ENHANCED FERTILITY ESTIMATION AND NOVEL ION CHANNELS IN BOAR SPERM

BY

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DISSERTATION

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ABSTRACT

Cryopreserved boar sperm have many potential advantages when used for artificial insemination (AI), including increased time for pathogen testing and ease of semen transport. However, frozen boar sperm are seldom used for AI due to decreased pregnancy rates and litter sizes as a result of cryo-induced damage to sperm. Estimating the fertility of samples prior to AI would allow disposal of semen with poor predicted fertility. Unfortunately, conventional laboratory analyses of semen do not provide accurate estimates of fertility. Assessing sperm traits that more accurately predict fertility would improve commercial use of frozen boar sperm. Our objective was to identify traits of cryopreserved sperm that are related to boar fertility by including novel sperm assays with traditional laboratory analyses that may collectively provide a more accurate model for estimating fertility. Semen from 14 boars of several breeds was cooled to 15°C for overnight shipping prior to freezing. Semen was thawed and motility was estimated and confirmed using Computer Automated Sperm Assessment (CASA). Sperm viability and acrosome integrity were measured at 0, 30 and 60 min post-thaw. In addition to traditional analyses, each sperm sample was tested by IVF in two to three independent replicates and fertilization, cleavage and blastocyst development were recorded. As an assessment of sperm reservoir formation, a sperm-oviduct binding assay was used to compare the number of sperm bound to epithelial aggregates harvested from gilt isthmus. Additionally, a competitive zona binding assay using two distinct fluorophores for sperm identification was employed to measure the number of sperm from each boar bound to the zona. Frozen sperm from the same ejaculates subjected to laboratory analyses were used to determine boar fertility. Fertility was measured by AI of mature gilts using 4.0 x 10⁶ sperm from one boar at 24 h and a second boar at 36 h after the onset of estrus and calculated as the percentage of the litter sired by each boar. AI order was
reversed in consecutive replicates so that order of insemination was evenly distributed among boar comparisons. Reproductive tracts were harvested at ~ 32 d after AI and the number of fetuses were recorded and sampled for paternity identification using microsatellite markers. The least-squared means (LSM) of each laboratory evaluation were modeled by boar using multiple regression analyses to test their collective values in predicting fertility. The model generated was highly predictive of fertility (P < 0.001, $r^2 = 0.87$) and included 5 traits; acrosome compromised sperm (0 and 30 min), percent live sperm (0 min), percent total motility (30 min) and the number of zona bound sperm. An additional model in which fertility was assessed by the number of piglets sired by boar also predicted fertility (P < 0.05, $r^2 = 0.57$) and shared many of the same traits including percent live sperm, motility and the number of sperm bound to zona. These models indicate that the fertility of cryopreserved boar sperm can be predicted using both traditional and novel laboratory assays that consider multiple functions of sperm. This model provides a method to cull low fertility cryopreserved boar semen more accurately, thus enhancing the potential adoption of semen cryopreservation by the swine industry.

Both models that successfully estimated boar fertility included sperm traits that evaluated binding ability to the zona pellucida. Further, binding to the oviduct epithelium was related to fertility. Impaired functions of sperm membrane proteins that are critical for normal sperm interactions within the female reproductive tract are not always measured during routine semen analyses and therefore likely contribute to idiopathic subfertility. Sperm must undergo important capacitation-like changes for normal fertilization such as a change in motility parameters and an increase in intracellular calcium. Transient receptor potential channel-2 (TRPP2) is a membrane protein with regulatory roles in ion homeostasis. TRPP2 is important for sperm motility, storage and male fertility in lower organisms (Drosophila melanogaster), but the protein has not been
described in mammalian sperm. My objective was to identify, localize and begin to determine the functions of TRPP2 in porcine sperm. TRPP2 was detected in both capacitated and non-capacitated boar sperm and was localized to the head and principle piece. Antisera to TRPP2 inhibited sperm movement and increased tail cross-beat frequency. Sperm incubated with TRPP2 antibody had lower levels of free intracellular calcium and did not experience a rise in calcium over time when maintained in capacitating conditions. We conclude that porcine TRPP2 is a previously unreported mammalian sperm ion channel that regulates capacitation-like changes in boar sperm.
DEDICATION

For my wife Sarah, who saved me from what I might have been and gave me everything to become what I hope to be. None of this is possible without you. Thank you.

……………. and I love you most.
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LIST OF ABBREVIATIONS

AI: artificial insemination
ADPKD: autosomal dominant polycystic kidney disease
AR: acrosome reaction
ART: assisted reproductive technologies
COC: cumulus oocyte complex(s)
FTS: frozen-thawed sperm
ICSI: intracytoplasmic sperm injection
IVF: in vitro fertilization
mTBM: modified-Tris buffered medium
NRR: nonreturn rates
PI: propidium idodide
TRPP2: transient receptor potential channel-2
ZP: zona pellucida
CHAPTER 1
INTRODUCTION

Frozen-thawed boar sperm are rarely used for artificial insemination (AI) in the swine industry because litter sizes and pregnancy rates are decreased compared to AI with cooled semen (Johnson et al., 2000; Knox et al., 2008; Rath et al., 2009). Reasons for reduced fertility are partially attributed to sperm damage induced by cryopreservation that impairs sperm function. In addition to male effects, a long duration of estrus, lengthy ovulation to estrus interval and producing litters (making litter size continuous data rather than simply being pregnant or cycling) all likely contribute to the reduction in fertility when using frozen sperm for AI. Identifying characteristics of sperm that accurately predict fertility would be useful to improve the success of porcine AI with frozen sperm.

The use of frozen boar sperm has many potential advantages over cooled semen. Some of these advantages include increased time for pathogen testing and extended periods for transportation. Although most frozen boar ejaculates result in fertility below industry accepted standards, some have the potential to provide desired results (Ruiz-Sánchez et al., 2006). Accurate fertility estimates of frozen-thawed samples prior to AI are highly desired for improving the success of using frozen boar sperm for commercial swine application. Traditional analyses of sperm quality such as motility and morphology are not adequate for estimating fertility (Gil et al., 2005). Novel assays that measure sperm function and assess multiple sperm traits may prove more accurate in detecting fertility differences among ejaculates. Assays that can identify samples with poor fertilizing ability would allow them to be discarded prior to AI.
Additionally, these assays may be useful for identifying molecular reasons for fertility reduction when using frozen boar sperm for AI.

**My overall goal was to identify characteristics of frozen-thawed boar sperm that provided accurate estimates of fertility.** We combined traditional sperm analyses with novel sperm binding assays that measure sperm function to determine which sperm traits could collectively predict fertility when included in multiple linear regression analyses. A sub-aim of this research was to identify traits of frozen-thawed boar sperm that were related to IVF success (Daigneault *et al.*, 2014).

The final goal of my research was to further examine molecular reasons for differences in boar fertility inspired by results from the preceding experiments. Fertility differences of boar ejaculates were partially explained by sperm binding to oviduct epithelial cells. Our findings revealed a decrease in the ability of frozen boar sperm to bind oviduct epithelial aggregates, but more importantly, a wide range in the number of sperm bound to aggregates was detected among different semen samples (Daigneault *et al.*, 2015). Sperm storage within the female reproductive tract is important for pig fertility (Waberski *et al.*, 2005) yet molecular mediators of this interaction are not fully known. These interactions were therefore a focus of following experiments.

Research conducted with *Drosophila melanogaster* identified TRPP2, (Amo) as a sperm plasma membrane protein required for sperm storage and fertility (*Gao et al.*, 2003; *Watnick et al.*, 2003; *Kottgen et al.*, 2011). *Amo* mutation revealed a novel motility phenotype that is previously undescribed in the animal kingdom (*Kottgen et al.*, 2011). TRPP2 is a membrane protein and has been well characterized for its importance in maintaining ion homeostasis on the
cilium of renal epithelial cells (Foggensteiner et al., 2000; Kottgen & Walz, 2005). I therefore hypothesized that TRPP2 may also be expressed in boar sperm and important for mammalian sperm function. **My objectives were to identify, localize and determine the function(s) of TRPP2 in boar sperm.** Our results demonstrate that TRPP2 is a previously undescribed ion channel regulator in mammalian sperm and is important for motility and calcium trafficking in boar sperm. TRPP2-inhibition prevents capacitation-like changes in boar sperm that are expected during normal sperm interactions within the female reproductive tract.

In conclusion, we defined a robust model for estimating the fertility of frozen boar sperm prior to AI and have identified a novel protein in boar sperm that regulates sperm functions important for fertility.
REFERENCES


CHAPTER 2
LITERATURE REVIEW

Frozen Sperm for Artificial Insemination

The practice of artificial insemination (AI) has made commercial livestock production significantly more efficient than live mating. Collecting semen and artificially depositing the ejaculate into the female reproductive tract improves animal management and allows for quality control analyses of semen among many other benefits. Cooling semen after collection increases the storage time for which sperm can remain viable and allows for ease of transportation. Cryopreservation of sperm further extends the storage time to indefinite. Freezing sperm was first accomplished using the rooster (Polge et al., 1949). Although reductions in fertility from frozen sperm were initially reported, the efficiency of livestock production for many species dramatically increased as methodology and technology improved. Currently, AI with frozen-thawed bull sperm is used almost exclusively in all commercial dairy cattle operations. The availability of frozen sperm makes logistics, cost and timing of insemination simpler than with fresh or cooled semen. Furthermore, ejaculates can be more rigorously tested for pathogens of concern. Conception and pregnancy rates following AI with frozen-thawed sperm across many species are acceptable and in some cases may be more practical than using fresh or cooled semen (Pickett et al., 2000).

The use of frozen-thawed sperm for AI has been comparatively slower for adoption by the swine industry than for other species due to decreased litter sizes as a result of cryopreservation-induced damage to sperm. In fact, conservative estimates of frozen sperm used for AI are less than 1% of all commercial porcine inseminations (Johnson et al., 2000; Knox et
al., 2008). Technological advances that have improved the post-thaw viability of sperm include automated programmable freezers and directional freezing methods (Arav, 2012). These technologies increase the post-thaw viability of sperm and aid in packing a more desirable concentration of sperm that is compatible with the final use. Research in this area has been focused on optimization of cooling rates, extender or diluent composition, cryoprotectant and stabilization of the sperm membrane (Sieme & Oldenhof, 2015). However, important species differences in sperm regarding the composition and content of sperm plasma membrane proteins and phospholipids contribute to varying degrees of cryopreservation success (Parks & Lynch, 1992). Furthermore, because pigs bear litters and have a long estrus to ovulation interval (Almeida et al., 2000), any reduction in semen fertility is more clearly manifested in lower fertility than in singleton-bearing species. Insemination volume, total sperm number, and frequency of inseminations create variability in fertility and contribute to the difficulties of using frozen boar sperm for AI (Spencer et al., 2010). Additionally, the timing of insemination relative to ovulation is difficult to optimize, especially considering the shortened lifespan of frozen sperm compared to fresh or cooled semen (Spencer et al., 2010).

Overall, the success of freezing boar sperm has improved (Okazaki et al., 2011; Didion et al., 2013; Estrada et al., 2014) but the smaller litter sizes produced from AI and poor fertility prediction of frozen ejaculates using traditional analyses have limited the use of frozen sperm by the swine industry (Gil et al., 2005; Ruiz-Sánchez et al., 2006; Lovercamp et al., 2007; Knox, 2011; Broekhuijse et al., 2012). The demand for frozen-thawed boar sperm to use in AI will likely increase as costly disease outbreaks in commercial swine operations continue to threaten. However, more accurate fertility estimates of frozen boar sperm are needed to maintain industry standards for litter size. The wide range in fertility and success of frozen ejaculates among and
within boar semen collections requires further investigation to allow for the identification of poor fertility samples that can be culled prior to insemination. Understanding molecular reasons for differences in fertility among ejaculates and developing sensitive assays to predict fertility are necessary in order to advance the use of frozen-thawed boar sperm for industry application.

*Effects of Cryopreservation on Sperm*

➢ *Membrane Structure*

Sperm have been cryopreserved for a number of domestic and wild species with varying degrees of success. Membrane damage that occurs during the cooling and thawing processes attributes to fertility reduction. The sperm plasma membrane is comprised of amphipathic lipids associated with proteins that play important biological functions within the female reproductive tract. Disturbances in the sperm membrane or structural composition are detrimental to overall sperm function.

The sperm plasma membrane consists of a fluid bilayer of amphipathic lipids that contain both integral and associated peripheral membrane proteins (Parks & Graham, 1992). There is a high degree of compartmentalization consisting of lipid-lipid and lipid-protein interactions that are unevenly distributed throughout the membrane (Parks & Graham, 1992). Sperm membrane function is regulated by compartmentalization factors such as lipid-lipid interactions, lipid immobilization, protein barriers and solubility of lipids and proteins within the membrane (Parks & Graham, 1992). Lipid-lipid interactions inhibit lateral diffusion of interacting lipids. Some lipids become immobilized when they are associated with membrane bound proteins. Membrane structure can be described as lateral organization and diffusion because interactions take place in a fluid membrane (Parks & Graham, 1992).
The sperm plasma membrane has vertical compartmentalization because it is comprised of two leaflets that are asymmetrical. The outer, or extra-cellular leaflet, contains lipids with positive and neutral charges such as phosphatidylcholine, sphingomyelin and glycosphingolipids. Phospholipids such as phosphatidylserine (PS) that do not have a charge, or are anionic, reside on the inner, or cytoplasmic-facing leaflet (Parks & Graham, 1992). Lipid aggregates also exist within membranes and play important transient roles during fusion events. They can form hexagonal arrangements that are not permeable and create tight seals around proteins to prevent lipids that prefer a bilayer structure from forming hexagons. Thus, the lipid bilayer of a membrane is formed and maintained (Parks & Graham, 1992).

Lipids and proteins within sperm membranes are non-covalently bound and are not intimately associated. Under physiological conditions, lipids are miscible unless the membrane is cooled and the lipid bilayer reorganizes. When sperm are cooled, lipids that reach levels below their transition temperature will change from a liquid-crystalline structure to a gel phase and begin to aggregate. Non-bilayer lipids likely undergo a phase transition first during cooling due to a lower phase transition temperature than bilayer lipids. Negative effects occur during warming when lipids integrate with other structures that they are not normally associated with. These interactions potentially disrupt the membrane changing lipid-protein interactions (Parks & Graham, 1992).

There are species differences in the membrane composition of sperm that make them unique. Major lipid classes include phospholipids, glycolipids and sterols. Phospholipids constitute the majority of the lipid bilayer. Boar and bull sperm contain about 50% of phospholipids (Parks & Graham, 1992). Sterol composition is constituted mainly of cholesterol in sperm and modulates bilayer fluidity and stability through interaction with membrane
phospholipids (Parks & Lynch, 1992). In general, mammals that have a high molar ratio of cholesterol to phospholipid have a greater tolerance for cold shock (Parks & Graham, 1992). Differences in composition and thermotropic phase behavior of glycolipids between rooster and mammalian sperm explain why rooster sperm are less susceptible to cold shock (Parks & Lynch, 1992).

- **Cryoprotectant Effects on Sperm**
  
  During freezing, sperm are subjected to many insults including cryoprotectant, changes in volume that affect membrane plasticity, dehydration, phase transitions in membrane phospholipids and intracellular ice formation (Parks & Graham, 1992). Glycerol is a common penetrating cryoprotectant used to dehydrate sperm before freezing. Alternative penetrating cryoprotectants include dimethyl sulfoxide, ethylene glycol and propylene glycol (Purdy, 2006). Although beneficial, glycerol can also have negative effects upon entering the cytoplasm and plasma membrane (Parks & Graham, 1992). Glycerol may bind to phospholipid head groups and interfere with membrane fluidity, membrane bound proteins and glycoproteins. Furthermore, the addition of glycerol to freezing diluents can reduce membrane electrical potential by rearrangement of membrane structures creating gap-junction-like structures (Kachar B, 1985) and cause the two bilayer leaflets to associate and change polymerization of microtubules (Parks & Graham, 1992). The latter effect of glycerol may have negative implications on membrane signal transduction, which is important for fertilization (Tapia et al., 2012). Sperm ATP consumption rises as a result of cryoprotectant due to an increase in ion membrane permeability and stimulation of ion pumps as a result of repeated phosphorylation and dephosphorylation of substrates (Hammerstedt & Graham, 1989; Hammerstedt & Graham, 1992). In contrast to boar sperm, the effects of glycerol are more tolerated by sperm from bucks (Purdy, 2006).
The potential for membrane damage to sperm occurs both during freezing and thawing processes of cryopreservation. Sperm are most susceptible to damage between temperatures of -15 to -60°C (Parks & Graham, 1992). During cooling, penetrating cryoprotectants optimally dehydrate spermatozoa via osmosis and eventually equilibrate with water resulting in similar intracellular and extracellular concentration and osmolarity. The freezing point of sperm is lowered due to less intracellular water and consequently less intracellular ice crystal formation, which is detrimental to sperm (Purdy, 2006). Penetrating cryoprotectants also act as solvents that dissolve sugars and salts in ice crystals (Purdy, 2006) resulting in higher concentration of salts in unfrozen water (Hammerstedt & Graham, 1992). The increased extracellular osmolarity causes cellular and plasma membrane dehydration followed by sperm becoming entrapped in channels of unfrozen solution between ice crystals. The rate at which sperm are cooled has profound effects on ice crystal formation, cell deformation and plasma membrane lateral phase separations or lyotropic phase transitions from lamellar to hexagonal phase (Parks & Graham, 1992; Parks & Lynch, 1992). Damage to sperm during cooling and freezing can be minimized with the addition of non-penetrating cryoprotectants such as egg yolk. Egg yolk contains low-density lipoproteins. Phospholipids within the low-density lipoprotein fraction aid in surrounding the plasma membrane and help to prevent cold shock (Parks & Graham, 1992).

**Cryopreservation of Boar Sperm**

Of all the domestic livestock in which sperm are frozen, the boar is perhaps the most challenging and least successful. Over 90% of all breeding within the swine industry utilizes artificial insemination (AI). Less than 1% of AI is accomplished with frozen sperm (Knox et al., 2008). Although changes in processing, handling and freezing techniques have been applied to boar sperm, low fertility limits industry use of frozen-thawed semen (FTS). Reasons for
reduced fertility of boar FTS remain unclear but may be attributable to distinct physiological characteristics of boar sperm and ejaculate composition. Furthermore, inconsistency of ejaculate quality among boars makes universal methods of handling sperm challenging. Boar breed confounds cryopreservation as demonstrated by the genetic potential of Landrace for ‘good’ freezability compared to Large White or Duroc (Thurston et al., 2002). Mice models also demonstrate that inter-individual variation in sperm freezability as well as differences between strains is due to a genetic component (Tada et al., 1990).

Boar sperm are particularly sensitive to cold shock. When sperm are cooled and reach the transition from liquid to gel phase, there is an increase in phospholipid hydrocarbon chains within the lipid bilayer of the plasma membrane. The result is a decrease in membrane fluidity and a reduction in the mobility of membrane components (Blanch et al., 2012). The variety of phospholipid families in boar sperm membranes cause phase transitions to occur over a wide temperature range. These changes cause lipids to aggregate into small domains within the fluid membrane. The lipids eventually are removed from the membrane causing alterations in lipid-lipid and lipid-protein interactions, which can change the permeability and function of the membrane (Blanch et al., 2012).

Many attempts have been made to improve the success of freezing sperm. The plasma membrane of boar sperm contains a comparatively higher content of protein than cholesterol, helping to explain sperm sensitivity to cold shock and cryopreservation due to membranes undergoing phase transition at relatively higher temperatures as compared to those of other domestic livestock species (Blanch et al., 2012). The cholesterol to phospholipid molar ratio of boar sperm is 0.26 compared to 0.45 for bulls (Parks & Lynch, 1992). The addition of cholesterol to boar sperm causes increased association with cholesterol and fatty acyl chains.
within the plasma membrane and prevents fatty acid interactions with other fatty acids when sperm are cooled (Blanch et al., 2012). Cholesterol loaded cyclodextrins deliver cholesterol into the sperm membrane via simple diffusion. Adding cholesterol results in lipid transition to gel state occurring at below normal temperatures for that particular lipid, thus preventing membrane damage that might occur within the fluid state (Blanch et al., 2012). The addition of cholesterol to boar sperm membranes has marginally improved viability but not the fertility of frozen sperm (Blanch et al., 2012). These characteristics of boar sperm membranes increase the likelihood of irreversible membrane disturbance during freezing and thawing processes (Parks & Graham, 1992).

In addition to physiological attributes and genetic components there are variations in freezability of sperm within the same boar (inter-ejaculate differences) (Pena et al., 2006; Roca et al., 2006). Epididymal sperm have an increased freezability as compared to ejaculated boar sperm and have improved post-thaw motility among boars previously identified as ‘poor’ freezers (Okazaki et al., 2012). For these reasons, seminal plasma has been identified by many as a key component that adds variability to post-thaw quality of sperm (Berger & Clegg, 1985).

Gram-negative and gram-positive bacteria are present in boar ejaculates. Although semen extenders contain antibiotics such as penicillin G, and amikamycin, they induce bacteriolysis. This event causes the release of lipopolysaccharides, which stimulate Toll-like receptor (TLR) 4 on sperm and, through a signaling cascade, induces sperm membrane abnormality and apoptosis (Okazaki & Shimada, 2012). The effects of seminal plasma on sperm sensitivity to cold shock and freezability are well documented but may prove of low significance considering that seminal plasma is removed prior to freezing. However, the length
of time that sperm are exposed to plasma and the variation in protein content due to nutritive differences among boars may induce important changes to acrosome integrity and motility.

