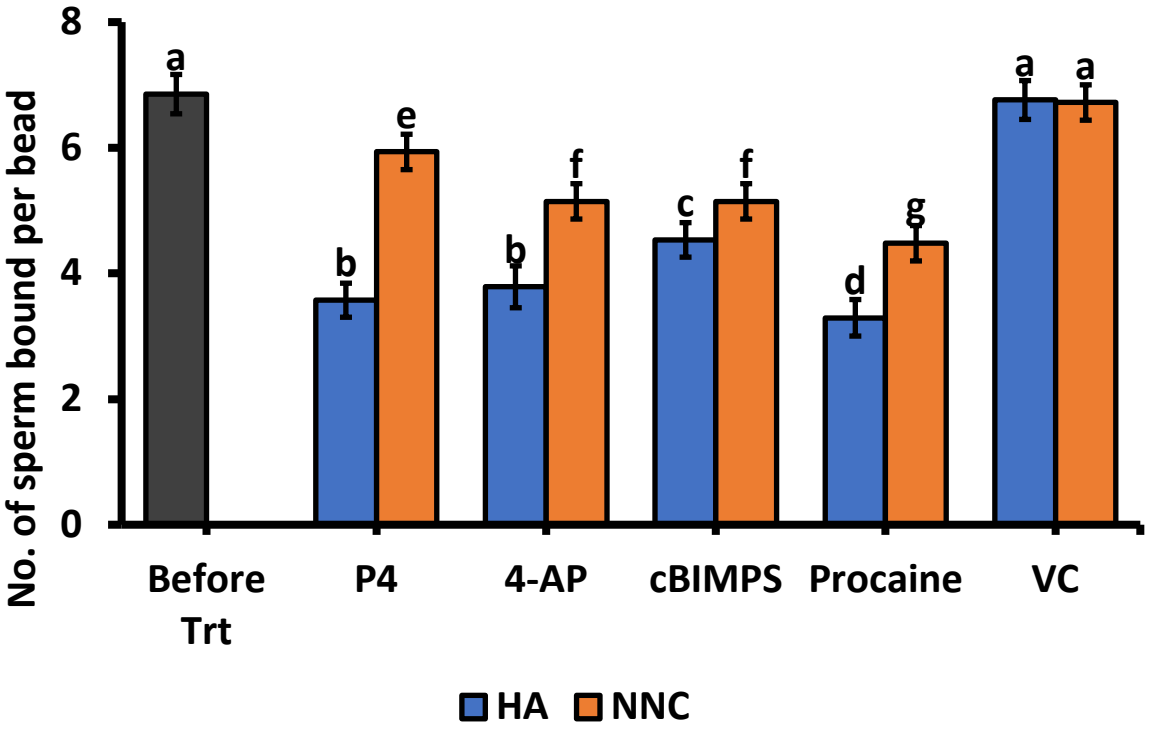

No. of sperm bound per bead
8
6
4
2
0
a
b
e
b
f
c
f
d
g
a
a
Before Trt
P4
4-AP
cBIMPS
Procaine
VC
HA
NNC