**Laboratory Estimates of Fertility for Fresh and Frozen Sperm**

Conventional estimates of male fertility such as sperm motility and morphology only provide a limited estimate of fertility and sperm function (Amann, 1989) and have not historically provided accurate estimates of fertility (Gil et al., 2005). Traditional analyses of boar sperm, such as motility, morphology and viability are only moderately useful for identifying boars of low fertility (Flowers, 1997; Gadea et al., 2004; Gadea, 2005; Alm et al., 2006; Lovercamp et al., 2007; Broekhuijse et al., 2012). Laboratory assays that test sperm functions may provide novel tools for estimating boar fertility. Assays that are designed to closely resemble sperm interactions within the female reproductive tract may be more predictive of in vivo function and useful when identifying reasons for reduced fertility (Fazeli et al., 1997; Braundmeier et al., 2004; Collins et al., 2008).

Numerous attempts have been made to identify in vitro sperm parameters that are directly related to fertility (Holt et al., 1997; Tardif et al., 1999; Larsson & Rodríguez-Martínez, 2000; Waberski et al., 2005; Alm et al., 2006; Broekhuijse et al., 2012; Schulze et al., 2013). Estimating boar fertility through multiple trait comparisons has been more effective than through single trait analysis (Holt et al., 1997; Foote, 2003). However, attempts to estimate fertility have failed to produce accurate models and leave much of differences in fertility among boars (80%) unexplained (Holt et al., 1997). Although traditional semen evaluation parameters such as motility and morphology are often included in models that are used to estimate fertility, there are usually poor correlation among these sperm characteristics and the ability to predict fertility (Gadea et al., 2004), especially when trying to rank boars of normal fertility (Alm et al., 2006).
Penetration assays that evaluate the number of sperm that penetrate the zona pellucida are thought to be the most commonly used marker of in vitro boar sperm fertilizing ability (Gil et al., 2008). Although high penetration rates are correlated with polyspermy (Suzuki et al., 2003; Gil et al., 2008), polyspermy does not always influence blastocyst development (Han et al., 1999). Confounding factors include breed influences, which were more prevalent than individual male variation in a study of 15 boars and 3 different breeds (Suzuki et al., 2003; Gil et al., 2008). Sperm from Large White boars penetrated at higher rates than Landrance or Duroc, resulting in higher polyspermic fertilization (Suzuki et al., 2003). The fraction of ejaculate also contributes to variability observed in sperm penetration and polyspermic fertilization, demonstrating that the first sperm rich fraction of the ejaculate (F1) should be used for IVF (Xu et al., 1996; Xu et al., 1996; Gil et al., 2008).

The Sperm Oviduct Reservoir and its Relationship to Fertility

Mammalian sperm that are deposited into the female reproductive tract are stored in the oviduct for varying time lengths that are species dependent. Sperm bind to epithelial cells in the lower portion of the oviduct, or isthmus, after semen deposition and are maintained in a quiescent state prior to capacitation and release. The formation of a functional reservoir has been indicated for pigs, mice, hamsters, rabbits, horses and cows (Petrunkina et al., 2002). The functions of a sperm reservoir include control of sperm transport, maintenance of sperm viability and modulation of capacitation (Petrunkina et al., 2002). The caudal region of the isthmus appears to be specialized for regulating events associated with capacitation in the pig.

Sperm-oviduct binding assays are sometimes used to determine differences in sperm binding ability among semen samples and have been used to predict reproductive performance of domestic livestock (De Pauw et al., 2002; Petrunkina et al., 2002; Braundmeier et al., 2004),
although few reports have thus far been conducted with cryopreserved sperm. Additionally, oviduct cell aggregates are more physiologic than using epithelial monolayers due to the increased polarity of aggregate structures (Petrunkina et al., 2002). These assays are frequently used with cooled semen, so there is little information available regarding the ability of cryopreserved sperm to bind to oviduct epithelium. However, others have demonstrated that cryopreservation induces precocious capacitation of boar sperm and/or acrosome damage (Bailey et al., 2008) and therefore reduces the ability of cryopreserved boar sperm to bind to oviduct cells (Lefebvre & Suarez, 1996; Fazeli et al., 1999; Bailey et al., 2008).

Sperm found in the oviduct tend to lack cytoplasmic droplets, have good morphology and are characterized by an intact acrosome and plasma membrane. Viable sperm usually bind deep within epithelial crypts whereas damaged sperm bind more towards the folds and lumen (Tummaruk & Tienthai, 2010). Sperm bound to oviduct epithelium have comparatively lower intracellular Ca$^{2+}$ compared to unbound sperm, suppressed tyrosine phosphorylation and normal chromatin structure; all characteristics of non-capacitated sperm (Petrunkina et al., 2001; Waberski et al., 2005). Selection of this sub-population of sperm by the oviduct suggests a critical role for the oviduct in fertility. Although release of sperm from the reservoir is not fully understood, hormonal fluxes in coordination with sperm activated modulation of oviductal gene expression results in changes of secretory proteome in the oviduct (Topfer-Petersen et al., 2008). Sperm release may also be mediated by factors originating from follicular fluid and COCs and is a current investigation of our laboratory (Machado et al., submitted).

Sperm adhere to the lower portion of the isthmus through a series of carbohydrate-mediated interactions. Sperm attach to the apical plasma membrane of both ciliated and secretory cells. Glycans, or sugars, on the apical portion of epithelium bind to proteins, or
lectins, on the sperm membrane. Sugar residues on glycoproteins that are thought to be partially responsible for sperm binding include terminal sialic acid for the hamster and rat, fucose in bull sperm and galactose in stallion sperm (Topfer-Petersen et al., 2008). Mannosyl glycoconjugates were previously thought to be main mediators of sperm-oviduct binding in the pig (Wagner et al., 2002). More recently, our lab has shown that glycans, such as biantennary sialylated lactosamine and sulfated Lewis X trisaccharide are more boar specific sperm glycan binding motifs (Kadirvel et al., 2012; Machado et al., 2014).

Bioassays that measure sperm binding to oviduct cells may be good indicators of the capacity for samples to form sperm reservoirs. The reservoir may be of greater significance for litter bearing species, females that display intermittent ovulation, prolonged estrus, long estrus to ovulation intervals or when semen is deposited well before ovulation. One such assay yielded a positive relationship with the field fertility of bulls expressed by the 56 day non-return rate (NRR) (De Pauw et al., 2002). Interestingly, the relationship between number of sperm bound and NRR was found only when the membrane integrity of the initial sperm sample was > 60%. Furthermore, sperm incubation time with epithelial extracts for 24 h was positively correlated with sire NRR (De Pauw et al., 2002). The sensitivity and more physiologic nature of oviduct binding assays may be useful for detecting differences in fertility among males, help to understand sperm binding and release and provide molecular understanding and clinical reasons for reduced fertility of AI with frozen boar sperm.

Sperm-Zona Interactions and their Relationship to Fertility

The zona pellucida (ZP) is a thin layer surrounding the oocyte and is comprised primarily of a glycoprotein matrix. The major functions of the ZP are to protect the oocyte from the surrounding environment, provide a binding surface for sperm and aid in prevention of
polyspermy. Capacitated and acrosome reacted sperm bind and penetrate the ZP during natural and artificial fertilization (Jin et al., 2011). The fertilizing potential of sperm is often measured by their ability to cross the ZP (Waberski et al., 2005). Sperm-zona binding, penetration and IVF are all ways of measuring sperm-zona interactions (Waberski et al., 2005).

Sperm may partially interact with the ZP through acrosin, a serine proteinase that is post-meiotically expressed in male germ cells and stored during differentiation within the acrosomal cap in its inactive form, proacrosin (Topfer-Petersen et al., 2008). Proacrosin has a high affinity binding activity ($K_d 10^{-8}$) to the sulfated polysaccharide fucoidan and some affinity for ZP-glycoproteins. Proacrosin conversion to the mature $\beta$ acrosin is autocatalytic, dependent upon pH and is regulated by zona peptides (Topfer-Petersen et al., 2008).

Sperm-zona binding is sometimes employed as a predictor for IVF and has been used in human clinics when determining IVF or intracytoplasmic sperm injection (ICSI) candidacy of sperm samples (Waberski et al., 2005). Sperm penetrating the ZP are indicative of a subpopulation of sperm capable of binding the zona, undergoing the acrosome reaction and entering the perivitelline space. However, it is possible that sperm penetration assays are more predictive of fertilizing potential than zona binding assays because less than 15% of sperm that bind the zona penetrate the oocyte (Lynham & Harrison, 1998; Waberski et al., 2005). Variation in success when using these assays to predict fertility with boar sperm has been attributed to experimental design, lack of assay validation and inconsistency with technique and boar collection (Waberski et al., 2005).

The hemizona assay (HZA) employs the use of two matching ZP halves allowing for a direct comparison of two sperm suspensions; a testing sample and an internal control (Burkman
et al., 1988; Waberski et al., 2005). Ranking boars on the ability of their sperm to bind zonae was not successful for one group due to large variations observed in the number of sperm bound to hemizona among three ejaculates from the same boar between replicates (Waberski et al., 2005). Cryopreserved sperm may have a decreased ability to bind to the zona due to precocious capacitation often observed in thawed samples (Bailey et al., 2008). Cryo-capacitation also reduces the ability of sperm to bind oviduct epithelial cells (Ivanova & Mollova, 1993; Lefebvre & Suarez, 1996; Fazeli et al., 1999; Bailey et al., 2008). The decreased ability of sperm to bind to either of these two matrices is plausible explanation for the reduced fertility observed when frozen boar sperm are used for AI.

Our laboratory has successfully employed zona binding assays using fresh boar semen that positively correlate with litter size ($r^2 = 0.62$) when using homospermic insemination. No relationship was detected between zona binding and farrowing (Braundmeier et al., 2004). In a different experiment, zona binding ability was not significantly correlated with fertility when fertility was determined with heterospermic AI and zona binding was used as a single predictor of fertility. However, zona binding ability of sperm was useful for predicting fertility when combined with other sperm traits (Collins et al., 2008). Similar studies suggest that litter size may be a better estimate of fertility than farrowing rate (Xu et al., 1998). Insemination dose related to litter size but not farrowing rate may be explained by the fact that farrowing rate only gives two possible outcomes (pregnant or not pregnant) whereas litter size has the potential for many measures (Flowers, 2002). Although successful in swine, our laboratory did not find correlation between zona binding and NRR in cattle (Braundmeier et al., 2002). These findings may be explained by comparison of a singleton versus litter bearing species where there are several estimates of fertility rather than one (Xu et al., 1998). Furthermore, for years the cattle
industry has selected against sub-fertile bulls resulting in a narrower range of male fertility compared to the swine industry (Braundmeier et al., 2002).

Commercial versions of sperm binding assays (SBA) show reasonable predictability of subfertile boars, although information may be of limited value due to cost effectiveness (Waberski et al., 2005). Importantly, these assays were only useful for indicating subfertile boars based on D 60 non-return rate and/or litter size, and are therefore not useful for prospective analyses of ejaculates (Waberski et al., 2005). The percentage of bound sperm was independent of motility and abnormal morphology. Lack of fertility prediction was attributed to ejaculate variability and sperm Percoll selection, which has been thought to remove seminal fluid contribution of adhesive proteins necessary for normal zona binding (Waberski et al., 2005). Low SBA sensitivity may also be explained by a high tendency for head-to-head agglutination and non-specific collision frequency of sperm resulting in non-physiological binding (Waberski et al., 2005).

**IVF with frozen-thawed boar sperm and its relationship to fertility**

Using frozen-thawed boar sperm for IVF produces inconsistent fertilization and developmental results (Nagai et al., 1988; Zheng et al., 1992; Abeydeera & Day, 1997a; Córdova et al., 1997; Rath & Niemann, 1997; Suzuki et al., 2005). Frozen–thawed boar sperm is sometimes used for IVF in attempt to reduce the effects of inter and intra-ejaculate variability and provide more opportunity for repeatability of experiments compared to liquid semen. However, the efficacy of porcine IVF, especially with frozen sperm, is hindered by variation between boars in freezability of their spermatozoa (Gil et al., 2008) and also due to factors unrelated to sperm (Xu et al., 1996; Long et al., 1999; Gil et al., 2008). Furthermore, optimal culture conditions play a large role in IVF success (Abeydeera & Day, 1997b; Abeydeera, 2002).
IVF conditions such as sperm number, caffeine concentration, and gamete co-incubation time are often optimized for each boar (Abeydeera, 2002). Although frozen sperm allows for a more consistent and reduced male factor, a great deal of variability both within and among boars still exists (Gil et al., 2008). Conventional evaluation of sperm parameters such as motility, morphology, viability and membrane integrity are less than adequate for predicting IVF success (Gil et al., 2008). For all of these reasons, there seems to be an inconsistent and often low correlation between IVF and fertility (Larsson & Rodríguez-Martínez, 2000; Ruiz-Sánchez et al., 2006).

Polyspermic fertilization is a frequent occurrence in porcine IVF. In mammals, cortical granule exocytosis, remodeling of the perivitelline space and modifying the ZP all prevent polyspermy (Abeydeera, 2002). These events are triggered by sperm penetration. Optimizing culture conditions and gamete co-incubation time decreases polyspermy (Abeydeera, 2002; Gil et al., 2008). Delayed zona reaction during IVF followed by simultaneous sperm penetration is a plausible explanation of polyspermy in porcine IVF (Wang et al., 2003). The sperm: oocyte ratio has been of interest to minimize polyspermy without decreasing penetration rates (Rath, 1992; Gil et al., 2008). However, IVF is a highly artificial system and there are a number of environmental factors that likely contribute to the variability in fertilization outcomes (Abeydeera & Day, 1997b). Interestingly, ovulated oocytes are less susceptible to polyspermy (28%) than in vitro matured oocytes (65%) following IVF (Wang et al., 1998). These data suggest that the oviduct may play critical roles in preventing polyspermy, as evidenced by changes in fertilization after transfer of in vitro derived oocytes to oviducts of gilts in heat (Day et al., 2000). Proper use of IVF medium also influences polyspermy. A modified Tris-buffered medium (mTBM) is more efficient than modified TALP at reducing polyspermy (Kidson et al.,
2001; Matiniez-Madrid et al., 2001). Relatively high Ca\(^{2+}\) concentration and lack of bicarbonate ions probably contribute to the superiority of mTBM as a fertilization medium by decreasing capacitation-like status and spontaneous acrosome reaction (Abeydeera & Day, 1997b).

A further hindrance to using frozen-thawed porcine sperm in IVF is increased production of reactive oxygen species (ROS) compared to fresh semen (Jones et al., 1979; Kessopoulou et al., 1992; Gil et al., 2008). Due to the toxic nature of ROS, some alleviation of detrimental effects has been observed by reducing gamete co-incubation time (Gil et al., 2008). Production of ROS may be more prevalent when using frozen sperm for IVF due to the increased incidence of dead sperm and comparatively high total sperm numbers used for semination as compared to fresh semen. Extracellular supplementation and intracellular glutathione production are proposed to regulate ROS (Abeydeera, 2002). Media supplementation with epidermal growth factor (EGF) and cysteine indirectly stimulate glutathione production, promote oocyte cytoplasmic maturation and reduce multiple pronuclei (Ding & Foxcroft, 1994). Alternative media supplement can be beneficial for aiding in capacitation-like status of sperm for IVF. Caffeine has been replaced with adenosine and fertilization-promoting peptide (FPP) in an attempt to minimize spontaneous acrosome reaction, which may be related to polyspermy (Funahashi et al., 2000; Gil et al., 2008). Hyaluronic acid seems to have similar affects and may be a more physiological compound because it is present in oviduct fluid and secreted by COCs during maturation (Tienthai et al., 2000; Gil et al., 2008). Sperm from different boars respond variably to these compounds and a supplement such as caffeine may be necessary for some but not for all boars (Gil et al., 2008).

Predicting male fertility with in vitro assays such as IVF is attractive considering cost and time effectiveness when compared to AI. There may be individual measures of IVF success that
are predictive of boar fertility when analyzed with traditional and novel sperm analyses (Ruiz-Sánchez et al., 2006). In boars, zona pellucida penetration rate and oocyte penetration have been successful for screening subfertile boars and ejaculates (Berger & Parker, 1989; Ivanova & Mollova, 1993; Gadea et al., 1998; Ruiz-Sánchez et al., 2006). Fertility has been partly explained by male pronuclear formation from sperm of superior boar ejaculates (Ruiz-Sánchez et al., 2006). Although correlation was low, the fertility model was strengthened by addition of multiple variables (Ruiz-Sánchez et al., 2006). Boar sperm IVF measures that were not affected by time and were different among boars had some predictive value for identifying subfertile boars with above average motility (Ruiz-Sánchez et al., 2006). Multivariate regression that includes IVF penetration measures may be instrumental for identifying subfertile boars to estimate fertility.

**Transient Receptor Potential Polycystin 2 (TRPP2)**

The polycystic kidney disease gene (PKD) encodes the polycystin-2 protein, also known as transient receptor potential polycystin 2 (TRPP2) (Mochizuki et al., 1996; Hofherr et al., 2014). TRPP2 belongs to the TRPP category, one of seven mammalian categories of transient receptor potential channels (TRPs). The TRPs span the plasma membrane six times and have both their NH₂ and COOH termini located in the cytoplasm. All proteins in the TRP family are categorized by their primary sequence (Montell, 2005) because many of them are activated by common modes. These proteins are activated by ligand binding and respond to sensory stimuli such as temperature, heat, pain, taste and pheromone signaling (Nilius & Owsianik, 2011). They play extensive roles in ion homeostasis and are also have motile functions, such as muscle contraction (Nilius & Owsianik, 2011). The major function of TRPP2 is as a non-selective cation channel; its function in human autosomal dominant polycystic kidney disease (ADPKD)
has been studied extensively (Ma et al., 2005; Köttgen, 2007; Cai et al., 2014), although founding knowledge was gained using *Drosophila* models (Cosens & Manning, 1969). Mutations of *PKD1* and *PKD2* are causes of fatal renal failure (Sutter & Germino, 2003) and together account for the most common inherited renal disease with PKD2 accounting for 15% of all persons with autosomal dominant PKD (Koptides et al., 1999). TRPP2 regulates permeation through the plasma membrane of calcium and magnesium by forming a heteromultimer functional ion complex with TRPP1 between transmembrane segments five and six (Tsiokas et al., 1997; Owsianik et al., 2006; Tsiokas, 2009; Zhu et al., 2011).

*Localization and Activation of TRPP2*

TRPP2 is well defined as a transmembrane protein but an intracellular localization has been described in several cells. Different cell lines and culture conditions have revealed the presence of TRPP2 at the basolateral plasma membrane, endoplasmic reticulum and primary cilium of renal epithelial cells (Foggensteiner et al., 2000; Kottgen & Walz, 2005). The trafficking of TRPP2 from the endoplasmic reticulum to the plasma membrane is dependent upon adaptor proteins that recognize the carboxy-terminal domain of TRPP2, directing it from the ER to the Golgi upon phosphorylation of TRPP2 at Ser^{812} (Kottgen et al., 2005; Morick et al., 2013). Furthermore, the subcellular localization of TRPP2 modulates its function (Fu et al., 2008). In primary cilia, the Ca^{2+} channel activity is mechanosensitive and can be stimulated by fluid shear stress causing an increase of intracellular Ca^{2+} (Nauli et al., 2003).

*TRPP2 Activation and Regulation*

TRPP2 can be activated by many different modes including mechanosensation, receptor operation, cell adhesion and potentially by changes in cellular voltage (Tsiokas, 2009). All of
these modes of activation may be experienced by sperm during critical reproductive events within the female reproductive tract. Furthermore, the functions of TRPP2 may occur at the plasma membrane, primary cilium or both. For example, fluid shear stress applied to ciliated renal epithelial cells caused a rise of intracellular Ca\(^{2+}\) that was attenuated by a TRPP2 specific antibody specific to the first extracellular loop (Nauli et al., 2003). TRPP2 also behaves as a receptor-operated (ROR) channel in that it can respond to GPCR/RTK-induced activation of second messengers, but not store depletion of Ca\(^{2+}\) (Tsiokas, 2009). Supporting examples of ROR TRPP2 are seen in response to extracellular epidermal growth factor (EGF) in the kidney epithelial cell line LLC-PK1 where addition of EGF in extracellular medium caused a rise in intracellular Ca\(^{2+}\), but did not activate store-operated Ca\(^{2+}\) channels (Ma et al., 2005; Tsiokas, 2009).

Although much of TRPP2 activation is associated in extracellular regions of transmembrane proteins, TRPP2 is also gated by an intracellular protein, mammalian Diaphanous related formin 1 (mDia1). mDia functions as an intracellular voltage-dependent gate for TRPP2 at the plasma membrane of LLC-PK1 cells and does so by binding to the COOH termini of TRPP2 during resting physiologic potentials (Bai et al., 2008). The activity of TRPP2 is blocked by mDia1 at negative potentials but can be released at positive potentials through EGF (Bai et al., 2008). However, the physiological relevance of this observation to sperm is unclear because sperm hyperpolarize during capacitation (Escoffier et al., 2015).

The activation of TRPP2 is not solely dependent upon its interaction with TRPP1. When stimulated by bradykinin (BK), TRPP2 forms a functional channel complex with members of nonhomologous groups of the TRP family, such as TRPC1 (transient receptor potential cation channels) (Tsiokas, 2009). The significance of this observation is that endothelial cells sense
fluid shear stress through the activation of BK-2 receptor (Chachisvilis et al., 2006). The TRPP2/TRPC1 complex has thus been proposed as a mechanosensor during mechanical bending of the primary cilium (Tsiokas, 2009) and bending of the cilium causes a rise in intracellular Ca\(^{2+}\) through the activation of phospholipase C (Praetorius & Spring, 2003). Because both TRPP2 and EGFR have been identified in renal cilium, TRPP2 may also function as an EGF-activated Ca\(^{2+}\) channel at this location (Tsiokas, 2009). Although many of these proposed models of TRPP2 activation seem plausible, it is important to note that most of them have not been thoroughly tested. However, given the diversity of interactions with other proteins but more importantly, the specificity of interactions at different locations on renal epithelium, TRPP2 on sperm may be activated at different times within the female reproductive tract and carry out more than one important function.

**TRPP2: Reproduction and Fertility**

Although much is known about the epidemiology of polycystic kidney disease, there is comparatively little information regarding direct implications of PKD2 mutations on reproduction, fertility and development. Most reports have focused on reasons for embryonic loss associated with polycystic kidney disease models. For example, mice that are homozygous for deletion of TRPP2 show abnormalities in left-right embryo axis patterning and abnormal heart loop morphology (Pennekamp et al., 2002). TRPP2 mutant embryos are typically lethal and conditional knockout mice models have provided insight to placental abnormalities that could be rescued by tetraploid aggregation (Garcia-Gonzalez et al., 2010).

At the sperm level, the plasma membrane association of TRPP2 with TRPP1 is currently the most likely interaction that is related to reproductive function and fertilization (see Fig. 2.1).
TRPP1 and TRPP2 are non-covalently bound and form a heteromultimeric functional ion channel complex at the COOH termini (Tsiokas et al., 1999; Wei et al., 2007). The extracellular portion of PKD1 contains several domains described in cell adhesion models (Tsiokas, 2009). The sea urchin REJ domain (receptor of egg jelly) has extensive homology with human TRPP1 and is thought to directly mediate sperm-egg interactions in sea urchins (Moy et al., 1996; Hughes et al., 1999). Interestingly, the heteromultimeric TRPP1/TRPP2 complex is found exclusively at the plasma membrane of the acrosomal complex in sea urchin sperm (Neill et al., 2004) and has been postulated to play roles in ion regulation that are important for the acrosome reaction. Further evidence for this hypothesis is supported by a single-cell green alga, chlamydomonas reinhardtii, where the TRPP2 homolog was identified on the flagellum and is essential for gamete mating (Pazour et al., 2005). RNAi-mediated PKD2 knockdown blocked early mating events but these events could be rescued downstream (Tsiokas, 2009). Early in mating, C. reinhardtii is activated by gamete cell adhesion. Given the evolutionary conservation of cilia and flagella (Pazour et al., 2005), it is plausible that mammalian TRPP2 on the sperm flagellum could also be activated by cell adhesion and activation could be dependent or independent of TRPP1. However, the exact mechanism of activation is likely to be different in mammals because most evidence in C. reinhardtii indicates a Ca^{2+} efflux upon TRPP2 activation (Bloodgood & Levin, 1983; Goodenough et al., 1993) from the cell wall and not the ER, rather than the characteristic Ca^{2+} influx observed in mammalian sperm during capacitation and the acrosome reaction. Furthermore, a minimal reduction in mating was reported upon chelation of extracellular medium with EGTA (Goodenough et al., 1993), which is again contradictory to what one would expect during mammalian fertilization.
These proposed models for TRPP2 activation are likely confounded by the location of TRPP2 on the specific cell of interest. For example, although the location of TRPP2 has been described on the plasma membrane, the function of this protein could be quite different when it is located on the cilium or flagellum depending upon its interaction with other proteins. One such model for TRPP2 function on the cilium suggests that Ca\(^{2+}\) induces a rise in cAMP, but that these events were localized to the flagellar membrane with no evidence of increased cytosolic free Ca\(^{2+}\) during mating-related events (Goodenough et al., 1993). Clearly, TRPP2 plays an important role during mating but how these events relate to mammalian sperm, whether they are consistent across kingdoms and if TRPP2 interaction with other proteins is dependent upon cellular location are all questions that still remain unanswered.

The only current evidence of TRPP2 function on sperm in any animal species has been described in *Drosophila melanogaster*. The location of TRPP2 was initially reported at the flagella tip of *d. melanogaster* sperm and targeted mutation of TRPP2 produced some very important phenotypes (Watnick et al., 2003). Male fruit flies continued to produce sperm but these mutant sperm remained in the uterus and were not present in the sperm storage areas after semen deposition. The TRPP2 mutation was thus named Amo (*almost there*) to describe this phenotype, and was identified as the homolog or human TRPP2. RT-PCR of sex-sorted flies revealed that Amo was male germ-line specific and that females completely lacked the protein. Amo (TRPP2) was disrupted by homologous and intra-genic recombination to produce male fruit flies that were severely subfertile. Although males were nearly infertile, the rate of flagella beating was not different than wild-type sperm. Mutant males also displayed normal mating behavior. In agreement with the above report, a different research group published identical findings on the same day in the same journal, (Gao et al., 2003) but indicated that Amo was also
identified on the head and acrosomal region of sperm and that the expression was found along the whole sperm in a punctuate pattern. Furthermore, rare mutant sperm which made it to sperm storage areas were able to fertilize eggs (3%) and produce viable young. More typically, sperm remained widespread in the uterus instead of congregating towards the anterior portion of the uterus before entering sperm storage areas, indicating that Amo had important function in directional sperm movement. The authors suggest that because Amo is expressed at different points along the flagellum, that activation of the protein could provide a signal for initiating asymmetric tail beating or steering by transient mediation of Ca$^{2+}$ influx. Further research described that the location of Amo in spermatocytes was restricted to the ER, but was again expressed at the tip of the tail of mature sperm (Kottgen et al., 2011). A more novel finding described that both wild type and Amo mutant sperm swim backwards in the female reproductive tract and is the first such report described for any species. The beat frequency of Amo mutant sperm in the female tract was similar to wild type, but failed to increase over time and the swimming speed was significantly slower than wild type sperm. All findings clearly conclude that Amo (TRPP2) is required for sperm storage in *d. melanogaster*.

The importance of TRPP2 for mammalian sperm has not been determined, but a few cases of male infertility have been described for humans with ADPKD (Kanagarajah et al., 2012). Most of these cases focus on men with seminal vesicle dilation (Reig et al., 2015) azoospermia, oligospermia, and ejaculatory duct obstruction likely due to cyst formation (Hendry et al., 1998; Orhan et al., 2000; Reig et al., 2015). However, the sperm of some patients were noted to contain flagellar defects related to the axoneme and microtubules that resulted in failed embryo development following ICSI (Okada et al., 1999). Other cases point to decreased pituitary function as the primary cause of infertility (Vecchi et al., 2003). Although these cases
of infertility are related to polycystic kidney disease, no studies have been reported regarding a possible function of TRPP2 in sperm from patients with this disease.

Pigs have been used as a model to study ADPKD and TRPP2 RNA is highly expressed in many tissues including the testis, but the protein has not been reported at this location (Wang et al., 2011). Porcine TRPP2 (NCBI Reference Sequence: NP_001232908.1) is a 970-amino acid sequence that has six transmembrane spans with intracellular amino and- and carboxy-termini (Mochizuki et al., 1996) and is 93% homologous to huTRPP2. To date, no prior information exists to identify the presence, localization or function of TRPP2 on boar sperm. Based on the importance of TRPP2 function in *d. melanogaster* sperm, and given the ability to recapitulate PKD in pigs, I am compelled to explore if TRPP2 is present in boar sperm and to identify possible novel functions of TRPP2 previously undescribed for mammalian sperm.
FIGURES

Figure 2.1. TRPP2 and TRPP1 interaction at the plasma membrane.

Adapted from Tsiokas, L. Am J Physiol Renal Physiol; 2009
REFERENCES


CHAPTER 3
NOVEL AND TRADITIONAL TRAITS OF FROZEN-THAWED PORCINE SPERM THAT ARE RELATED TO IN VITRO FERTILIZATION SUCCESS

ABSTRACT

Cryopreserved semen allows the use of single ejaculates for repeated analyses, potentially improving in vitro fertilization (IVF) consistency by eliminating inter-ejaculate variability observed with fresh semen. However, the freezing and thawing processes result in compromised sperm function and IVF success. Semen samples are often screened for motility prior to use for IVF. Samples that fall below a designated motility threshold may be discarded. Our objectives were to determine if post-thaw sperm motility, other traits that may be indicative of sperm function or a novel assay of oviduct binding were related to IVF success. Semen from 16 boars was cooled to 15°C for overnight shipment prior to cryopreservation. Semen was thawed and motility was recorded microscopically and confirmed using Computer Automated Sperm Assessment (CASA). Each sample was tested by IVF in two to three independent replicates. Regression and correlation analyses were employed to determine the interrelationships between sperm traits and the relationships between post-thaw motility, sperm-oviduct binding and IVF outcomes. Among sperm traits examined, sperm acrosome integrity was negatively correlated with post-thaw motility ($r^2 = 0.64$) but not with IVF results. The number of sperm bound to oviduct aggregates was correlated with IVF polyspermy rates ($r^2 = 0.62$, $P < 0.05$) but less with overall IVF fertilization rates ($r^2 = 0.31$, $P > 0.10$). There was some relationship of post-thaw motility with IVF monospermic fertilization ($P = 0.06$, $r^2 = 0.08$) but not to other IVF outcomes. Our results indicate that post-thaw motility of frozen-thawed boar sperm is strongly related to

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acrosome integrity but has limited use for predicting IVF success. The number of sperm bound to oviduct cells was related to IVF polyspermy rates and may be more indicative of in vitro sperm function than traditional sperm motility and acrosome status evaluation.

Keywords: IVF, sperm, cryopreserved, motility, oviduct, acrosome

INTRODUCTION

Frozen-thawed porcine semen has advantages over fresh and cooled semen when used for in vitro fertilization (IVF). Semen can be used from a single ejaculate, thereby eliminating sperm source variability and improving consistency in experimental parameters. Frozen-thawed semen can be used repeatedly and for multiple experimental objectives. However, IVF using cryopreserved boar semen often results in lower fertilization rates than fresh semen [1-5] because boar sperm are susceptible to damage during cryopreservation and thawing [6].

Although moderate and varied success has been achieved when using frozen-thawed boar sperm for IVF, [2, 7-9] average IVF success and embryonic development rates with porcine cryopreserved semen are lower than other domestic species, such as cattle [10]. The reason for the low adoption of frozen semen for IVF is likely the same as the cause for low adoption of frozen semen for AI, the reduced fertility caused by cryopreservation [11-13]. Furthermore, the efficacy of porcine IVF with frozen-thawed semen is often hindered by the large male-to-male variability in the success of maintaining sperm function following cryopreservation [6]. The difference in IVF success is notable even among ejaculates from the same boar [14-16]. An additional challenge is the high polyspermy rates with swine gametes, regardless of whether sperm have been cryopreserved [6, 17, 18].
Identification of sperm traits that predict IVF success would be useful for screening frozen-thawed boar ejaculates for compatibility with IVF. Currently, damage to cryopreserved sperm is commonly assessed by visual observation of post-thaw motility [6]. A semen sample containing sperm with motility below some threshold is typically discarded rather than used for IVF. However, evaluating semen based upon motility and morphology may be inadequate to assess boar sperm for use in IVF [3, 19, 20]. Even including other measurements such as viability and acrosome integrity still does not predict IVF success adequately [6].

To develop more effective screening assays to assess the potential IVF success with frozen boar sperm, assays of additional traits necessary for fertility may be needed. Cryopreservation of boar sperm appears to induce a precocious capacitation state [21] that may render sperm less able to bind to the oviduct epithelium to form a reservoir [22, 23]. The oviduct epithelium plays an important role in regulating normal fertilization [24, 25]. Thus, an assay that measures the ability of sperm to bind the oviduct was employed to assess sperm function, in vitro [26].

Our objectives were to determine if post-thaw motility of frozen-thawed boar sperm was related to IVF success and if additional sperm parameters such as acrosome integrity and sperm binding to oviduct cell aggregates might also be useful.

MATERIALS AND METHODS

Experimental Approach

The University of Illinois Animal Care and Use Committee approved use of animals in these experiments.
**Sperm Collection and Freezing**

Ejaculates from Landrace, Large White, Composite and Duroc boars from a total of 19 commercial boars within the United States were used for all experiments. The sperm rich fraction of one ejaculate from each boar was collected using the gloved hand technique. Semen was diluted using Androstar® Plus (Minitube of America, Verona, WI) at 37°C, cooled to 5°C over 2 h and then shipped to the USDA facility in Ft. Collins, Colorado for processing within 24 h of collection. Sperm were separated from seminal plasma and frozen in 0.5 mL straws at a final concentration of $1.4 \times 10^9$ sperm/mL in egg yolk and glycerol [27]. Straws were then shipped in liquid nitrogen vapor to the University of Illinois for these experiments.

Boar semen was also collected at the University of Illinois Imported Swine Research Lab from a single Yorkshire boar throughout the duration of the experiment to serve as a cooled, internal system control. The control boar was 18 months of age at the beginning of the experiment and was collected 1-2 times per week. The sperm rich fraction of semen was collected by hand manipulation using the double-gloved method. The gel portion was discarded through filtration and raw semen transported for extension within an hour of collection. Semen was extended using 1.175 g Androhep EnduraGuard™ in 25 mL of water at approximately 27°C. Raw semen was added at a 1:5 dilution (semen:extender) regardless of sperm concentration and stored at approximately 15°C for 2-3 days before use.

**Motility and Membrane Analyses of Sperm**

Post-thaw motility, viability and acrosome integrity of boar sperm were evaluated for three independent samples of each boar ejaculate used in these experiments by light microscopy. Motility was verified by a separate facility using computer automated sperm assessment (CASA,
Hamilton Thorne, Beverly, MA). The procedures in both laboratories were the same. Straws were thawed at 50°C for 20 s and the contents of the straws expelled into glass tubes at 37°C. Samples were diluted in Androhep CryoGuard Thawing Extender (Minitube of America, Verona, WI) at 1:40 for motility evaluation. Evaluation was performed at 0, 30, and 60 min after thawing. Samples were examined under a phase contrast microscope with a 37°C heated stage at 200X magnification. Ten fields were examined to evaluate 100 sperm/slide and motility was expressed as a percentage of the total number of sperm cells. Motility analysis was confirmed for accuracy (± 5 %) on all samples using CASA. The CASA was set up as follows: 30 frames acquired, frame rate of 60 Hz, minimum contrast of 55, minimum cell size of 5 pixels, VAP cutoff of 20 μm/s, progressive minimum VAP cutoff of 45 μm/s, VSL cutoff of 5 μm/s, static head size of 0.53 to 4.45, and magnification of 1.89. A minimum of 7 fields and 1,000 sperm were observed for motility analysis.

Propidium iodide staining (PI) was performed to determine membrane integrity (PI, Sigma Aldrich, St. Louis, MO). Acrosome integrity was assessed using Fluorescein-labeled Peanut agglutinin (FITC-PNA, Sigma Aldrich, St. Louis, MO). For assay, samples were thawed and 10 μl was diluted in 0.5 mL Beltsville Thawing Solution® (Minitube of America, Verona, WI) at 26°C and co-incubated with PI (7 μL of a 2.5 mM solution in water), and FITC-PNA (15 μL of a 1 mg/mL solution in water) for 15 min. The final concentration of PI was 32.9 μM and of FITC-PNA was 28.2 ng/mL. Sperm were subsequently fixed with 0.4% paraformaldehyde in PBS (a concentration that does not permeabilize sperm, [26]) at 0, 30 and 60 min after thawing and evaluated. A total of 300 sperm were evaluated using a Carl Zeiss AxioCamHRc (Carl Zeiss Microscopy, LLC. Thornwood, NY) at 400X magnification. Those sperm that excluded PI were
considered live. Sperm that were labeled with FITC-PNA were considered to have compromised acrosomes.

**Oocyte Recovery and Maturation**

Sow ovaries were collected from a local abattoir and transported at approximately 31°C in a dry thermos. Ovaries were rinsed with 0.9 % saline at 37°C, and follicles of approximately 3 – 10 mm in size were aspirated from normal, healthy appearing follicles and ovaries. Oocytes with a minimum of two layers of cumulus cells surrounding a homogeneous cytoplasm were selected in SOF HEPES with 0.1 % BSA [28]. Selected oocytes were washed and cultured in 500 µL maturation medium composed of TCM199 (Life Technologies, Grand Island, NY) with Earle’s salts and L-glutamine, 10 % (v:v) porcine follicular fluid, 3.05 mM glucose, 0.91 mM pyruvate, 0.57 mM cysteine, 1.0 % (v:v) PSA (10,000 IU/mL penicillin, 10,000 µg/mL streptomycin, and 25 µg/mL amphotericin), pLH (0.01 U/mL), pFSH (0.01 U/mL) and EGF (10 ng/mL) at pH 7.35 in groups of approximately 50 per well in 6 % CO₂ and air at 38.7°C and 100 % relative humidity for 41 h [28].

**Sperm Preparation for IVF**

Frozen sperm were thawed at 50°C for 20 s in a water bath. A total of two straws (1 mL) were used to test a single ejaculate on a given day. Straw contents were dispersed into 12 x 75 mm glass culture tubes held at 37°C on dry heat for ~ 7 min. Control and frozen sperm were layered onto 45:90 % Percoll step gradients in 15 mL polystyrene tubes maintained at 37°C. Samples were centrifuged at 700 x g for 20 min at ~ 25°C. After centrifugation, the supernatant was removed and the sperm pellet enriched for motile sperm was resuspended in a total volume of 5 mL of Hepes-buffered saline (126 mM NaCl, 50 mM KCL, 15 mM HEPES) held at 37°C.
Sperm were washed twice by centrifugation at 950xg for 5 min, resuspending the pellet in mTBM [29]. The final sperm pellet was re-suspended in 100 µL of mTBM and held at 6% CO₂ and air at 38.7°C until oocyte semination.

Gamete Co-incubation

After 41 h of maturation, oocytes were denuded in HEPES with hyaluronidase (100 µg/mL, 80-160 U/mL) by vortexing for 90 s. Oocytes were washed and selected based on morphology including homogenous cytoplasm and membrane integrity. For semination, oocytes were cultured in 100 µL droplets of modified-Tris-buffered medium (mTBM) [29] containing 5 mM caffeine and 0.02% BSA under mineral oil (16-17 oocytes per droplet). The concentration of frozen sperm for IVF was optimized in a separate preliminary experiment prior to the start of this experiment by pooling sperm from three different ejaculates. We reduced the number of frozen-thawed sperm we used for IVF to the minimal number needed to provide adequate numbers of developed embryos so that meaningful development data would be generated. The final concentration of frozen and control sperm for gamete co-incubation was 500,000 total sperm/mL and 250,000 total sperm/mL, respectively. Gametes were co-incubated at 6% CO₂ in air at 38.7°C for five hours.

Embryo Culture

Embryos were cultured in 50 µL droplets of North Carolina State University 23 (NCSU23) culture medium [28, 30] prepared under mineral oil and equilibrated at 6% CO₂ and 10% O₂ for a minimum of 4 h prior to culture. A total of 37 zygotes per boar were randomly selected and placed into NCSU23 droplets for culture in groups of 9-10 per drop. The remaining
13 zygotes (approximately 25%) were maintained in wash drops of NCSU23 and fixed for evaluation of fertilization. Zygotes were cultured at 6% CO$_2$ and 10 % O$_2$ at 38.7°C.

**Fertilization, Cleavage, Blastocyst Development and Cell Number Evaluation**

Twelve hours after semination, zygotes selected for fertilization assessment were mounted onto glass slides in groups of ~13 by mounting a square coverslip with paraffin/wax to the slide and applying a glue sealant and allowing ~ 60 s for drying. The slides were submerged in a 3:1 mixture of 70% ETOH and glacial acetic acid for 4-5 days to allow sufficient time for lipids to clear. After removal from fixative, 1 % aceto-orcein solution (Sigma Aldrich, St. Louis, MO) was applied to stain nuclear content by pulling the solution under a coverslip mounted to a glass slide containing presumptive zygotes. Fertilization and meiotic maturation was evaluated by observing the number of pro-nuclei or stage of meiosis using phase contrast microscopy.

Mitotic cleavage was assessed 48 ± 2 h post insemination. Blastocysts were evaluated morphologically 144 h after transfer to culture (6 days post fertilization). Blastocysts were described as early, expanded or hatching/ed. Following evaluation, blastocysts were placed into 25 µg/mL Hoechst 33342 in 100% ETOH solution and held at 5°C for 24-48 h. Embryos were then placed in a small droplet of glycerol on a glass slide and covered with a coverslip. The total number of cells per embryo was counted with fluorescent microscopy using a DM 400 UV2A filter (EX 330-380 and BA 420; Nikon, Düsseldorf, Germany). A blastocyst was defined as having 18 or greater cells.

**Oviduct-Sperm Binding Assay**

Frozen-thawed sperm from seventeen different boar ejaculates were used for this experiment, nine of which were also used for the IVF experiments. Boars were assigned
randomly for comparison in triplicates using a loop design model. Sperm from three different boars were tested independently on an experimental day. One of those boars was tested with two other boars on the next experimental day and one of those two tested with two more boars, and so on, until semen from all boars was tested. In the end, each boar was compared directly to two other boars and then indirectly to the remainder of the boars so that they could be ranked by the average least-squared mean (LSM) number of sperm bound to aggregates.

Epithelial sheets were harvested from the isthmus portion of gilt oviducts obtained from a local abattoir. Epithelial cells were concentrated by centrifugation (200g for 30 sec) and disrupted by pipetting. Disrupted cells were incubated in dmTALP [26] at 39°C for 1.5-2 h to allow cell reaggregation. Three dimensional aggregates with a diameter of ~ 150-200 µm were selected, washed through three consecutive droplets of dmTALP and placed into a final droplet in groups of 10 for co-incubation with sperm. Three separate sperm-oviduct cell incubation droplets were used for each boar and droplet was considered the experimental unit for statistical analyses so that each boar was tested a minimum of three times.