FIGURE 4.4.C

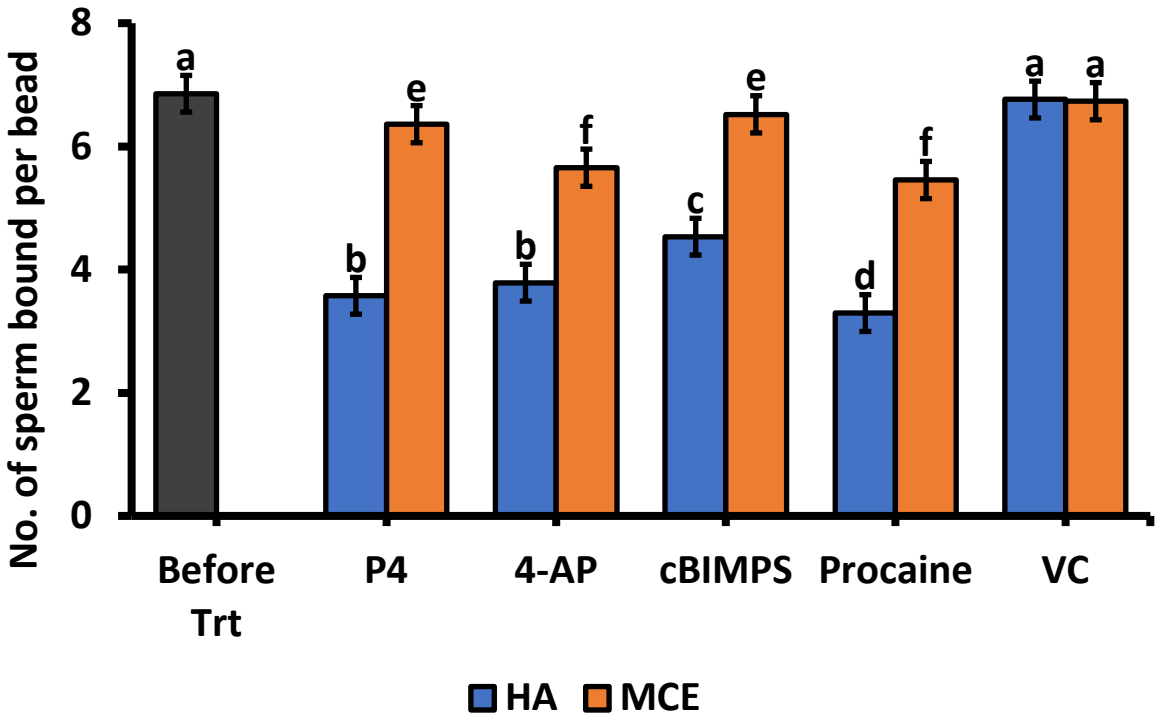


Figure 4.4. Hyperactivation activates the release of sperm from immobilized oviduct glycans in the presence of progesterone receptor, CatSper, and proteasome inhibitors. The number of sperm bound per bead incubated with oviduct glycans (suLe^X, bi-SiaLN) immobilized on beads. cBiMPS (100 μM), 4-AP (4mM), procaine (5 mM), and progesterone (80 nM) were used to induce sperm release from immobilized oviduct glycans. **A,** MAFP had a minimal effect on release triggered by 4-AP, cBIMPS, and procaine. MAFP blocked sperm release induced by progesterone. . **B,** NNC 055-0396 diminished sperm release induced by progesterone, 4-AP, cBIMPS, and procaine. **C,** A cocktail of proteasome inhibitors (MCE) diminished sperm release induced by 4-AP and procaine and blocked all release induced by progesterone and cBIMPS. Different letters indicate significant differences between treatments and controls, n=3.

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CHAPTER 5

OVERALL CONCLUSIONS AND FUTURE DIRECTIONS

5.1. OVERALL CONCLUSIONS

Sperm reservoir formation in the female reproductive tract is used as a strategy by many animal species to allow some asynchrony between mating and ovulation. Adhesion to the oviduct increases sperm lifespan, regulates capacitation and prevents polyspermy in mammalian species. This dissertation clarifies the effects of sperm binding to oviduct glycans and their release mechanism. The results of this study indicate that progesterone, CatSper, hyperactivation and the ubiquitin-proteasome system (UPS) all play a role in the release of sperm from the glycan facilitated boar sperm oviduct storage

Capacitation-related events like an increase in sperm protein tyrosine phosphorylation, an ability to undergo an induced acrosome reaction and changes in motility pattern were not altered or delayed by sperm binding to oviduct glycans. Verifying the overall status of capacitation in boar sperm is still something which needs to be pondered as most individual assays for facets of capacitation are not indicative of the complete capacitation process. The assays that have been established so far focus on one aspect of capacitation rather than all aspects that encompasses capacitation. Thus, verifying the capacitation status to this point has been difficult.