Sperm were thawed at 50°C for 20 sec., held at 37°C for ~7 min and centrifuged on a Percoll cushion containing non-capacitating (NC) dmTALP with no BSA or bicarbonate [26]. Following a second wash by centrifugation, sperm were held in dmTALP NC and counted by hemocytometer. Final sperm suspensions in dmTALP were made to achieve 2 x10^6 sperm/mL in 100 µL droplets with aggregates. Sperm and aggregates were co-incubated at 39°C and 99% humidity for 30 min to allow sufficient time for binding. Aggregates were gently washed by hand pipette using 35 µL of dmTALP to remove loosely adherent sperm and then mounted by droplet in groups of 10 on a glass slide with coverslip. Sperm tightly bound to the periphery of aggregates were counted using phase contrast microscopy. Images of aggregates were captured
using a Zeiss Axioskop and AxioCam HRc digital camera (Carl Zeiss, Thornwood, NY). The circumference of each aggregate was measured using AxioVision V 4.5 software (Carl Zeiss) and the number of sperm bound to aggregates was normalized by dividing the number of sperm bound by the length of the perimeter of the aggregate. The mean number of sperm bound to a mm of aggregate surface for each droplet was used for statistical analyses.

**Statistical Analyses**

Boars were ranked on the ability of sperm to bind oviduct epithelial aggregates based on the calculated least squared means (LSM) of the number of sperm bound/aggregate for each boar using a mixed linear model in SAS 9.0. Data were log transformed for normalization and the number of sperm bound to aggregates was tested for main effects while replicate and droplet were considered random effects.

IVF data were collected in two to three replicates for each ejaculate. Droplet was considered the experimental unit for developmental data and embryo the experimental unit for cell data. Values were determined to be statistically significant when $P \leq 0.05$. The MIXED and GLM procedures of SAS were used to analyze IVF data and generate LSM for regression analyses and the coefficient of regression (SAS 9.0, SAS Institute, Cary, NC). Replicate and boar were considered random variables.

Regression analysis and Pearson’s correlation coefficients were used to determine relationships between the number of sperm bound to oviduct cell aggregates and IVF outcomes as well as additional sperm traits using the Anti-Log (e) values from the number of sperm bound to aggregates. The percent of acrosome-compromised sperm after thawing was analyzed and
tested for correlation with post-thaw total motility using the Proc Corr procedure in SAS 9.0 after testing for outliers.

RESULTS

Sperm viability, motility and acrosome integrity were determined after thawing sperm from 19 different boars by evaluating each parameter at 0, 30 and 60 min of incubation after thawing. The number of ejaculates used for all assays varied from 9 to 19 due to limitations in sperm numbers. Sperm viability, tested by PI exclusion, ranged from 14-57% and was correlated with motility at each time point (P<0.05; data not shown). Acrosome integrity was assessed using FITC-PNA. Post-thaw total motility at time = 0 (no incubation) was negatively correlated with the percent of acrosome-compromised sperm at time = 0 from respective samples ($r^2 = 0.64$, Figure 3.1) but not after incubation for 30 or 60 min (data not shown).

Frozen-thawed sperm from 16 boars were subjected to IVF (Table 3.1). Monospermic fertilization was confirmed by the presence of two pro-nuclei and polyspermic fertilization by three or greater pro-nuclei. Total fertilization considered all oocytes fertilized that contained a minimum of two pro-nuclei. IVF results herein using frozen-thawed semen were comparable to previous reports from our laboratory using cooled semen [28, 31, 32]. The mean percentage of fertilized oocytes using frozen-thawed sperm from all boars was 67 % (least square means, LSM). Forty-two percent of oocytes were fertilized by one sperm and 25 % of oocytes were fertilized by multiple sperm (polyspermy). Embryo development was assessed by recording 2-cell embryo cleavage and blastocyst development (Table 3.2). Mean embryo cleavage and blastocyst development (LSM) from 16 boars was 54 % and 16 %, respectively. Of the embryos that cleaved, 25 % developed into blastocysts. Day 6 blastocysts had an average of 53 cells per
blastocyst (Table 3.2). The observation that these IVF results were similar to those previously published by our lab using cooled semen [28, 31, 32] indicates that frozen-thawed semen yielded normal IVF results and that the semen samples were representative of those from a typical group of commercial boars.

Straws of sperm from the same collections of the 16 boars used for IVF were thawed and analyzed to assess total motility. The relationship of post-thaw motility to IVF measures was evaluated by regressing motility values against fertilization measures including the percentage of oocytes fertilized (total, monospermic and polyspermic fertilization). Motility was also regressed against embryo cleavage, blastocyst development, and blastocyst cell number using the total number of oocytes exposed to sperm. No significant relationship was found between post-thaw motility and IVF measures except for a tendency ($P = 0.06$) for motility to be related to monospermic fertilization (Figure 3.2). Considering the possibility that the number of immature oocytes may not have been equal in each group, fertilization success was also calculated after discarding degenerate oocytes or those that had not reached meiotic maturation at the time of fertilization. However exclusion of this group had no effect on any of the relationships tested (data not shown).

Frozen-thawed sperm from 17 different boars were used in a sperm-oviduct binding assay to assess the ability of sperm to bind with oviduct cell aggregates in vitro. When the results from each boar were included in an overall model, the model allowed ranking of the boars successfully to calculate the mean (LSM) number of sperm bound to aggregates, generating an $r^2 = 0.73$ for model fitness (Figure 3.3A). Nine of the seventeen boars tested in this assay were also used in the previous IVF experiment. The mean (LSM) number of sperm bound to oviduct aggregates was tested for correlation with IVF outcomes from those nine boars to determine the
relationship between sperm-oviduct binding and IVF (Figure 3.3 B-D). There was no relationship between oviduct binding and monospermic fertilization. However, a positive linear relationship was observed between the number of sperm bound to oviduct aggregates and IVF polyspermic fertilization ($r^2 = 0.62$, $P < 0.05$, Figure 3.3D). Although not significant ($P = 0.12$), there was a tendency for oviduct binding to be related to overall IVF fertilization rates ($r^2 = 0.31$) but this was due to the relationship between oviduct binding and polyspermic fertilization.

**DISCUSSION**

The overall IVF results achieved with frozen-thawed boar sperm were comparable to results achieved in our laboratory using fresh or cooled semen [28, 31, 32]. The range in post-thaw motility (21-56 %) represented low and high motility samples from boars whose fresh liquid semen was used for commercial AI. Although we expected less variability with IVF outcomes when using frozen sperm from a single ejaculate, inter and intra-assay variability was still observed when using cryopreserved sperm for IVF. The variation between each boar, regardless of semen characteristics prior to freezing, was likely a contribution to the variability in IVF outcomes. This observation is consistent with another report in which variation between boars, regardless of pre-freeze characteristics, accounts for 70% of the variation in post-thaw quality, estimated by motility and viability [19]. Our data also show that there is considerable variability in IVF results using the same cryopreserved ejaculates. Thus, factors other than semen sample and individual boar contribute considerable variation to IVF outcomes.

Post-thaw motility values of boar sperm at time = 0 were mildly related to the rate of monospermic fertilization achieved with IVF ($P = 0.06$). The motility values regressed against monospermic fertilization revealed that higher motility sperm tended to have slightly increased
frequencies of normal fertilization. This correlation may have been reduced because we used a discontinuous Percoll gradient to prepare sperm, a method that removes moribund cells. Percoll washing was done to remove dead sperm that can depress IVF success [5]. For the oviduct binding assays, we added a constant number of sperm to oviduct cells so that the sperm we added was a true sample of all sperm in the semen straw. This avoided the complication of “correcting” for motility differences by selecting only motile sperm for the assays. Ultimately, although the relationship was not robust, we found that cryopreserved sperm with higher motility yielded a slightly higher percentage of normally fertilized oocytes.

Sperm samples with higher motility also had fewer acrosome-compromised sperm, suggesting less cryo-induced damage. However, it was surprising that acrosome integrity was not related to IVF outcomes.

In addition to the relationship between sperm motility and monospermic fertilization, the number of sperm bound to oviduct cells was related to IVF polyspermic fertilization results. We predicted that if cryopreservation caused precocious capacitation, both oviduct binding and IVF success would be reduced [21]. We expected that oviduct binding would be reduced because of the evidence that sperm capacitation reduces oviduct binding [22, 23, 33]. Finally, we expected that IVF rates would be reduced because sperm that are precociously capacitated more readily undergo premature spontaneous acrosome reactions prior to zona binding [21, 34]. The higher rates of polyspermy from sperm samples that bound in greater number to oviduct cells is consistent with the premise that sperm more able to bind the oviduct have less functional damage and are more effective at fertilization, both polyspermic and monospermic [23, 35-39]. Previous reports with bovine sperm showed that the population of sperm that bound the oviduct was more effective at IVF than sperm that did not bind oviduct cells [40]. However, the observation that
oviduct binding was not related to monospermic fertilization is inconsistent with this conclusion. We considered the possibility that the relationship between oviduct binding and polyspermy was explained because each was related to motility. But in fact, sperm motility was not significantly correlated with oviduct binding, making this explanation unlikely. Taken as a whole, these results indicate that the number of sperm bound to oviduct aggregates is related to at least one IVF outcome, polyspermy rate. The biological explanation for the relationship between oviduct binding and polyspermy is unclear. Regardless, polyspermy is a major cause of poor developmental problems with IVF in swine and being able to predict it would be useful to estimate IVF outcomes.

CONCLUSIONS

Analysis of cryopreserved sperm from 17 boars indicated that the acrosome integrity of frozen-thawed sperm is strongly correlated with post-thaw motility. Post-thaw motility of cryopreserved boar sperm had limited value in predicting IVF success but was somewhat useful for predicting the rate of monospermic fertilization. Although the number of sperm bound to oviduct aggregates was not related to monospermic fertilization rates, it was related to polyspermic fertilization rates. Post-thaw motility and sperm binding to oviduct aggregates are moderately useful indicators of different IVF outcomes using cryopreserved boar sperm. This is the first indication that sperm binding to oviduct cells is related to IVF.

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FIGURES AND TABLES

Table 3.1. Overall in vitro fertilization results using frozen-thawed sperm from 16 boars.

<table>
<thead>
<tr>
<th>No. of oocytes</th>
<th>% Fertilized Oocytes</th>
<th>% Monospermic (2PN) Fertilization</th>
<th>% Polyspermic Fertilization</th>
</tr>
</thead>
<tbody>
<tr>
<td>n = ~500</td>
<td>67.4 ± 4.3</td>
<td>41.9 ± 3.3</td>
<td>25.4 ± 3.7</td>
</tr>
</tbody>
</table>

Sow oocytes collected from a local abattoir, matured and incubated with frozen-thawed boar sperm. Twelve hours after initial gamete co-incubation, approximately 25% of oocytes exposed to sperm from each ejaculate were mounted on slides and fixed. Fertilization was measured by staining pronuclei with 1% aceto-orcein. Oocytes with two pro-nuclei were categorized as normally fertilized whereas those with three or greater pro-nuclei were considered polyspermic. Values are expressed as LSM ± SEM.
Table 3.2. Overall in vitro development of oocytes seminated with frozen-thawed sperm from 16 boars.

<table>
<thead>
<tr>
<th>No. of oocytes</th>
<th>% Cleaved</th>
<th>% Blastocyst</th>
<th>% Blast by Cleaved</th>
<th>Average Blastocyst Cell No. (n = 239 blastocysts)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1586</td>
<td>53.9 ± 3.2</td>
<td>15.7 ± 2.4</td>
<td>24.9 ± 3.4</td>
<td>52.6 ± 3.0</td>
</tr>
</tbody>
</table>

After initial gamete co-incubation, mitotic cleavage and blastocyst development were assessed at 48 h and 144 h (6 days) and calculated as a percentage of all oocytes exposed to sperm. Oocytes that were not at the proper stage of development for fertilization were discarded for analyses. Blastocyst development was also calculated by measuring the percentage of cleaved embryos that became blastocysts (% Blast by Cleaved). Blastocyst cell number was determined by staining blastocysts with Hoechst 33342. Values are expressed as LSM ± SEM.
Figure 3.1. Post-thaw motility of boar sperm is related to sperm acrosome integrity.

Semen from 19 different boars was thawed and total motility was assessed using light microscopy and confirmed with CASA. Acrosome integrity was evaluated for each ejaculate using Fluorescein-labeled Peanut agglutinin (FITC-PNA), which detects sperm with compromised acrosomes. The percentage of FITC positive sperm (acrosome-compromised) was correlated with post-thaw motility values (P < 0.05). All observations depicted were recorded at time = 0 min after thawing.
Frozen semen from 16 boars was thawed and held at 37°C. Total motility was recorded 5 min after thawing using light microscopy. In vitro fertilization measures using sperm from each boar ejaculate (A, total fertilization rate; B, monospermic fertilization rate; C, polyspermic fertilization rate) were regressed against post-thaw motility values for each collection. There was a tendency for post-thaw motility to be related to monospermic fertilization (P = 0.06). Motility was not related to total or polyspermic fertilization (P > 0.1).
Sperm from 17 boars (of which 9 were also used for IVF) were thawed and allowed to bind oviduct cell aggregates. Loosely bound sperm were washed away and the number of sperm bound to the periphery of each aggregate was counted. The number of sperm bound to each mm of oviduct cell aggregate was calculated for each boar (A). A mixed linear model was used to calculate the average number of sperm bound to aggregates ($r^2 = 0.73$ for the model). Nine different boars that were subjected to both IVF and oviduct cell binding assays were analyzed for correlation between the number of sperm bound to oviduct epithelial aggregates and IVF outcomes utilizing LSMs from both assays (B, total fertilization rate; C, monospermic fertilization rate; D, polyspermic fertilization rate). The number of sperm bound to oviduct aggregates was related to polyspermic fertilization ($P = 0.01$) but not related to total or monospermic fertilization ($P > 0.1$).
REFERENCES


CHAPTER 4

ENHANCED FERTILITY PREDICTION OF CRYOPRESERVED BOAR SPERM USING NOVEL SPERM FUNCTION ASSESSMENT

SUMMARY

Due to reduced fertility, cryopreserved semen is seldom used for commercial porcine artificial insemination (AI). Predicting the fertility of individual frozen ejaculates for selection of higher quality semen prior to AI would increase overall success. Our objective was to test novel and traditional laboratory analyses to identify characteristics of cryopreserved sperm that are related to boar fertility. Traditional post-thaw analyses of motility, viability and acrosome integrity were performed on each ejaculate. IVF, cleavage and blastocyst development were also determined. Finally, sperm-oviduct binding and competitive zona binding assays were applied to assess sperm adhesion to these two matrices. Fertility of the same ejaculates subjected to lab assays was determined for each boar by multi-sire AI and defined as (1) the mean percentage of the litter sired and (2) the mean number of piglets sired in each litter. Means of each lab evaluation were calculated for each boar and those values were applied to multiple linear regression analyses to determine which sperm traits could collectively estimate fertility in the simplest model. The regression model to predict the percent of litter sired by each boar was highly effective ($p < 0.001$, $r^2 = 0.87$) and included 5 traits; acrosome-compromised sperm, percent live sperm (0 and 60 min post-thaw), percent total motility and the number of zona-bound sperm. A second model to predict the number of piglets sired by boar was also effective ($p < 0.05$, $r^2 = 0.57$). These models indicate that the fertility of cryopreserved boar sperm can be predicted effectively by including traditional and novel laboratory assays that consider functions of sperm.

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INTRODUCTION

The use of cryopreserved sperm has many advantages compared to fresh or extended semen for artificial insemination (AI). Some advantages include increased time to test for pathogens and fertility and additional time for distribution of semen with high genetic merit (Tribout et al., 2010; Knox, 2011). However, less than 1% of porcine artificial inseminations (AI) use frozen-thawed boar sperm because the economic advantages do not compensate for the reduction in fertility caused by cryopreservation damage (Johnson et al., 2000; Knox et al., 2008; Rath et al., 2009). Identifying cryopreserved ejaculates of acceptable fertility prior to use would be beneficial given the interest for enhancing the commercial use of frozen-thawed semen by the swine industry (Didion et al., 2013; McNamara & Knox, 2013; Ringwelski et al., 2013; Tomás et al., 2013; Estrada et al., 2014). Conventional laboratory evaluation of frozen-thawed boar sperm has not historically provided accurate estimates of fertility (Gil et al., 2005). We hypothesized that inclusion of novel assays of sperm function would improve fertilization prediction of frozen-thawed boar semen.

Cryopreservation-induced damage to boar sperm results in reduced motility, viability and litter size (Rath et al., 2009; Didion et al., 2013; Estrada et al., 2014). The average litter size produced when using frozen-thawed sperm is below what most of the industry will accept (Ruiz-Sánchez et al., 2006). Traditional analyses of boar sperm that include motility, morphology and viability, are only moderately useful for identifying boars of low fertility (Flowers, 1997; Gadea et al., 2004; Gadea, 2005; Alm et al., 2006; Lovercamp et al., 2007; Broekhuijse et al., 2012). Knowledge of the causes of reduced fertility due to cryopreservation is incomplete, although there are reductions in routinely measured sperm traits. There are likely additional types of
cellular damage due to cryopreservation that are not detected in standard semen analysis, given the low correlation between fertility and traditional microscopic evaluation. Numerous attempts have been made to identify in vitro sperm parameters that are directly related to fertility (Holt et al., 1997; Tardif et al., 1999; Larsson & Rodríguez-Martínez, 2000; Waberski et al., 2005; Alm et al., 2006; Broekhuijse et al., 2012; Schulze et al., 2013). However, novel assays that measure sperm interactions within the female reproductive tract and with oocytes may be more predictive of in vivo function as well as useful when identifying reasons for reduced fertility (Fazeli et al., 1997; Braundmeier et al., 2004; Collins et al., 2008).

Our objective was to identify traits of cryopreserved boar sperm that collectively predict the fertility of cryopreserved semen accurately. To be practical, the number of traits that would need to be evaluated to predict fertility should be minimal. We used multiple linear regression to identify that model. Various routine and novel assays were tested for their relationship to fertility as assessed by competitive insemination of gilts. In addition to traditional post-thaw analyses of sperm function (motility, viability and acrosome integrity), we assessed oviduct binding because cryopreservation may cause precocious capacitation and hinder the ability of sperm to bind to the oviduct epithelium and form a reservoir (Lefebvre & Suarez, 1996; Fazeli et al., 1999; Bailey et al., 2008). A competitive zona binding assay using two different fluorophores for boar identification was employed to detect differences in the number of sperm bound to the zona of oocytes, a potential indicator of sperm damage. In vitro fertilization was used as a tool to collect multiple measures of fertilization and embryo development. Finally, multiple linear regression analyses were performed that included these novel and conventional post-thaw measures of sperm attributes to identify those characteristics that collectively and non-redundantly were related to fertility.
MATERIAL AND METHODS

Experimental Approach

The University of Illinois Animal Care and Use Committee approved use of animals in these experiments.

Sperm Collection and Freezing

Ejaculates from 19 commercial boars (Landrace, Large White, Composite and Duroc) from the United States were used for all experiments. The average age of boars was 1-2 years and body condition score was assessed on each boar. The number of boars used differed slightly between experiments. The sperm rich fraction of one ejaculate from each boar was collected using the gloved hand technique. Semen was diluted using Androstar® Plus (MOFA) at 37°C, cooled to 17°C over 2 h and then shipped to the USDA in Fort Collins, Colorado for processing within 24 h of collection. Sperm were separated from seminal plasma and further processed for freezing (Almlid & Johnson, 1988) in 0.5 mL straws at a final concentration of 1.4 x 10⁹ sperm/mL in egg yolk and glycerol (Ringwelski et al., 2013). Straws were then shipped in liquid nitrogen vapor to the University of Illinois.

Motility and Membrane Analyses of Sperm

Post-thaw motility, viability and acrosome integrity of boar sperm were evaluated using three independent samples from each boar ejaculate by light microscopy. Straws were thawed at 50°C for 20 s and the contents of the straws expelled into glass tubes at 37°C. Samples were diluted in Androhep CryoGuard Thawing Extender (MOFA, Verona, WI) at a 1:40 dilution for motility evaluation. Evaluation was performed at 0, 30, and 60 min after thawing. Samples were examined under a phase contrast microscope with a 37°C heated stage at 200X magnification. Ten fields were examined to evaluate 100 sperm/slide and motility was expressed as a
percentage of the total number of sperm. Motility analyses were confirmed for accuracy (± 5 %) on all samples using CASA (Hamilton Thorne, Beverly, MA). The CASA was set up as follows: 30 frames acquired, frame rate of 60 Hz, minimum contrast of 55, minimum cell size of 5 pixels, VAP cutoff of 20 μm/s, progressive minimum VAP cutoff of 45 μm/s, VSL cutoff of 5 μm/s, static head size of 0.53 to 4.45, and magnification of 1.89. A minimum of 7 fields and 1,000 sperm were observed for motility analyses.

Propidium iodide staining (PI) was performed to determine membrane integrity (PI, Sigma Aldrich, St. Louis, MO). Acrosome integrity was assessed using fluorescein-labeled peanut agglutinin (FITC-PNA, Sigma Aldrich, St. Louis, MO). For assay, samples were thawed and 10 μL was diluted in 0.5 mL Beltsville Thawing Solution® (MOFA) at 26°C and co-incubated with PI (7 μL of a 2.5 mM solution in water), and FITC-PNA (15 μL of a 1 mg/mL solution in water) for 15 min. The final concentration of PI was 32.9 μM and of FITC-PNA was 28.2 ng/mL. Sperm were subsequently fixed with 0.4% paraformaldehyde in PBS (a concentration that does not permeabilize sperm, (Kadirvel et al., 2012) at 0, 30 and 60 min after thawing and evaluated. A total of 300 sperm were evaluated using a Zeiss AxioCamHRc (Zeiss Microscopy, LLC, Thornwood, NY) at 400X magnification. Those sperm that excluded PI were considered live. Sperm that were labeled with FITC-PNA were considered to have compromised acrosomes.

**Estrus Synchronization and Artificial Insemination**

Post-pubertal terminal line gilts (Genetiporc USA, Alexandria MN) between 147 and 180 d of age that exhibited estrus during fence line boar exposure were placed into gestation stalls for synchronization of estrus. Gilts were fed 15 mg×gilt⁻¹×d⁻¹ of MATRIX (Altrenogest 2.2 mg/mL, Merck Animal Health, Summit, NJ) for 14 d as a top-dress on a standard sow gestation diet.
Estrous detection was performed twice daily at 12 h intervals beginning on the third day following last MATRIX feeding.

To be certain the boars sampled represented a wide range of fertility, motility of ejaculates was evaluated immediately after thawing and ranged from 21 to 46%. Gilts (n =136) were inseminated with frozen-thawed sperm from one boar at 24 and a different boar at 36 h (AI-1 and AI-2) after onset of estrus. The boar semen AI sequence was always reversed in a different gilt(s) for each pair of boars tested to avoid confounding fertility with AI order. The mean number of gilts inseminated with sperm from each boar was 5.9 ± 0.78. Each insemination contained 4.0 billion total sperm (live + dead). For inseminations, straws of boar sperm were thawed at 50°C for 20 s and the contents expelled into 100 mL plastic AI bottles containing 80 mL of Androhep CryoGuard Thawing Extender held in a 26°C water bath. Intracervical insemination was performed within 15 min of thawing using polygel-tipped AI catheters (MOFA).

**Reproductive Tract Processing and Fetal Paternity**

Gilts were euthanized at 31 to 35 d following AI and reproductive tracts were collected to assess pregnancy status and number of fetuses (McNamara & Knox, 2013). Prior to AI, DNA was obtained from boar semen and gilt blood. A liver sample was obtained from each fetus for DNA genotyping to determine paternity as described by (Ringwelski et al., 2013). A panel of 14 microsatellite markers was chosen for PCR and products were combined, purified and sequenced as described by (Meyers et al., 2010). Alleles were identified using GeneMarker software (SoftGenetics, LLC, State College, PA) and confirmed manually. Fetal parentage was determined using genotypes from the dam and two potential sires.
**Oocyte Recovery and Maturation for IVF**

Sow ovaries were collected from a local abattoir and transported at approximately 31°C in a dry thermos. Ovaries were rinsed with 0.9 % saline at 37°C, and follicles of approximately 3 – 10 mm in size were aspirated from normal, healthy appearing follicles and ovaries. Oocytes with a minimum of two layers of cumulus cells surrounding a homogeneous cytoplasm were selected in SOF HEPES with 0.1 % BSA (Yuan & Krisher, 2012). Selected oocytes were washed and cultured in 500 µL maturation medium composed of TCM199 (Life Technologies, Grand Island, NY) with Earle’s salts and L-glutamine, 10 % (v:v) porcine follicular fluid, 3.05 mM glucose, 0.91 mM pyruvate, 0.57 mM cysteine, 1.0 % (v:v) PSA (10,000 IU/mL penicillin, 10,000 µg/mL streptomycin, and 25 µg/mL amphotericin), pH (0.01 U/mL), pFSH (0.01 U/mL) and EGF (10 ng/mL) at pH 7.35 in groups of approximately 50 per well in 6 % CO₂ and air at 38.7°C and 100 % relative humidity for 41 h (Yuan & Krisher, 2012).

**Sperm Preparation for IVF**

Frozen sperm were thawed at 50°C for 20 s in a water bath. A total of two straws (1 mL) were used to test a single ejaculate on a given day. Straw contents were dispersed into 12 x 75 mm glass culture tubes held at 37°C on dry heat for ~ 7 min. Control and frozen sperm were layered onto 45:90 % Percoll step gradients in 15 mL polystyrene tubes maintained at 37°C. To generate the gradient, a 10X modified sperm TALP (30.9 mM KCl, 2.9 mM NaH₂PO₄, 0.8 M NaCl, 99.9 mM HEPES) was diluted 1:10 in a 45 mL Percoll solution (2.6 mM CaCl₂, 0.39 mM MgCl₂, 0.37% (v:v) Lactic Acid, 24.9 mM NaHCO₃). Forty-five percent Percoll solution was achieved by 1:1 dilution of 90% Percoll solution to HEPES-buffered saline (126 mM NaCl, 50 mM KCl, 15 mM HEPES). Samples were centrifuged at 700 g for 20 min at ~ 25°C. After centrifugation, the supernatant was removed and the sperm pellet was resuspended in a total
volume of 5 mL of HEPES-buffered saline held at 37°C. Sperm were washed twice by centrifugation at 950 \( g \) for 5 min, resuspending the pellet in mTBM (Abeydeera & Day, 1997b). The final sperm pellet was re-suspended in 100 µL of mTBM and held at 6 % CO\(_2\) and air at 38.7°C until oocyte semination.

**Gamete Co-incubation**

After 41 h of maturation, oocytes were denuded in HEPES (Yuan & Krisher, 2012) with hyaluronidase (100 µg/mL, 80-160 U/mL) by vortexing for 90 s. Oocytes were washed and selected based on homogenous cytoplasm and membrane integrity. For semination, oocytes were cultured in 100 µL droplets of modified-Tris-buffered medium (mTBM) (Abeydeera & Day, 1997b) containing 5 mM caffeine and 0.02% BSA under mineral oil (16-17 oocytes per droplet). The concentration of frozen sperm for IVF was determined in a separate preliminary experiment based on sperm pooled from three different ejaculates. We reduced the number of frozen-thawed sperm to the minimal number needed to provide adequate numbers of embryos so that meaningful embryonic development data would be generated. The final concentration of frozen sperm for gamete co-incubation was 500,000 total sperm/mL. Gametes were co-incubated at 6% CO\(_2\) in air at 38.7°C for five hours.

**Embryo Culture**

Embryos were cultured in 50 µL droplets of North Carolina State University 23 (NCSU23) culture medium (Petters & Wells, 1993; Yuan & Krisher, 2012) under mineral oil and equilibrated at 6% CO\(_2\) and 10% O\(_2\) for a minimum of 4 h prior to culture. A total of 37 zygotes per replicate were randomly selected and placed into NCSU23 droplets for culture in groups of 9-10 per drop. The remaining 13 zygotes (approximately 25%) were maintained in wash drops
of NCSU23 and fixed for evaluation of fertilization. Zygotes were cultured at 6% CO₂ and 10% O₂ at 38.7°C.

**Fertilization, Cleavage, Blastocyst Development and Cell Number Evaluation**

Twelve hours after semination, zygotes selected for fertilization assessment were mounted onto glass slides in groups of ~13 by mounting a square coverslip with paraffin/wax to the slide, applying a glue sealant and allowing ~ 60 s for drying. The slides were submerged in a 3:1 mixture of 70% ETOH and glacial acetic acid for 4-5 days to allow sufficient time for lipids to clear. After removal from fixative, 1% aceto-orcein solution (Sigma Aldrich, St. Louis, MO) was applied to stain nuclear content by pulling the solution under a coverslip mounted to a glass slide containing presumptive zygotes. Fertilization and meiotic maturation were evaluated by observing the number of pronuclei or stage of meiosis using phase contrast microscopy at 400x magnification.

Mitotic cleavage was assessed 48 ± 2 h post insemination. Blastocysts were evaluated morphologically 144 h after transfer to culture (6 days post fertilization). Blastocysts were described as early, expanded or hatching/ed. Following evaluation, blastocysts were placed into 25 μg/mL Hoechst 33342 in 100% ethanol solution and held at 5°C for 24-48 h. Embryos were then placed in a small droplet of glycerol on a glass slide and covered with a coverslip. The total number of cells per embryo was counted by fluorescence microscopy using a DM 400 UV2A filter cube set (excitation filter 330-380 nm, dichroic mirror 400 nm and barrier filter > 420 nm; Nikon Instruments, Melville, NY). A blastocyst was defined as having a visually identifiable blastocoel cavity and 18 or more cells.
**Oviduct-Sperm Binding Assay**

Frozen-thawed sperm from seventeen different boar ejaculates were used for this experiment, nine of which were also used for the IVF experiments. Boars were assigned randomly for comparison in triplicates using a loop design. Sperm from three different boars were tested independently on an experimental day. One of those boars was tested with two other boars on the next experimental day and one of those two tested with two more boars, and so on, until semen from all boars was tested. In the end, each boar was compared directly to two other boars and then indirectly to the remainder of the boars so that they could be ranked by the average least squares mean (LSM) number of sperm bound to aggregates.

Epithelial sheets were harvested from the isthmus portion of gilt oviducts obtained from a local abattoir. Epithelial cells were concentrated by centrifugation (200 g for 30 s) and disrupted by pipetting. Disrupted cells were incubated in dmTALP (Kadirvel et al., 2012) at 39°C for 1.5-2 h to allow cell reaggregation. Three-dimensional aggregates with a diameter of ~ 150 µm were selected, washed through three consecutive droplets of dmTALP and placed into a final droplet in groups of 10 for co-incubation with sperm. Three separate sperm-oviduct cell incubation droplets were used for each boar and droplet was considered the experimental unit for statistical analyses so that each boar was tested a minimum of three times.

Sperm were thawed at 50°C for 20 s, held at 37°C for ~7 min and centrifuged on a Percoll cushion containing non-capacitating dmTALP (dmTALP in which BSA was replaced with an equal mass of PVP and bicarbonate was replaced with an equimolar amount of HEPES) (Kadirvel et al., 2012), hereafter referred to as TALPNC. Following a second wash by centrifugation, sperm were held in TALPNC and counted by hemocytometer. Final sperm suspensions in dmTALP were made to achieve 2 x 10⁶ sperm/mL in 100 µL droplets with
aggregates. Sperm and aggregates were co-incubated at 39°C and 99% humidity for 30 min to allow sufficient time for binding. Aggregates were gently washed by Stripper pipette using 35 µL of dmTALP to remove loosely adherent sperm and then mounted by droplet in groups of 10 on a glass slide with a coverslip. Sperm tightly bound to the periphery of aggregates were counted using phase contrast microscopy at 400x magnification. Images of aggregates were captured using a Zeiss Axioskop and AxioCamHRc digital camera (Zeiss, Thornwood, NY). The circumference of each aggregate was measured using AxioVision V 4.5 software (Zeiss, Thornwood, NY) and the number of sperm bound to aggregates was normalized by dividing the number of sperm bound by the length of the perimeter of the aggregate. The mean number of sperm bound to a mm of aggregate surface for each droplet was used for statistical analyses.

**Zona Pellucida Binding Assay**

Oocytes from gilts were obtained from a local abattoir. Follicles measuring 3-8 mm were manually aspirated using a 12 mL plastic syringe and 18 ga. needle. Aspirate was placed in 50 mL conical tubes and TALPNC was added to facilitate oocyte pelleting. Oocytes were selected in TALPNC without BSA and vortexted at moderate speed (3) for 5 min with 0.1% hyaluronidase (v:v) to remove cumulus cells. An additional 700 µL of TALPNC was added followed by centrifugation at 10 000 g for 15 s. The supernatant was removed and the remaining oocyte and cumulus pellet was placed into TALPNC for selection. Oocytes with no cumulus cells were washed three times in droplets of TALPNC and then placed in 0.5% paraformaldehyde for 5 min in HEPES buffered saline (125 mM NaCl, 50 mM KCl, 15 mM HEPES). Fixed oocytes were then washed through five consecutive droplets of TALPNC and stored in TALPNC with mineral oil at 5°C until use for experiments.
Sperm from 18 different ejaculates were randomly assigned to a system of pairwise comparisons using a loop design. That is, among each pair of boars tested, sperm from one boar was stained with 20 µM of Cell Trace™ Oregon Green and sperm from the other boar was stained with 500 nM Cell Tracker™ Orange (Molecular Probes® Life Technologies™, Eugene, Oregon). The dye assignment was switched so that each boar tested received each dye. A total of three boars were compared on a given day (A:B, B:C) so that a single ejaculate served as an internal control for each experiment. Prior to experiment, dyes were determined to have no effect on sperm motility or oocyte binding.

Straws of sperm were thawed at 50°C for 30 sec and placed on dry heat at 37°C for 7 min. Samples were centrifuged on a Percoll cushion containing dmTALP (Kadirvel et al., 2012). The sperm pellet was washed in dmTALP and the resulting pellet was held in TALP and sperm counted by hemocytomter. Sperm were placed in dmTALP suspensions and one of two fluorophores was added to each sample. Final sperm suspensions contained 20 x 10^6 sperm/mL in 300 µL volumes. Samples were incubated for 30 min at 39°C and 99% humidity to facilitate fluorophore staining. Sperm were then centrifuged at 10,000 g for 30 sec, the supernatant was removed and 298 µL of TALP was added back to the sperm pellet.

Optimal sperm numbers for semination were determined in preliminary experiments by pooling frozen-thawed sperm from three distinct ejaculates to determine the number of sperm for semination needed to obtain an average of 15 sperm bound per oocyte (data not shown). A sperm suspension for semination was made to achieve 6.0 x 10^6 total sperm/mL by combining equal numbers of sperm from each boar to sperm suspension containing dmTALP. Sperm tubes for semination were mixed by inversion and 50 µl of sperm in solution was added to 50 µL droplets containing 10 oocytes each to achieve a final concentration of 3 x 10^6 sperm/mL. A
total of three droplets (30 oocytes) were used for each replicate. Gametes were returned to incubation at 39°C for 15 min to allow binding. Oocytes were moved to wash drops and rinsed with 35 µL of TALP to remove loosely adherent sperm. The oocytes were then placed in 0.5% paraformaldehyde for 2-5 min to ensure that bound sperm remained fixed to the zona for evaluation. Ten oocytes were mounted on a glass slide with coverslip and the number of bound sperm was counted using an inverted Zeiss Axioskop 25 (Zeiss, Thornwood, New York) with fluorescent filters (excitation 450-490 nm, emission >515nm and excitation band pass 546/12, emission >590 nm) for green and orange fluorophore detection, respectively.

Statistical Analyses

All analyses were generated using statistical software from SAS 9.0 and 9.3 (SAS Institute, Cary, NC). Boar fertility for both average litter size and the percent of litter sired by boar were calculated using a mixed linear model. When litter size was 0, both boars were scored as 0% contribution to litter. The order of insemination (AI-1 and AI-2) was tested for main effects and the effects of gilt and post-thaw motility categorization (G, M, and P) were considered random. Fixed effects not found to be significant were removed from models and boars were ranked by least squares means (LSM) for fertility.

Boar ejaculates were ranked for zona binding ability by LSM calculated using ANOVA of the average number of sperm bound/oocyte for each boar. Ejaculates of sperm that were not compared directly were compared indirectly by comparison to shared competitors. A mixed linear model was employed by testing the main effects of boar, dye and the interaction; replicate and droplet were considered random effects. Droplet was used as the experimental unit and post hoc analysis was performed using Tukey’s HSD. Similarly, boars were ranked on the ability of sperm to bind oviduct epithelial aggregates based on the calculated LSM of the number of sperm.
bound/aggregate for each boar using a mixed linear model. Data were log (base) transformed consistent with Normality assumption and the number of sperm bound to aggregates was tested for the main effects of boar while replicate and droplet were considered random effects.

IVF data were collected in two to three replicates for each ejaculate. Droplet was considered the experimental unit for developmental data and embryo the experimental unit for cell count data. A mixed linear model was used to analyze all data and generate LSMs. Replicate and boar were considered random variables. An arcsine or square root transformations were applied for the Normality assumption, when appropriate.

Multiple linear regression was employed in the final stage of the analyses to determine which sperm traits were useful for estimating boar fertility. Boar fertility was first calculated using data generated from AI of gilts. Least squares mean estimates of sperm quality that included in vitro assay data as well as post-thaw analyses were then regressed against boar fertility values. Values from missing observations were imputed when correlation was greater than 0.70 for two independent variables by using the relationship between those two variables (y = intercept + slope(x) as determined by the CORR procedure in SAS. Stepwise regression using the REG procedure in SAS was used for analyses with variable entry set to $\alpha$ p-value $\leq 0.15$. Collinearity among variables in the initial regression model that tested all variables was evaluated. Regressors with a variance inflation factor $\geq 7.0$ or a variance proportion $\geq 0.5$ were considered for removal from final analyses. Seventeen variables were regressed against fertility values (percent of litter sired and litter size) using LSMs from previously modeled experiments. The starting full models used to estimate fertility prior to objective exclusion of variables that did not meet defined criteria for entry into final models were:
Fertility (litter size or no. piglets/litter) = acrosome compromised (ACT0) * + AC30 + AC60 + membrane intact (MI0) + M30 + M60 + motility (MOT0) + MOT30 + MOT60 + fertilized oocytes + monospermy + polyspermy + cleaved + blastocyst + percent of cleaved oocytes that developed to blastocyst + oviduct bound + zona bound + random error.

(* T = time after post-thaw)

Additional regression analyses and Pearson’s correlation coefficients were used to determine relationships between the number of sperm bound to oviduct cell aggregates and IVF outcomes as well as other sperm traits. The percent of acrosome reacted sperm after thawing was analyzed and correlated with post-thaw total motility using the CORR procedure after testing for outliers.

RESULTS

Frozen boar sperm were evaluated for viability, motility and acrosome integrity at 0, 30 and 60 min of incubation after thawing (Table 4.2). The number of different boars used for all assays varied from 14-18 due to limitations in sperm numbers and/or inadequate number of semen straws for all experiments. In the end, a total of 14 boars were represented in every experiment including AI and therefore, were subjected to final regression analyses. Sperm viability tested by PI exclusion ranged from 19- 57% and at 0 and 60 min post-thaw, was predictive of fertility in multiple models. Acrosome integrity was assessed using FITC-PNA and ranged from 12-36% and at 0 and 30 min post-thaw was negatively correlated to fertility (p < 0.01). Total motility ranged from 13-50% and at 30 min post-thaw was positively correlated with fertility (p < 0.01) for both fertility models (Table 3.1 A-B).
A sperm-oviduct cell binding assay was employed to measure the ability of frozen-thawed boar sperm to bind to gilt oviduct epithelium. Sperm from 17 boars were introduced to epithelial aggregates and the number of sperm bound specifically to the periphery of aggregates was counted. Least squares means (LSM) were calculated to rank boars based on the average number of sperm bound to aggregates by both direct pairwise and indirect comparisons. The number of sperm bound ranged from 6-57 sperm per mm aggregate circumference (Fig 4.1 and Fig 1 in Daigneault et al., 2014).

Eighteen boars were subjected to a competitive zona binding assay where equal numbers of sperm from two different boars were added to droplets containing fixed oocytes and ejaculates were identified using green and orange fluorophores. The effect of dye on sperm binding and motility (data not shown) was not significant (Fig 4.2 A). Boars were ranked by the number of sperm bound to the zona pellucida (Fig. 4.2 B). Although the effect of dye on motility and number of sperm bound to zona was not significant in preliminary experiments, a boar*dye interaction was observed during overall ranking (p < 0.01). The LSM number of sperm bound by boar ranged from 14-22 sperm per oocyte (Fig. 4.2 B). Additionally, after testing for outliers and removal of two observations, the number of sperm bound to both oviduct aggregates and zona pellucida were highly correlated (R = 0.94) (Fig 4.2 D).

In vitro fertilization using 16 boars was conducted to determine the relationship of IVF to fertility and other sperm traits. Fertilization was defined as the formation of more than one pronucleus. The percentage of oocytes fertilized (total, monospermic and polyspermic) were calculated and plotted (Fig. 4.3 A-C). Developmental data that were assessed included embryo cleavage, blastocyst development and blastocyst cell number. There were no relationships detected among any of the IVF data generated and fertility estimates (p > 0.15). However,
analyses of covariates revealed relationships between monospermic fertilization and motility ($p = 0.06$), motility and acrosome-compromised sperm ($r^2 = 0.62$) and polyspermic fertilization and oviduct binding ($r^2 = 0.64$) (See Fig 1 in Daigneault et al., 2014). Considering the possibility that some droplets had degenerate oocytes that affected fertilization success, we alternatively calculated IVF fertilization percentage by discarding degenerate oocytes or those that had not reached meiotic maturation at the time of fertilization. Exclusion of this group did not significantly improve any relationship to fertility. Due to the lack of a relationship to fertility, IVF results were not included in the final regression equations.

Boar fertility was assessed using two different approaches, calculating the average number of piglets sired per litter and the average percent of littered sired by each boar. The model for each calculation included order of insemination (AI-1 or AI-2) as a fixed effect and initial motility and gilt as random effects. The order of insemination was not significant ($p > 0.01$). A total of 136 gilts were inseminated resulting in an average litter size for each boar of $4.2 \pm 0.8$. The mean litter size for each boar ranged from $0.6 – 10.0$ piglets per litter and the range in the mean percent of litter sired by boar was 7.0-94% (Fig. 4.4 A-B). Litter size was highly correlated ($R = 0.91$) with the percent of litter sired for each boar (Fig. 4.4 C).

To determine if any laboratory traits could individually or collectively predict fertility, fertility data (percentage of pigs sired and the number of pigs sired by each boar) from AI trials were regressed against oviduct binding, zona binding, IVF and other post-thaw analyses by stepwise multiple linear regression. Seventeen independent variables were entered into both fertility models. Stepwise multiple linear regression removed variables that were collinear and those that were not significantly related to fertility. As mentioned, none of the IVF results were related to fertility so they were excluded from analyses ($p > 0.15$). The percentage of acrosome-
compromised sperm was measured at 3 time points. There was a high degree of collinearity among the 30 min and 60 min post-thaw acrosome-compromised sperm measurements and thus, the 60 min observation was removed due to a high variance inflation factor (0.7), a quantification of the inflation of estimated coefficients due to collinearity. Similarly, oviduct binding data were not used for analyses because of the robust relationship with zona binding (R = 0.94; Fig. 4.2 D).