Proteasomes played a key role in sperm release from the oviduct reservoir and their inhibition reduced progesterone-induced release. The key proteins (likely sperm surface glycan receptors) that are the target of proteasomes need to be identified to understand how sperm proteasomes are enabling sperm to release from oviduct glycans in the reservoir.

Boar sperm “full-type” hyperactivation can be induced independent of capacitation and is sufficient to induce sperm release from oviduct reservoir to allow sperm to advance to the ampulla. This release is dependent on CatSper and the sperm proteasomal system. Therefore, both the hyperactivation generated force and protein degradation have a significant role in sperm release. I speculate that sperm hyperactivation must generate forces strong enough for sperm to be released from the oviduct epithelia where they are bound to the glycans via their surface receptors. Identification of the force generated by full-type hyperactivation of porcine sperm and comparison to the affinity of boar sperm to oviduct glycans will be important in determining the role of hyperactivation in sperm release.

This knowledge is applicable in the industry when it comes to identifying factors which affect fertility, abnormal fertilization (polyspermy), identifying molecular targets for contraceptives (e.g. CatSper and Proteasomes), and help in decreasing the number of inseminations at estrus during artificial insemination.

5.2. FUTURE DIRECTIONS

Do anchored oviduct glycans have more effects on sperm than soluble glycans?

Oviduct glycans have been known to improve sperm lifespan and viability. The assays done in this study have shown no effect of soluble glycans on sperm capacitation. Studying the effect of immobilized glycans on sperm might give a better insight on their influence on sperm function based on their anchoring effect which will mimic more in vivo glycan-sperm interaction in the oviduct.

Better assay development for capacitation detection

A further problem is that of measuring capacitation. The development of better capacitation detection assays is vital for making productivity of the industry better in terms of developing new methods to improve the semen lifespan and quality. This complicated process includes many individual steps that are not always coincident with each other. Deciding which of these many steps to measure to gain an overall indication of capacitation is not obvious. Although some of the most common assessments of capacitation were used in this study, no effect of soluble glycans was observed. These soluble oviduct glycans may have other effects on sperm that were not measured. A broader assessment of capacitation may detect effects of oviduct glycans.

Identify sperm proteins that are targets for degradation by proteasomes

Recent research and this study have shown that proteasomes may be essential for the degradation of membrane-associated proteins during the remodeling of the sperm plasma membrane occurring during capacitation. The exact target of sperm proteasomes can be found by doing ubiquitin (chaperone-protein) pull-down experiments on whole sperm protein lysate. This will help in identifying the proteins which are tagged by ubiquitin for degradation by sperm proteasomes. This information will determine which proteins might be degraded and clarify the role of sperm proteasomes in release from the oviduct.

Determine the location within sperm of ubiquitin tagging and the stage of sperm maturation in which tagging occurs.

The ubiquitin-proteasome pathway is a regulated method for substrate-specific protein degradation. It is important to understand the complete ubiquitination–degradation cycle to identify the specific enzymes responsible for tagging the proteins for degradation. It will be noteworthy to study when and where the sperm proteins are being marked/flagged for degradation (before or after capacitation). Moreover, it will also be interesting to see if the addition of sperm proteasome inhibitors is causing accumulation of ubiquitin on sperm and at which stage of maturity (before or after capacitation).

Develop more sophisticated methods to analyze the sperm flagellar beat in aqueous and viscous media.

Passage of sperm through the viscoelastic cervical mucus is a useful indicator of natural fertility. Investigating how viscosity differences affect sperm motility during and after capacitation may hold keys for new diagnoses and treatments. The CASA system has been used in research for a long time to look at basic sperm motility parameters by only following sperm head motion. But now in basic research, modern digital high-speed imaging alongside fluid mechanic analysis of the flagellar waveform has provided a tangible way to study the organization of the flagellar beat and to potentially assess detailed media (viscous, non-viscous) effects on sperm motility. These systems can also help us understand how changes to the flagellar beat can cause different types of hyperactivation motility patterns in sperm and their

causes. Incorporating mathematical modeling along with high-speed imaging which will give us more objective results compared to CASA systems.