Fertility, assessed by the percent of pigs sired in litters, was highly predicted by laboratory assays (p < 0.001, \( r^2 = 0.87 \)). The final model included five traits; acrosome compromised sperm (0 and 30 min), percent live sperm (0 min), percent total motility (30 min) and the number of zona bound sperm (Table 4.1 A). An additional model in which fertility was assessed by the number of piglets sired by boar (Table 4.1 B) was predicted by a group of laboratory assays that included percent live sperm (0 and 60 min), percent total motility (30 min) and the number of zona bound sperm (p < 0.05, \( r^2 = 0.57 \)). Several of the same traits were in both final models including percent live sperm, percent total motility and the number of sperm bound to zona.

**DISCUSSION**

The objective of this work was to test traditional and novel sperm traits as possible predictors of fertility after boar sperm cryopreservation and to develop a simple model that could be used to predict fertility feasibly. Remarkably, a model including five traits, acrosome compromised sperm (0 and 30 min), percent live sperm (0 min), percent total motility (30 min) and the number of zona bound sperm, strongly predicted the percentage of a litter sired by a boar in mixed insemination (\( r^2 = 0.87 \)). We reported the adjusted \( r^2 \) of 0.87 from 0.92 (Table 4.1 A) because one can expect the \( r^2 \) to increase by chance with the addition of variables to the model.
However, the adjusted $r^2$ was virtually unchanged when adding any additional variables to the five included in the final model.

The maximum $r^2$ value for the relationship of an individual trait to fertility was 0.23. These results are strikingly similar to those reported for motion characteristics of fresh boar sperm that were related to fertility (Holt et al., 1997). Thus, inclusion of several traits in the final model increased the $r^2$ value markedly compared to a single sperm characteristic. Others have also shown that fertility prediction for fresh or frozen semen can be improved by multiple trait comparisons rather than through single trait analysis (Holt et al., 1997; Foote, 2003). An objective analysis of fresh boar sperm to evaluate the relationship of sperm motion parameters to fertility from AI trials (Holt et al., 1997) indicated that the most robust regression model that related fertility with semen quality only accounted for up to 20% of the variability in boar fertility. The authors noted that up to 80% of differences in fertility were attributed to other factors that could be related to sperm function. Likewise, others have described poor correlation between semen individual characteristics and fertility (Gadea et al., 2004), although in this report, motility was significantly related to fertility in one model, as was the case in both of our models. The inclusion of several assessments of sperm function (membrane and acrosome integrity and zona binding) likely strengthened our final model. To our knowledge, the combination of these five sperm characteristics provides the most robust fertility prediction model reported.

Although there are many reports showing that some sperm traits are retrospectively correlated with fertility, prospectively predicting fertility is a greater challenge (Amann & Hammerstedt, 1993). The evaluations reported herein were performed on samples of the same frozen-stored collection prior to the time fertility was known, preventing any possible bias of the
laboratory assessments; they tested the ability of sperm attributes to predict fertility prospectively.

Sperm require a number of attributes to fertilize eggs and have normal fertility. They must be motile and able to survive in the female genital tract. They must have the ability to bind and penetrate through the zona pellucida, fuse with the oolemma, activate the egg, contribute functional DNA and trigger zygotic development. A deficiency in any one of these attributes would be expected to reduce fertility. But testing all these attributes on a specific semen sample is not practical. We sought to identify the minimal traits needed to provide an accurate estimate of the fertility of a given semen sample. We expected that attributes included in the final model would have adequate variation. If there were little variation between semen samples, that trait could not distinguish high and low fertility samples. We found variation among samples in all the traits we tested. Furthermore, because they would have a significant affect on fertility, only attributes necessary for fertility would be expected in the final model. We also expected that attributes that were frequent causes of infertility would be included in the final model, because they would reduce fertility of many samples. Attributes that were highly correlated with each other were not both expected to be in the model because the second attribute would not contribute more predictive ability to the model than the first attribute. All these considerations contributed to the final model derived from the data. Indeed, the sperm attributes in the final model, intact functional acrosomes, live and motile cells and ability to bind the zona pellucida, all are consistent with these predictions.

This work was all performed using cryopreserved sperm. Previous results from our laboratory found that zona pellucida binding was positively correlated with litter size when using fresh liquid semen and homospermic insemination (Braundmeier et al., 2004). When fresh
semen was tested and fertility determined by heterospermic AI, zona binding ability alone was not significantly correlated with average litter size, but when added to other sperm assessments such as motility, morphology and acrosome integrity, fertility of fresh semen could be predicted successfully (Collins et al., 2008). However, these are the first experiments to predict fertility of cryopreserved porcine sperm by assessing zona pellucida binding. We suspected that assays to predict fertility of cryopreserved sperm might be different that those that predicted fertility of fresh liquid semen because of the damage done by cryopreservation. Furthermore, using cryopreserved semen allowed us to use the same semen collection in all assays that was used to determine fertility, which eliminates ejaculate variation that was present in the previous studies that used multiple ejaculates (Braundmeier et al., 2004; Collins et al., 2008).

The requirement for oocytes and greater skill to complete zona pellucida binding assays makes this assay challenging when using fresh semen. However, this assay and the other assays measuring motility, viability and acrosome integrity can be completed on cryopreserved semen due to the extended time semen can be stored before use for AI. Fertility could be estimated using these calculations before semen is shipped. So this assay of sperm binding may be particularly valuable for cryopreserved sperm.

Some assays described herein that were tested for their ability to predict fertility have been in use for decades (motility and viability) whereas other measurements are more novel (oviduct binding, zona binding and IVF). In total, the assays tested for fertility prediction assess sperm viability and motility, the function of the sperm surface, acrosomal stability, the ability to complete fertilization, activate eggs and promote embryonic development. The function of the sperm surface was assessed by two separate criteria, oviduct cell binding and zona pellucida binding. Curiously, samples with sperm that bound more frequently to oviduct cells also bound
more frequently to the zona pellucida. Perhaps both are good general measures of the function of the cell surface and, for that reason, only one of these apparently redundant attributes was necessary in the model to predict fertility. Although there is less information on the effect of cryopreservation on sperm binding to the zona pellucida, it is known that cryopreservation induces precocious capacitation of boar sperm and/or acrosome damage (Bailey et al., 2008) and reduces the ability of sperm to bind oviduct epithelial cells (Lefebvre & Suarez, 1996; Fazeli et al., 1999). This reduction is probably because premature capacitation and acrosomal damage can reduce oviduct cell and zona pellucida binding (Ivanova & Mollova, 1993; Bailey et al., 2008).}

The final model included an inverse relationship between the percentage of acrosome compromised-sperm after 30 min (T30) and percentage of piglets sired by a specific boar (Table 3 A) but, in contrast, a positive relationship between acrosome-compromised sperm at T0 and fertility (Table 3 A). This result emphasizes that the interaction of independent variables complicates the analysis of individual coefficients with fertility.

In vitro fertilization using frozen-thawed boar sperm achieved similar results to that of cooled boar semen used in our laboratory (Yuan et al., 2011; Silva et al., 2012; Yuan & Krisher, 2012). Although frozen-thawed sperm are often used for IVF to improve the consistency and repeatability of experiments, we observed a considerable amount of variation unrelated to sperm, similar to IVF results with cooled semen (Xu et al., 1996; Long et al., 1999; Gil et al., 2008). The overall success of IVF using frozen-thawed boar sperm has been somewhat inconsistent (Nagai et al., 1988; Zheng et al., 1992; Abeydeera & Day, 1997a; Córdova et al., 1997; Rath & Niemann, 1997; Suzuki et al., 2005). However, our objective was to determine if IVF was related to fertility. The minimal relationship between IVF and fertility may be partially explained by sperm preparation procedures for IVF that select for an enriched population of
fertile sperm by removing some non-viable and/or less motile sperm (Abeydeera & Day, 1997a; Abeydeera & Day, 1997b). Furthermore, IVF is a highly artificial system with high sperm:egg ratios and a number of environmental factors that likely contribute to the variability in fertilization outcomes (Abeydeera & Day, 1997b). This may explain the low correlation between IVF and fertility, which has been previously reported (Larsson & Rodríguez-Martínez, 2000; Ruiz-Sánchez et al., 2006).

CONCLUSION

Multiple linear regression analyses of both novel and traditional sperm traits were highly predictive of the fertility of frozen-thawed boar sperm for AI. Although no one sperm attribute predicted fertility with high reliability, a model including five traits, acrosome compromised sperm (0 and 30 min), percent live sperm (0 min), percent total motility (30 min) and the number of zona bound sperm, predicted highly the percentage of piglets in a litter sired by a boar in mixed insemination ($r^2 = 0.87$). These assays and predictive models could be used as a screen to detect poor quality frozen porcine semen prior to use and thus, improve AI success.

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AUTHOR’S CONTRIBUTIONS

B.W.D., D.J.M., R.V.K. and R.L.K contributed to experimental design of the research. B.W.D., K.A.M. and P.H.P. collected data. B.W.D. and S.R.Z. performed the statistical analyses. B.W.D. prepared the manuscript. All authors analyzed the data, contributed to revisions and approved the submitted manuscript.
FIGURES AND TABLES

Table 4.1(A). Multiple linear regression analysis to assess the relationship of sperm traits to boar fertility as determined by the mean percent of pigs in each litter sired for each boar.

<table>
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<th>Variable</th>
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<th>p-Value</th>
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<td>Acrosome compromised % (T0)</td>
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<td>-6.11</td>
<td>&lt; 0.01</td>
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<td>0.41</td>
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<tr>
<td>Average sperm bound to zona</td>
<td>-7.18</td>
<td>1.70</td>
<td>-4.23</td>
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Model Summary

<table>
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<th>R-Square</th>
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<th>p-Value</th>
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<tbody>
<tr>
<td>0.92</td>
<td>0.87</td>
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Partial values for each variable included in the final model.

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Table 4.1(B). Multiple linear regression analysis to assess the relationship of sperm traits to boar fertility as determined by the mean number of piglets sired per litter

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<th>Variable</th>
<th>Estimate</th>
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Model Summary

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Partial values for each variable included in the final model.

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<th>Model R-Square</th>
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Multiple linear regression using the least squares mean from laboratory assays and post-thaw analyses of cryopreserved boar ejaculates was used to predict boar fertility. Fourteen boar ejaculates were subjected to a sperm-oviduct binding assay, competitive zona-binding and IVF,
as well as routine post-thaw analyses. Boar fertility was determined by AI. The LSMs for each boar from the above sperm assays and post-thaw analyses were regressed in two models against fertility measures from AI to determine which sperm characteristics could estimate boar fertility. The same single ejaculate for each boar that was used to determine boar fertility was also used for all laboratory assays. A total of seventeen sperm traits were regressed against two fertility measures (A. mean percent of litters sired for each boar and B. mean number of piglets per litter for each boar) using stepwise linear regression.
An example of sperm bound to an aggregate of oviduct cells harvested from the isthmus (scale bar = 50 µm). Sperm were enumerated and the aggregate circumference measured to determine binding density.
Competitive zona pellucida binding assay to quantify differences among boars in the binding ability of frozen-thawed sperm. (A) Sperm were incubated with either green or orange fluorophore for boar identification (20 μM of Cell Trace™ Oregon Green; 500 nM Cell Tracker™ Orange, Molecular Probes® Life Technologies™ Eugene, Oregon) and validated to determine effect of dye on sperm binding. Equal numbers of sperm labeled with each fluor were added to droplets containing fixed oocytes and the number of sperm bound to the periphery of the zona was counted. Data were expressed as the percentage of bound sperm from each sample. (B) Sperm from 18 different ejaculates were tested in a competitive zona binding assay and then ranked by boar for the average (LSM ±SEM) number of sperm bound to the periphery of zona. (C) Representation of sperm from two different ejaculates identified by either fluorophore that were bound to the zona pellucida (scale bar =100 μm). (D) A strong relationship (R = 0.94) was identified among the number of sperm bound to oviduct aggregates and the number of sperm bound to the zona pellucida in two separate assays. Sperm from the same frozen ejaculate of twelve different boars were compared among assays after exclusion of two outliers.
Variation in fertilization outcomes using frozen-thawed sperm from sixteen different boars. Gametes were allowed to co-incubate for 5 h and returned to incubation for 7 h. Approximately 25% of oocytes exposed to sperm from each replicate were removed and mounted on a glass slide followed by fixation (70% ethanol and acetic acid) for 4-5 days. Presumptive zygotes were stained with 1% aceto-orcein solution (Sigma Aldrich, St. Louis, MO) and boars were ranked by LSM from a minimum of 2-3 replicates to determine the range in variation of fertilization outcomes for the (A) total percentage of oocytes with two or more pronuclei, (B) monospermic fertilization (two pronuclei) and (C) polyspermic fertilization (more than two pronuclei). Fertilization and stage of meiosis were evaluated using phase contrast microscopy. The data are ranked from lowest to highest to show variation; the number on the abscissa does not represent the same boar for each evaluation (A-C).
Fertility of each boar following artificial insemination of gilts with frozen-thawed boar sperm was similar when calculated in two separate models. (A) The average number of piglets sired per litter by each boar. (B) The mean percentage of piglets in a litter sired by each boar. A total of 136 gilts were inseminated with 4.0 billion sperm 24 and 36 h (AI-1 or AI-2) after the onset of estrus using a single ejaculate from one of two boars at each insemination. AI order was reversed among boar pairs in the following replicates. Results are expressed as the LSM ± SEM using a mixed linear model and boar ranking IDs reflect the same boar for (A) and (B). (C) The mean number of piglets sired by a specific boar in each litter and the mean percent of offspring in each litter sired by the specific boar were highly correlated (R = 0.91).
Cryopreserved ejaculates from a single collection of 14 different boars were thawed and evaluated for motility, viability and acrosome integrity (Ac. Int.) at 0, 30 and 60 min after thawing. Samples were thawed at 50°C for 20 sec and motility was recorded microscopically and confirmed (± 5%) using Computer Automated Semen Assessment. Sperm viability was determined by membrane exclusion of propidium iodide (PI, Sigma Aldrich, St. Louis, MO) and acrosome integrity was assessed using fluorescein-labeled peanut agglutinin (FITC-PNA). Values reported represent the mean of three independent samples for a single ejaculate.

<table>
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<tr>
<th>Boar I.D.</th>
<th>Motility % (T0)</th>
<th>Motility % (T30)</th>
<th>Motility % (T60)</th>
<th>Live % (T0)</th>
<th>Live % (T30)</th>
<th>Live % (T60)</th>
<th>Ac. Int. % (T0)</th>
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REFERENCES


CHAPTER 5
TRPP2 REGULATES MOTILITY AND CALCIUM TRAFFICKING OF PORCINE SPERM

ABSTRACT

Transient receptor potential channel-2 (TRPP2) is a membrane protein important in regulation of calcium homeostasis in renal epithelial cells. Mutations in human TRPP2 cause enlarged cystic kidneys and contribute to polycystic kidney disease (PKD). Male Drosophila with mutated TRPP2 display a mild sperm motility phenotype but have a drastic reduction in fertility due to failed sperm migration and storage within the female tract. Although TRPP2 has critical roles for Drosophila sperm function, the protein has not been described in mammalian sperm. Herein, we report the expression of TRPP2 in porcine sperm and have identified functions of TRPP2 in regulating capacitation-like changes important for fertility. TRPP2-inhibited porcine sperm swim significantly slower than control samples and have an increase in tail cross-beat frequency when sperm are maintained in capacitating conditions. Sperm incubated with TRPP2 antibody also showed a significant decrease in intracellular calcium levels and failed to experience a transient rise in calcium over time that is characteristic of capacitating sperm. Porcine TRPP2 is a previously unreported sperm membrane protein that appears to function as an ion channel to regulate capacitation-like changes in porcine sperm.

Keywords: PKD2, capacitation, swine, ion
INTRODUCTION

Sperm plasma membrane proteins play critical roles as sperm respond to extrinsic signals within the female reproductive tract prior to fertilization. The plasma membrane is critical as sperm undergo a number of physiological and metabolic changes during maturation, or capacitation, including increased cytosolic calcium, hyperactivation, and the acrosome reaction (Florman & First, 1988). Many of these events are regulated in part by sperm membrane proteins through direct ligand interactions and activation of second messengers that are not completely understood. Sperm interaction within the female genital tract may be regulated by proteins that have the potential to perform multiple functions. The transient receptor potential family of proteins (TRPs) are likely candidates for directing important sperm functions due to their well-characterized role in ion regulation and ligand-induced activation during reproductive events described in model organisms (Goodenough et al., 1993; Gao et al., 2003; Watnick et al., 2003; Kottgen et al., 2011; Nilius & Owsianik, 2011).

The TRP family of proteins has been characterized into seven sub-families that are based on protein homology (Wu et al., 2010; Nilius & Owsianik, 2011). The transient receptor potential polycystic (TRPP) sub-family is unique in that many of these proteins can be activated by several different modes (Venkatachalam & Montel, 2007; Tsiokas, 2009). In particular, TRPP2 is well-studied for its role in human polycystic kidney disease (Mochizuki et al., 1996; Ma et al., 2005; Köttgen, 2007; Cai et al., 2014). Mutations of TRPP2 account for 15% of all persons with autosomal dominant polycystic kidney disease (ADPKD) (Koptides et al., 1999). Congenital and de-novo mutations of TRPP2 result in disruption of ion homeostasis within the renal epithelium causing enlarged cystic kidneys (Tsiokas et al., 1997; Owsianik et al., 2006; Tsiokas, 2009; Zhu et al., 2011).
The importance of TRPP2 for reproductive events is relatively unknown except for some sperm functions that have been demonstrated using model organisms. TRPP2 interacts with TRPP1 to form heteromultimeric complexes that function as ion channels (Hanaoka et al., 2000). TRPP1 contains leucine-rich repeats and a REJ domain (receptor egg-like jelly) that has been implicated in sea-urchin fertilization (Moy et al., 1996; Hughes et al., 1999) and TRPP1/TRPP2 complexes are found exclusively at the plasma membrane of the acrosome (Neill et al., 2004). The single-cell alga *chlamydamonus reinhardii* expresses a TRPP2 homologue (crTRPP2) that is localized to the flagellum and is important for early mating events (Pazour et al., 2005). Much of what is known about the function of TRPP2 in relation to sperm has been described in *Drosophila melanogaster* (Gao et al., 2003; Watnick et al., 2003; Kottgen et al., 2011). Sperm from *D. melanogaster* with mutated TRPP2 (Amo) are subfertile, swim slower in the female reproductive tract and fail to occupy the sperm storage areas, indicating a functional requirement of TRPP2 for sperm storage in this species (Kottgen et al., 2011). Substantially less information is available regarding the importance of TRPP2 in humans with ADPKD, but clinical cases citing impaired reproductive performance have been documented (Okada et al., 1999; Kanagarajah et al., 2012; Reig et al., 2015).

Porcine (*sus scrofa*) TRPP2 is a 970-amino acid plasma membrane protein that has been described in renal epithelium and other tissues (Mochizuki et al., 1996; Wang et al., 2011; Morick et al., 2013). Although TRPP2 seems to play important reproductive functions in lower organisms, no prior reports exist to demonstrate the expression of TRPP2 in mammalian sperm. We hypothesized that TRPP2 was important for mammalian sperm capacitation-related events such as motility and intracellular calcium regulation. Our overall goal was to detect, localize and identify putative functions of TRPP2 in porcine sperm. Antibody-mediated inhibition of TRPP2
demonstrated a novel motility phenotype and suppression of intracellular Ca\textsuperscript{2+} in porcine sperm that is previously unreported in any mammalian species. These results demonstrate a conserved role for TRPP2 in mammalian sperm functions that are important for normal sperm interactions within the female reproductive tract.

**MATERIALS AND METHODS**

**Experimental Approach**

Porcine semen was collected from adult commercial boars of various breeds in accordance with their routine schedule.

**Semen Collection and Processing**

Semen from three fertile boars was collected and diluted in BSA-free Preserv Xtra extender (Reproquest, Fitchburg, WI, USA) and maintained at approximately 17°C until further use. Equal volumes of semen from three different boars were pooled and passed through a Percoll gradient containing dmTALPNC (Tyrodes-based medium with albumin, lactate, pyruvate) to remove extender (Kadirvel *et al.*, 2012). Briefly, 3 mL of semen was placed on top of a Percoll gradient containing 5.4 mL of Percoll, 0.6 mL of 10X PBS (1.3 M NaCl, 40 mM, KCl, 10 mM CaCl\textsubscript{2}, 5 mM MgCl\textsubscript{2}, 140 mM fructose and 0.75 M BSA) and 4 mL of dmTALPNC (non-capacitating): (2.1 mM CaCl\textsubscript{2}, 3.1 mM KCl, 1.5 mM MgCl\textsubscript{2}, 100 mM NaCl, 0.29 mM KH\textsubscript{2}PO\textsubscript{4}, 0.36% lactic acid, 0.17 uM polyvinylpyrrolidone, 1 mM sodium pyruvate, 35 mM HEPES, pH 7.4). Sperm were centrifuged at 800 g for 10 min followed by removal of supernatant and resuspension of the sperm pellet in TALPNC. Sperm were then washed twice by centrifugation at 600 g for 5 min. For sperm collected under capacitating conditions, pooled samples were subjected to dmTALP containing 0.91 mM BSA, 10mM NaHCO\textsubscript{3} and 25 mM HEPES for 4 hr.
Protein Extraction from Whole Sperm Lysate

Cooled semen from three boars was concentrated by centrifugation through a Percoll cushion under non-capacitating and capacitating conditions followed by a second wash in PBS. Sperm pellets were diluted 1:1 (v:v) with RIPA (50 mM TRIS HCL, 150 mM NaCl, 1% NP40, 0.5% Na deoxycholate, 0.1% SDS) with protease inhibitor (SIGMAFAST™ EDTA free). Samples were placed on ice and maintained at 4°C for 30 min and vortexed every 10 min. After incubation, samples were centrifuged at 10 000 g for 5 min to pellet the insoluble debris. Supernatant was removed and aliquots of sperm lysate were frozen on dry ice using methanol and held at -20°C. Protein from sperm lysate used for assay was quantitated using the Pierce® BCA Protein Assay Kit (Thermo Scientific, Rockford, IL) using a BSA standard.

TRPP2 Anti-serum Production

TRPP2 anti-serum was prepared and provided as described by (Watnick et al., 2003). To generate the antiserum, a fragment encoding Drosophila melanogaster TRPP2 (Amo) amino acids 288-555 (first extracellular loop) was subcloned into pRSETB (Invitrogen) to create a HIS-tagged fusion protein that was expressed in E. coli (BL21), purified with nickel resin (Amersham), and injected into rabbits (Covance). Sequence alignment of sus scrofa polycystin-2, (TRPP2) NCBI: NP_001232908.1 revealed a 970 - amino acid protein with ~ 22% homology to Drosophila melanogaster 924 amino acid Amo, (TRPP2) NCBI: AAF53183.2 and ~ 30% homology in the last 149 amino acid sequence corresponding to the antibody specific region. TRPP2 antiserum targets the first extracellular loop allowing functional analysis.

TRPP2 Detection by SDS-page and Western Blot

Protein from sperm lysate (20 µg) was solubilized in 2x Laemmli buffer (Bio-Rad Laboratories Inc., Hercules, CA) by dilution to 1x in sample with 5% β-mercaptoethanol. Samples were heated at 70°C for 10 min and resolved by gel electrophoresis in 7.0% gels (Mini-
PROTEAN® TGX™, Bio-Rad) for 1.5 hr at 95 V. The resolved proteins were transferred onto a nitrocellulose membrane in a transfer buffer (48 mM Tris base, 139 mM glycine, 20% methanol, and 0.025% SDS) at 95 V for 1 hr. The membrane was blocked at room temperature using 5% nonfat milk for 1 hr. After blocking, the membrane was washed three times for 10 min each using Tris-buffered saline and Tween20 (TBST; 20 mM Tris Base, 137 mM NaCl, 0.1% Tween20). The membrane was divided into three sections and probed separately with TRPP2 antibody (1:1000) and rabbit serum (1:1000; Vector Laboratories, Inc., Burlingame, CA) in 2.5% BSA overnight at 4°C. Membranes were then washed three times in TBST and incubated 1:5000 with goat anti-rabbit IgG polyclonal horseradish peroxidase-conjugated antibody (Thermo Fisher Scientific, Rockford, IL) for 30 min in 2% BSA. After washing, bound antibody was visualized by incubation with ECL western blotting substrate (Pierce Biotechnology) using Image Quant 2000 software and 20 sec of exposure.

**Localization of TRPP2 by Immunofluorescence**

Cooled semen from three boars was pooled and separated from extender through Percoll centrifugation as previously described in TALPNC and TALP for non-capacitating and capacitating conditions, respectively. For capacitating conditions, sperm were incubated in TALP containing BSA and bicarbonate for 4 hr at 39°C at a concentration of 100 x 10⁶ sperm/mL in 300 µl. TRPP2 anti-serum was added at a concentration of 1:500 and samples were returned to incubation at 39°C for 45 min. Propidium iodide (PI: MP Biomedicals, Santa Ana, Ca) was prepared from a 1.5 mM solution in DMSO, diluted in TALPNC and then added to sperm at a final concentration of 12 µM for 15 min at 39°C to assess membrane integrity. Sperm samples were then centrifuged at 10 000 g for 30 sec and resuspended in TALPNC to the original volume (~298 µl). Secondary goat anti-rabbit IgG polyclonal horseradish peroxidase-
conjugated antibody (Thermo Fisher Scientific, Rockford, IL) was added at a concentration of 1:500 and sperm were returned to incubation at 39°C for an additional 30 min. Samples were then centrifuged at 10 000 g for 30 sec and resuspended to original volume in TALPNC. Sperm were placed on a glass slide with a coverslip, viewed at 630 X under oil and evaluated using a Zeiss Axioskop 25 and Axio-CamHRc (Carl Zeiss Microscopy, LLC, Thornwood, NY, USA) with fluorescent filters (excitation band pass 546/12, emission >590 nm). Sperm that excluded PI were considered live.

**Sperm Motility Assessment**

Semen pooled from at least 3 different boars was stored up to 48 hr and passed through a Percoll gradient containing TALPNC to remove extender as described. Sperm were aliquoted into three different treatments containing 50 x 10^6 sperm/mL in a total volume of 500 µl of TALPC (BSA and bicarbonate): 1) sperm extended in TALPC only 2) addition of TRPP2 antiserum (1:500) and 3) addition of rabbit serum (1:500 - Vector Lab). Sperm were incubated at 39°C for 20 min to allow binding of antibody and motility was assessed using CASA (Computer Aided Sperm Analysis; Hamilton, Thorne, Beverly, MA, USA). A minimum of 5 fields and 400 spermatozoa were observed on a 37°C heated stage by acquiring 30 frames at a rate of 60Hz with minimum contrast of 55, minimum cell size of five pixels, VAP cutoff of 20 µm/s, progressive minimum VAP cutoff of 45 µm/s, VSL cutoff of 5 µm/s, static head size of 0.53-4.45 and magnification of 1.89. Samples were measured every 30 min after the initial antiserum incubation time for 4 hr and returned to incubation at 39°C between measurements. Parameters measured included total motility, progress motility, VAP (average path velocity), VSL (straight-line velocity), VCL (curvilinear velocity), STR (straightness = VSL x 100/VAP),
LIN (linearity = VSL X 100/VCL), WOB (wobble = VAP X 100/VCL), BCF (beat cross frequency) and ALH (amplitude of lateral head displacement).

**Measurement of Free Ca$^{2+}$ in Sperm**

Semen was collected from 3 mature boars (Prairie State Semen, Inc., Champaign, IL) for each replicate and was extended, cooled and used with 24-48 hr of collection. Only samples with 80% or greater motility and < 20% abnormal morphology were used. Pooled semen was passed through a Percoll gradient to remove extender, the supernatant was removed and the remaining sperm pellet was resuspended in 2.5 mL of non-capacitating TALPNC (no BSA or bicarbonate). A Ca$^{2+}$ sensitive reporter, Fluo-4, AM (Thermo-Fisher), was added at a final concentration of 4 µM by the addition of 20 µl of Fluo-4-AM in DMSO from a 0.5 mM stock solution to 2.5 mL of sperm suspension. The sample was incubated in the dark at room temperature for 30 min and then washed by centrifugation for 5 min at 300 g with the addition of 2.5 mL of TALPNC. The sperm pellet was resuspended in TALP (with BSA and bicarbonate) and sperm concentration determined by hemocytometer. The final concentration of sperm was adjusted to 5 x 10$^6$ sperm/mL in a total volume of 500 uL of TALP. Samples were then incubated at room temperature for an additional 20 min to allow complete hydrolysis of the acetoxymethyl (AM) groups by cytoplasmic esterases. TRPP2 antiserum, rabbit serum and ADAM 5 antiserum were added at a 1:500 dilution 5 min prior to the first sample analyses. Sperm levels of free cytoplasmic Ca$^{2+}$ were measured by spectrofluorometric analyses using an argon-ion laser excitation at 494 nm and emission at 515 nm in a QuantaMaster 4CW fluorescence spectrophotometer (Photo Technology International, NJ). Calcium measurements were taken ~ 5 min after the addition of antibody and every 30 min for up to 120 min. Sperm samples were maintained at 37°C between measurements to facilitate capacitation.
Statistical Analyses

Differences among means for motility and calcium experiments were determined by a one-way analysis of variance using SAS statistical software (v. 9.0 SAS Institute, Inc, Gary, NC). The MIXED procedure in SAS was used to carry out all analyses and replicate was considered a random effect for all experiments. Interactions between time and treatment were included as fixed effects and repeated measure analyses were conducted for experiments measured over time. The results are shown as means ± SEM and treatments were considered different when p < 0.05. Post-hoc analysis was conducted using Tukey’s HSD for multiple comparisons when data were consistent with normality assumptions. Motility data were arcsine transformed. Square root and log(base) transformations were applied to sperm velocity data when normality assumptions were not met (p > 0.05). Quantification of TRPP2 bands from blot analyses was conducted using GraphPad Software. TRPP2 bands were normalized against a serum protein (~ 225 kD) and ratios were analyzed for significance (p < 0.05) by unpaired t test.

RESULTS

TRPP2 RNA has previously been described in porcine testes (Wang et al., 2011) but the protein has not been reported in sperm of any mammal. We sought to determine if TRPP2 was expressed in porcine sperm and to identify the locations of expression. Pooled samples of whole sperm lysate from multiple boars were collected under non-capacitating and capacitating conditions to probe for TRPP2. A band of approximately 110 kD that was specific to TRPP2 antibody was detected in sperm from both conditions (Fig 5.1). Other bands found in the lanes detected with TRPP2 antiserum were also observed when the membrane was probed with control antiserum. This is a size (110 kD) that is observed when porcine kidney tissue is probed with an antibody to TRPP2 (Wang et al., 2011).
To determine the location of TRPP2 in porcine sperm, live sperm were incubated with TRPP2 antibody and abundant staining was observed along the plasma membrane and post-acrosomal region of membrane-intact sperm maintained in non-capacitating conditions (Fig. 5.2 A). TRPP2 was also expressed along the anterior portion of the principal piece in a punctate pattern. Samples were double stained with PI to identify membrane-compromised sperm, and those sperm that were PI-positive exhibited weak TRPP2 staining (Fig. 5.2 B). The abundance of TRPP2 was distinctly attenuated and much less consistent among the whole population of sperm tested after incubation in capacitating conditions (Fig 5.2 C).

Possible functions of TRPP2 were explored by first determining if antibody-mediated inhibition of TRPP2 affected sperm motility parameters (Table 5.1). Pooled samples of porcine sperm were held in capacitating conditions for 4 hr and objective motility measurements were acquired through CASA every 30 min. The percent of total and progressive motile sperm were not significantly changed (p > 0.05) by TRPP2 antiserum or rabbit serum that was used as a vehicle control. However, the velocity measures (Fig 5.3) were changed when TRPP2 antiserum was added (p < 0.05) for many different sperm parameters including VAP (average path velocity), VSL (straight-line velocity) and VCL (curvilinear velocity). Interactions over time were not identified for most treatments (p > 0.05). Conversely, the beat cross frequency (BCF) of sperm was significantly higher (p < 0.05) for samples incubated with TRPP2 antibody and increased over time. The difference in BCF between treatments was lost at 3.5 h of incubation (p > 0.05) (Fig 5.3).

Because incubation with TRPP2 changed sperm kinematics and intracellular free calcium regulates sperm motility, the intracellular calcium content of TRPP2-inhibited sperm was measured over time. Pooled samples of sperm were loaded with Fluo4 AM in capacitating
conditions and intracellular Ca\textsuperscript{2+} was measured indirectly by spectrophotometry for 1.5 hr at 30 min intervals. The control sample, serum (vehicle control) and ADAM5 antisera-treated sperm (epitope is intracellular so this was an antibody control) showed an increase in intracellular Ca\textsuperscript{2+}, consistent with the development of porcine sperm capacitation. Samples treated with TRPP2 antiserum contained lower intracellular calcium levels than controls and showed no increase in intracellular Ca\textsuperscript{2+} over time (Fig 5.4).

**DISCUSSION**

The biological roles of human TRPP2 are well described primarily for their importance in renal function. Mutations in the gene encoding TRPP2 cause cyst development in many organs including the kidney that result in polycystic kidney disease (Koptides et al., 1999) due to disruption of ion homeostasis (Owsianik et al., 2006). TRPP2 is localized to the primary cilium of renal epithelial cells and can be activated by fluid shear stress, ligand binding, cell adhesion and voltage (Tsiokas, 2009). The reproductive functions of TRPP2 have been described in many model organisms including *D. melanogaster, C. reinhardii, C. elegans* and sea urchins (Watnick et al., 2003; Neill et al., 2004; Pazour et al., 2005). For all of these organisms, TRPP2 has been localized on the flagellum (or cilia) or the periacrosomal region. Inhibition or null mutation of TRPP2 results in severe subfertility, mating disruption and changes in directional swimming of sperm. Although mammals are phylogenetically distant from the aforementioned organisms, many of the proteins expressed on cilia seem to be well conserved and carry out similar functions (Pazour et al., 2005). However, the expression of TRPP2 in sperm has not been reported in mammals prior to these findings.
There are few clinical reports that implicate any TRPPs with impaired reproductive function in patients with PKD. General observations include cystic seminal vesicles attributed to mutated TRPPs, but no data has conclusively identified a role for TRPP2 in reproductive function or expression in mammalian sperm. The pig has been used as a model to study human PKD but TRPP2 protein has not been described in *sus scrofa* testis or sperm (Mochizuki et al., 1996; Wang et al., 2011; Morick et al., 2013). We detected a band of ~ 110 kD in porcine sperm that was consistent with the expected weight of TRPP2 reported in multiple porcine tissues (Wang et al., 2011) and that of other species (Torres et al., 2001; Koulen et al., 2002). TRPP2 was heavily localized to the apical plasma membrane and post-acrosomal ridge of sperm held in non-capacitating medium with punctuate staining patterns on the anterior region of the principle piece. However, the staining pattern of sperm held in capacitating conditions for 4 hr was much less consistent and weaker than sperm incubated in medium without BSA or HCO$_3$-; both of which are necessary for capacitation and consequent membrane destabilization (Parks & Graham, 1992). Data from immunoblots confirmed that TRPP2 was less abundant ($P = 0.03$) in capacitated sperm than sperm incubated under conditions that do not allow capacitation. The sperm plasma membrane undergoes a significant number of modifications and changes in protein interactions during sperm capacitation (Parks & Lynch, 1992). The cause of the decrease in TRPP2 in sperm is unclear. It may perhaps be due to proteolysis of the protein that results in a disappearance of the 110 kD band in western blots and a corresponding loss in immunofluorescence. The premise that TRPP2 localization is dependent upon plasma membrane integrity is supported by weak or non-existent TRPP2 staining on sperm positive for propidium iodide, a stain indicative of membrane damage and non-viable sperm. Disruption of the plasma
membrane due to changes during capacitation or loss of viability altered TRPP2 abundance in porcine sperm.

Because TRPP2 acts as a cation channel and calcium is a central regulator of sperm function, we thought it likely that TRPP2 may regulate intracellular calcium and sperm function. However, based on the mild phenotype in motility parameters of TRPP2-null Drosophila sperm, we also hypothesized that impaired TRPP2 function in mammalian sperm may not be detected when sperm were subjected to routine analyses. As predicted, the total and progressive motility of TRPP2-inhibited sperm was unchanged over time when compared to controls or sperm incubated in capacitating-medium only (TALPC). Although not directly quantified, the viability of sperm was not noticeably different among treatments. However, consistent with reports of Drosophila sperm, the velocity of TRPP2-inhibited sperm was significantly slower than control groups. These changes in motility parameters of sperm do not necessarily indicate a relationship to fertility, but they do provide useful information regarding important physiological changes of sperm prior to fertilization and thus possible functions of TRPP2. A number of the motility parameters that we reported in TRPP2-inhibited sperm are consistent with sperm that are not hyperactivated when held in a medium that promotes capacitation (Suarez et al., 1992; García Herreros et al., 2005), a physiological process required for zona penetration. Motility parameters for porcine sperm that clearly identify hyperactivity and capacitation are loosely defined with some contradiction due to differences in experimental conditions (White & Aitken, 1989; Suarez et al., 1993; Ho et al., 2002; Miller et al., 2014). However, TRPP2-inhibited sperm displayed many of the same characteristics as porcine sperm in a non-hyperactive state (decrease in sperm velocity, increase in beat-cross frequency)(Garcia Herreros et al., 2005). Thus, TRPP2 appears to be required for the development of hyperactivated motility in media that promote capacitation.
Changes in sperm velocity and tail beat patterns are indicative of changes in intracellular Ca\(^{2+}\) (Suarez et al., 1992; Schmidt & Kamp, 2004). An increase in sperm intracellular Ca\(^{2+}\) precedes both capacitation and the acrosome reaction prior to fertilization (Mizuno et al., 2009; Mizuno et al., 2012; Miller et al., 2014). The intracellular Ca\(^{2+}\) of porcine sperm was measured over time when samples were maintained in the same conditions as those for motility experiments. A slight rise in Ca\(^{2+}\) was observed over 90 minutes for sperm held in serum and control samples, as is consistent with mammalian sperm undergoing capacitation (Yanagimachi & Usui, 1974; Florman et al., 2008; Bailey, 2010). Conversely, TRPP2-inhibition resulted in significantly lower intracellular Ca\(^{2+}\) compared to other treatments and a decrease of Ca\(^{2+}\) content over time. Although ion regulation across the plasma membrane was a proposed function for TRPP2 in sperm, the marked suppression of Ca\(^{2+}\) was somewhat surprising due to the well-recognized function of CatSper in sperm Ca\(^{2+}\) regulation (Yanagimachi & Usui, 1974; Bailey, 2010). TRPP2 function on the cilia of *C. reinhardii* suggests that Ca\(^{2+}\) induces a rise in cAMP, but these events were localized to the flagellar membrane with no evidence of increased global cytosolic free Ca\(^{2+}\) during mating-related events (Miller et al., 2014). Given the evolutionary conservation of cilia and flagella (Goodenough et al., 1993), it is plausible that mammalian TRPP2 on sperm flagellum could also be activated by cell adhesion and activation could be dependent or independent of TRPP1. However, the mechanism of activation is likely to be different in mammals because most evidence in *C. reinhardtii* indicates a Ca\(^{2+}\) efflux upon TRPP2 activation (Pazour et al., 2005) from the cell wall and not the ER, rather than the characteristic Ca\(^{2+}\) influx observed in mammalian sperm during capacitation and the acrosome reaction. Furthermore, a minimal reduction in mating was observed with the chelation of extracellular Ca\(^{2+}\), (Goodenough et al., 1993), which is again contradictory to what one would
expect during mammalian fertilization. The proposed models for TRPP2 Ca$^{2+}$ regulation and activation are likely dependent on the context (i.e. co-factors, etc.) in which TRPP2 is found. TRPP2 function is dependent upon activation and activation is regulated by proteins and co-factors, ligand binding and cell adhesion (Bloodgood & Levin, 1983; Goodenough et al., 1993). Therefore, TRPP2 activation and function on the head of sperm may be different than that on the principle piece. Additionally, TRPP2 activation at distinct locations on sperm could also occur independently and at different times during transit within the female reproductive tract. TRPP2 regulatory proteins, timing of activation and cellular location of TRPP2 interactions with other proteins requires additional exploration to support further functions of sperm TRPP2 during fertilization. TRPP2 plays important functions during gamete transfer, but how these events relate to mammalian sperm, whether they are consistent across species and if TRPP2 interaction with other proteins is dependent upon cellular location are all questions that still remain unanswered.

CONCLUSION

TRPP2 is found in porcine sperm where it regulates sperm motility and intracellular calcium. Blocking TRPP2 resulted in a decrease in sperm velocity, increase in tail beat frequency and suppression of intracellular calcium, traits that normally occur during capacitation of porcine sperm. Previous data in non-mammalian sperm combined with our new data in porcine sperm suggest important functions for TRPP2 during mammalian fertilization.

ACKNOWLEDGEMENTS

The authors wish to acknowledge the donation of antibody from the Baltimore Polycystic Kidney Disease Research and Clinical Core Center (P30DK90868). Porcine semen was provided by Prairie State Semen, Champaign, IL.
(A) Lysate was collected from porcine sperm incubated in capacitating (C) and non-capacitating (NC) conditions and resolved by gel-electrophoresis for TRPP2 detection. Following transfer, the membrane was divided into three portions. One portion of membrane was probed with TRPP2 antiserum (1:1000) and the other with normal rabbit serum (1:1000) overnight at 4°C and then with goat-anti-rabbit secondary HRP for 30 min prior to 1 min of chemiluminescent substrate and a 60 sec exposure. The control portion of the membrane was probed with secondary antibody only. (B) Bands of ~110 kD were quantified for comparison between NC and C sperm lysate. TRPP2 expression was more abundant ($p = 0.03$) in non-capacitated than capacitated sperm.
Porcine sperm were held in non-capacitating conditions and incubated with diluted (1:500) TRPP2 antiserum and then Alexa 488-labeled secondary antibody followed by imaging with fluorescence microscopy. (A) TRPP2 is localized to the plasma membrane on the apical ridge and post-acrosomal region of sperm with some staining on the proximal portion of the principal piece. (B) Sperm stained with propidium iodide (PI) weakly express TRPP2 as indicated by white arrows. (C) Sperm in capacitating conditions have less defined TRPP2 expression compared to non-capacitated sperm. Membrane compromised sperm stained with PI also show low TRPP2 expression.
Figure 5.3

Porcine sperm were held in capacitating medium (TALPC) and incubated 1:500 with TRPP2 antiserum or rabbit serum (control). Computer Assisted Sperm Analysis was used to measure sperm kinematics every 30 min for 4 hr (A, C, E). The mean (± SEM) of each sample was calculated at the 4 hr time point (B, D, F). Sperm incubated with TRPP2 antibody had a significantly slower (p ≤ 0.05) average path (B) and curvilinear velocity (D) compared to normal serum and medium-only control samples. TRPP2 inhibition resulted in higher (p < 0.05) tail beat cross frequency than serum or sperm held in medium only (TALPC). (E) The BCF of sperm was not different (p > 0.05) among groups after 210 min of incubation.
Porcine sperm were loaded with Fluo-4-AM and then incubated with antisera against TRPP2, ADAM5 or normal rabbit serum (1:500 dilutions) in capacitating medium at 37°C. Spectrofluorometric reading were obtained every 30 min for 90 minutes. Samples of sperm in all treatments except TRPP2 demonstrated a rise in Ca\(^{2+}\) over time. Differences in intracellular Ca\(^{2+}\) among treatments were detected at 90 min after incubation (p < 0.05). No time by treatment interactions (p > 0.05) were observed. Different superscripts indicate differences among treatments at individual time points (p ≤ 0.05).
Table 5.1. Motility and kinematic parameters of sperm incubated with TRPP2, normal rabbit serum (vehicle control) or control medium (TALPC). Samples were held in TALPC at 39°C and measurements were taken every 30 min for 4 hr. A minimum of 400 sperm were evaluated at each time point using CASA. Values reported are the mean ± SEM of all observation over 4 hr for each parameter. Each replicate included pooled semen from 3 different boars. n =3 replicates.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>TRPP2</th>
<th>Serum</th>
<th>TALPC</th>
</tr>
</thead>
<tbody>
<tr>
<td>TM (%)</td>
<td>62.3 ± 3.8</td>
<td>63.7 ± 3.2</td>
<td>69.3 ± 3.5</td>
</tr>
<tr>
<td>PM (%)</td>
<td>33.1 ± 4.5</td>
<td>34.7 ± 3.5</td>
<td>45.9 ± 4.1</td>
</tr>
<tr>
<td>VAP (µms⁻¹)</td>
<td>59.5 ± 3.2*</td>
<td>74.5 ± 2.7</td>
<td>73.5 ± 2.7</td>
</tr>
<tr>
<td>VSL (µms⁻¹)</td>
<td>41.3 ± 4.0</td>
<td>48.3 ± 3.0</td>
<td>53.5 ± 3.1</td>
</tr>
<tr>
<td>VCL (µms⁻¹)</td>
<td>139.5 ± 2.2*</td>
<td>164.8 ± 1.7</td>
<td>161.0 ± 2.4</td>
</tr>
<tr>
<td>BCF (Hz)</td>
<td>42.6 ± 0.5*</td>
<td>40.6 ± 0.6</td>
<td>40.7 ± 0.5</td>
</tr>
<tr>
<td>LIN (%)</td>
<td>29.4 ± 2.0</td>
<td>29.6 ± 1.8</td>
<td>33.0 ± 1.6</td>
</tr>
<tr>
<td>STR (%)</td>
<td>68.4 ± 2.2</td>
<td>64.4 ± 1.82</td>
<td>72.1 ± 1.7</td>
</tr>
<tr>
<td>WOB (%)</td>
<td>42.5 ± 1.6</td>
<td>45.4 ± 1.6</td>
<td>45.6 ± 1.1</td>
</tr>
</tbody>
</table>

* p < 0.05 compared with control (TALPC). Motility and sperm kinematic parameters: TM (total motility), PM (progressive motility), VAP (average-path velocity), VSL (straight-line velocity), VCL (curvilinear velocity), BCF (beat cross frequency), LIN (linearity), STR (straightness) and WOB (wobble).
REFERENCES


Ho HC, Granish KA & Suarez SS. (2002) Hyperactivated motility of bull sperm is triggered at the axoneme by Ca2+ and not cAMP. *Dev Biol* 250, 208-217.


Summary

Commercial use of frozen boar sperm for artificial insemination is rare because fertility is reduced. The use of frozen sperm has many potential advantages compared to cooled semen such as additional time to test for pathogens and increased transport time. Laboratory assays that estimate the fertility of sperm prior to AI are necessary to help promote industry use of frozen boar sperm by identifying samples of low fertility. Evaluating assays by multiple linear regression analyses, we combined novel assays with traditional laboratory analyses of semen to identify sperm traits that collectively predicted fertility. A model that included five traits (acrosome compromised sperm (0 and 30 min), percent live sperm (0 min), percent total motility (30 min) and the number of zona bound sperm) predicted highly the percentage of piglets in a litter sired by a boar in mixed insemination ($r^2 = 0.87$). An additional model in which fertility was assessed by the number of piglets sired by boar ($r^2 = 0.57$) included many of the same traits as the previous model. Some laboratory assays also identified sperm traits that were related to IVF success. The post-thaw motility of cryopreserved boar sperm was somewhat useful for predicting the frequency of monospermic fertilization. The number of sperm bound to oviduct aggregates was highly correlated with zona binding ($R = 0.94$) and also related to IVF polyspermic fertilization ($p < 0.05$). Because motility and sperm binding were indicators of in vitro and in vivo fertilizing ability, we investigated the possibility that a sperm membrane putative ion channel, TRPP2, was present in porcine sperm and maintained conserved functions important for fertility. We identified TRPP2 in boar sperm and observed expression on both the head and principal piece. More importantly, TRPP2 inhibition changed the motility parameters
of sperm and inhibited intracellular calcium influx when challenged in capacitating conditions over time. This is the first report of TRPP2 for any mammalian sperm and suggests it has a function in ion transport and capacitation.

In conclusion, results from these studies provide: 1) the first indication that the number of boar sperm bound to oviduct cells is related to IVF measures, 2) identify the most robust model reported for estimating the fertility of cryopreserved boar sperm and 3) describe the first report of TRPP2 on mammalian sperm as well as functional roles in capacitation.

Future Directions

This research addressed a critical need for estimating the fertility of cryopreserved boar sperm prior to insemination. Our models indicated that fertility could be predicted by assays used in our laboratory. Most of the sperm traits identified include routine semen analyses that could be replicated in many laboratories. The critical aspect to repeating these experiments would require a standardized protocol for freezing, thawing and performing these laboratory assays. I have developed a working template that could be implemented as a computer program that would help to streamline this process and encourage consistency when analyzing samples.

The zona binding assay that we use could be improved to make the assay more adaptable for industry use. Previous work for our laboratory has identified oviduct-specific glycans that mediate sperm binding. Glycan coated beads in which sperm binding could be measured using computer automated sperm analyses (CASA) may be the first step in making zona binding a more user-friendly assay. Alternatively, it may be easier to load sperm into a chamber at one end and allow them to swim to the opposite end and observe binding to a glycan coated matrix (similar to a microscope slide used for CASA). This slide could potentially be loaded into a
CASA after a fixed time and used to count the number of sperm bound to the surface of interest. It could possibly replace the need for beads.

One of the limitations of the oviduct binding assay was the anecdotal observation that sperm from samples with poor fertility bound in high numbers to aggregates, probably due to the increased number of non-viable sperm. As a result, it was quite easy to identify sperm of poor fertility but perhaps more difficult to accurately rank those of acceptable fertility when using this assay alone. It may be possible to challenge sperm by increasing the distance which sperm must travel before binding to oviduct aggregates or a similar matrix. I have developed a prototype (Ovi-Zone) in which sperm would have to travel a greater distance before binding to oviduct aggregates and or zona (see Appendix C). Adding a distance component to this assay without significantly increasing incubation time may help to more accurately rank boars of moderate or high fertility.

Ideally, a single slide that contained multiple chambers for simultaneous testing of samples would help to mimic sperm competition parameters that we used in some of these assays. We may be able to combine motility, acrosome integrity, live-dead staining and glycan binding into one commercial slide compatible for use with CASA and the computer program template that I would like to develop (Appendix C.). Sperm could potentially be loaded into a chamber for CASA that measured all of the sperm parameters included in our assay that successfully explained fertility. Each chamber would be compartmentalized into small but continuous sections in which sperm are exposed to propidium iodide, FITC-PNA and then a fluorescinated glycan-coated matrix (see Appendix). The CASA is already designed to contain filter sets that detect all of these stains and can also count the number of sperm within a given area. Such a slide would be of great potential value for estimating fertility of animals and men.
Regarding TRPP2 research, there are many avenues that I encourage the pursuit of given the potential implications of this protein on sperm functions. The role(s) that TRPP2 plays in sperm may be direct or also indirect due to the many proteins that TRPP2 interacts with. First, it would beneficial to know if TRPP2 is localized to the plasma membrane directly over the acrosome. Although qualitatively it appeared that TRPP2 expression was attenuated in capacitated sperm, the expression was not completely lost. As a follow up experiment, I would be very interested to know if TRPP2 is necessary for the acrosome reaction, because of its potential role in calcium regulation. My overall hypothesis is that TRPP2 is important for multiple sperm functions that are critical for fertilization. Some of those functions we have begun to establish, such as a change in motility and regulation of intracellular calcium during capacitation. However, because TRPP2 has been identified in the acrosomal complex of other organisms, I find it likely that TRPP2 is also localized to mammalian sperm plasma membrane directly over the acrosome and may be playing an important role during the acrosome reaction that is independent of previously described functions.

The most interesting work that I would like to follow up on with TRPP2 is the different locations that the protein is expressed on mammalian sperm and more importantly, how (if) protein interactions change dependent upon localization. Although the TRPP2/TRPP1 complex is well described as a functional ion channel, TRPP2 is also known to form heteromultimers with other proteins. If TRPP2 does indeed interact with proteins independent of TRPP1, one might expect that the function of TRPP2 would be altered. Additionally, an intracellular interaction of TRPP2 with one protein has been suggested to serve as a voltage gate for TRPP2 activation. Because sperm usually hyperpolarize during capacitation and TRPP2 seems important for capacitation-like changes, the activation of TRPP2 may be dependent upon a shift in resting
potential of sperm. If any of these hypotheses prove true, TRPP2 could be a novel protein that is activated temporally during transit within the female reproductive tract and the functions of TRPP2 may be dependent upon more than one protein. Such a finding would be unique if TRPP2 was shown to regulate sperm motility, capacitation and the acrosome reaction. Furthermore, TRPP2 could be activated at different locations on sperm simultaneously, but be carrying out very different functions. However, the most obvious experiment to perform would be the production of TRPP2 conditional knockout mice (because the KO is embryonic lethal) to determine if fertility is reduced.

An additional experiment that I think is important would be to determine if TRPP2-inhibited sperm experience a rise in intracellular calcium when shear stress is applied. The justification for doing such an experiment is because the mechanism(s) of sperm release from the oviduct are still not known. TRPP2 localized to the cilia of renal epithelial cells responds to shear stress by regulating calcium. Others have demonstrated that only non-capacitated sperm bind to oviduct epithelial cells and that release of sperm from the oviduct coincides with capacitation and a rise in intracellular calcium. Oviductal fluid may very well play a role in sperm release by placing shear stress on the sperm flagellum and activating TRPP2.

Lastly, others in our laboratory have recently identified PKDREJ as a testis-specific lectin on boar sperm that may bind oviductal glycans to aid in the formation of an oviduct reservoir. PKDREJ is related to TRPP1 and also sea urchin REJ3 (suREJ3). TRPP2 and suREJ3 associate in the plasma membrane directly over the acrosome. It is possible that TRPP2 dimerizes with PKDREJ and that this relationship may be important for sperm binding to the oviduct and the acrosome reaction.
APPENDIX A

BOAR SPERM TRAITS EVALUATED BY MOTILITY CATEGORY

Fig A.1. Sperm were thawed at 50°C for 20 sec and samples were categorized as good (> 40%), moderate (26-39%) or poor (16-25%) motility. Acrosome - compromised sperm were identified at 0, 30 and 60 min post-thaw using FITC- PNA. Differences indicated are between motility categorizations at respective time points. n= 16 different boars.

\[\text{a,b,c values with different superscripts between motility categories are different (P } \leq 0.05).\]
Fig A.2. Sperm viability was measured at 0, 30 and 60 min after thawing samples at 50°C for 20 sec. Samples were categorized as good (> 40%), moderate (26-39%) or poor (16-25%) motility. Those sperm that stained positive with PI were considered dead. Differences indicated are between motility categorizations at respective time points. n= 16 different boars.

\[\text{a,b,c}\] values with different superscripts between motility categories are different. \(P \leq 0.05\).
Table A.1. In vitro fertilization with frozen-thawed boar sperm

Fertilization of oocytes after insemination with frozen-thawed boar sperm from 16 individual ejaculates categorized by post-thaw motility. Data was calculated using all oocytes exposed to sperm. Values are expressed as LSM ± SEM. n = ~ 500 oocytes

<table>
<thead>
<tr>
<th>Motility</th>
<th>No. of Replicates</th>
<th>% Fertilized Oocytes</th>
<th>% Non-Fertilized Oocytes</th>
<th>% Monospermic (2PN) Fertilization</th>
<th>% Polyspermic (PPN) Fertilization</th>
</tr>
</thead>
<tbody>
<tr>
<td>Poor</td>
<td>14</td>
<td>46.8 ± 8.4&lt;sup&gt;b&lt;/sup&gt;</td>
<td>53.1 ± 8.4&lt;sup&gt;b&lt;/sup&gt;</td>
<td>28.5 ± 5.6</td>
<td>18.3 ± 5.1&lt;sup&gt;a,b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Moderate</td>
<td>16</td>
<td>73.0 ± 3.9&lt;sup&gt;a&lt;/sup&gt;</td>
<td>27.0 ± 3.9&lt;sup&gt;a&lt;/sup&gt;</td>
<td>40.3 ± 4.2</td>
<td>33.0 ± 3.4&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Good</td>
<td>14</td>
<td>56.9 ± 6.9&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>43.0 ± 6.9&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>41.5 ± 5.0</td>
<td>15.3 ± 4.5&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a,b,c</sup> values with different superscripts are different (P ≤ 0.05). Motility categorization: Poor: (16-25%), Moderate (26-39%) and (Good > 40%).

2PN = 2 pronuclei; PPN = polypronuclear
Table A.2. Embryo development with frozen-thawed sperm

Embryo cleavage and blastocyst development following insemination with frozen-thawed boar sperm from 16 individual males categorized by post-thaw motility. Embryo development was calculated using the total number of oocytes exposed to sperm. Values are expressed as LSM ± SEM.

<table>
<thead>
<tr>
<th>Motility</th>
<th>n</th>
<th>% Cleaved</th>
<th>% Blastocyst</th>
<th>n</th>
<th>Average cell no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Poor</td>
<td>504</td>
<td>44.6 ± 3.1(^b)</td>
<td>13.7 ± 2.5(^b)</td>
<td>62</td>
<td>51.2 ± 3.2</td>
</tr>
<tr>
<td>Moderate</td>
<td>578</td>
<td>65.0 ± 2.8(^a)</td>
<td>20.4 ± 2.4(^a)</td>
<td>114</td>
<td>50.8 ± 2.2</td>
</tr>
<tr>
<td>Good</td>
<td>504</td>
<td>51.6 ± 3.7(^b)</td>
<td>13.2 ± 2.5(^b)</td>
<td>63</td>
<td>55.7 ± 3.5</td>
</tr>
</tbody>
</table>

\(^{a,b,c}\) values with different superscripts are different (P ≤ 0.05). Motility categorization expressed as percent total motility: Poor (16-25%), Moderate (26-39%) and Good (> 40%).
Table A.3. Embryo development following insemination with frozen-thawed boar sperm.

<table>
<thead>
<tr>
<th>Motility</th>
<th>2 cell</th>
<th>≥ 4 cell</th>
<th>Blastocyst</th>
</tr>
</thead>
<tbody>
<tr>
<td>Good</td>
<td>22.6 ± 2.2&lt;sup&gt;b,c&lt;/sup&gt;</td>
<td>7.1 ± 3.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>13.2 ± 2.5&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Moderate</td>
<td>16.5 ± 1.8&lt;sup&gt;a&lt;/sup&gt;</td>
<td>10.6 ± 3.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>20.4 ± 2.4&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Poor</td>
<td>18.3 ± 2.0&lt;sup&gt;a,c&lt;/sup&gt;</td>
<td>7.2 ± 3.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>13.7 ± 2.5&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Total (n)</td>
<td>278</td>
<td>569</td>
<td>178</td>
</tr>
</tbody>
</table>

<sup>a,b,c</sup> values with different superscripts are different (P ≤ 0.05). Motility categorization expressed as percent total motility: Poor (16-25%), Moderate (26-39%) and Good (> 40%).
Figure A.3. Variability of total (A) and monospermic (B) fertilization outcomes from frozen boar sperm subjected to IVF. Results are expressed as the LSM ± SEM of oocytes fertilized by one or more sperm with a minimum of two to three replicates per boar. n = ~ 500 oocytes, 16 different boars.
Figure A.4. Variability of polyspermic fertilization outcomes from frozen boar sperm subjected to IVF. Data are expressed as the LSM ± SEM of oocytes fertilized by one or more sperm with a minimum of two to three replicates per boar. \( n = \sim 500 \) oocytes, 16 different boars.

Figure A.5. Average polyspermic fertilization by post-thaw motility category of sperm. The LSM ± SEM from oocytes inseminated with sperm from 16 different boars. A total of \( \sim 500 \) oocytes were evaluated following fertilization. Motility categories with different superscripts are different (\( P < 0.05 \)) among treatments. Motility categorization expressed as percent total motility: Poor (16-25%), Moderate (26-39%) and Good (> 40%).
Figure A.6. Variability of cleavage (A) and blastocyst (B) outcomes from frozen boar sperm subjected to IVF. Data are expressed as the LSM ± SEM of oocytes fertilized by one or more sperm with a minimum of two to three replicates per boar. *n* = 1586 embryos, 16 different boars.
APPENDIX B
PROTOCOLS

Oviduct Binding Protocol

1. Collect Epithelial Cells
   1. Separate isthmus from ampulla with a sharp pair of scissors (isthmus is thicker, proximal to the uterine horn and narrows proximal to the ampulla); 20 isthmus yields approximately 120 aggregates.
   2. Make 0.5L of PBS (450ml H20 and 50ml 10x PBS). Rinse isthmus’ in 50 ml of PBS 3 times in a 50ml conical tube. Place conical tube with PBS into small Styrofoam box on ice.
   3. Add ~ 10ml of PBS at into a large petri dish (100 mm).
   4. With forceps, grab 1 end of the isthmus, submerge the whole isthmus in the petri dish with PBS and remove epithelial cells by pressing down on one end of the isthmus with a glass slide held at a 45° angle and extracting cells out the other end. Repeat process until all cells are extracted. Collect cells from two isthmuses each time.
   5. Gently aspirate clumps of extracted epithelial cells from the petri dish using a 200µl pipette.
   6. Place aspirated cells in a 15ml centrifuge tube on ice by slowly dispensing cells as close as possible to the bottom of the tube at a 45° angle.
   7. Dump the contents of the petri dish into a waste container and add 10ml of PBS and repeat process for all isthmus.
   8. Allow the pellet of epithelial cells to settle for 5 min or centrifuge at speed 1 for 30 sec using a table top centrifuge.
   9. Aspirate the supernatant and resuspend pellet in a total volume of 1 ml dmTALP capacitating medium.
  10. Gently pipette pellet up and down ten times within the tube using a 1000µl pipette to disaggregate cells.
  11. Resuspend further in dmTALP capacitating medium for a total volume of 5mL.
  12. Centrifuge for 1 min at speed 1 or allow pellet to settle. Aspirate supernatant and resuspend in 1 ml of dmTALP capacitating medium.
  13. Disaggregate cells very slowly using a 22 gauge needle and 1ml serological syringe 10 times.
  14. Resuspend disaggregated cells in 9mL of dmTALP C in the same tube.
  15. Transfer 3mL of cells and TALP mixture to 100mL petri dishes for a total of 3 dishes. Add 2 mL of TALP to each dish for a total of 5ml per dish. (Transfer equal-amounts of cells to 3 dishes for a total of 5 mL after adding TALP)
16. Incubate dishes containing cells at 39°C for 1.5-2hr at ~99% humidity for cells to reaggregate and form spherical, three dimensional structures. Observe the status of reaggregation at 1 hr. to determine if sufficient aggregates exist for the experimental design.

2. Droplet Co-Incubation Preparation
Before culture time is finished, begin preparing droplets in petri dishes for aggregate co-incubation with sperm

1. In a 100ml petri dish, prepare a 3x3 droplet design per treatment
   a. Prepare 2 rows and 3 columns of 100µl droplets of dmTALP C for selecting and washing aggregates
   b. Prepare the 3rd row with 3, 40µl droplets. Each droplet will ultimately contain 10 aggregates used for binding sperm. The final volume of the incubation droplet will be 50 µL, including semen.
   c. Place a small amount of mineral oil around the droplets to maintain the footprint but not to submerge the droplets

![Diagram of droplet co-incubation process]

3. Remove cells from incubator and select spherical aggregates approximately ~150-200µm in size using a 3µl Drummond with a microcapillary pipette with ~275µm of internal diameter so as not to dissipate spheres when selecting. Choose approximately 15 aggregates to add to the first wash droplet. Select 10 aggregates for a final wash placing them in the second droplet. Remove the 10 aggregates from the second droplet and place them into the third droplet to await co-incubation with sperm. You may randomize aggregate selection by selecting aggregates for the first column of every treatment from Petri Dish 1, the second column from Petri Dish 2 and the third from Petri Dish 3.
• Prepare Sperm

(Sperm preparation may be accomplished before or after aggregate selection depending upon design)

1. Prepare Percoll cushion in a 15ml conical tube for each sperm treatment
   Percoll 5.4ml
dmTALP N/C 4.0ml (this does not contain NaHCO3 or BSA)
10X HBS 0.6ml
2. Invert tube 4 times and place 1-3ml of sperm at 45° angle on top of Percoll
3. Centrifuge 10 min at 500xG (setting 5 on tabletop centrifuge)
4. Aspirate supernatant and resuspend pellet in 5ml of dmTALP NC solution
   a. add fluorophore here and incubate if necessary
5. Centrifuge 5 min at 400 x G (setting 3)
6. Aspirate supernatant and resuspend in 100µl of dmTALP NC (the vol. can be adjusted depending on pellet size)
7. Determine concentration of sperm using hemocytometer

-Hemocytometer Counting Instructions

  - Make a 1:100 dilution of sperm and water
  - Count 5 center squares in a diagonal on both sides of the hemocytometer and calculate the average from both sides (i.e. (45 sperm + 52sperm)/2 = 48.5
  - Calculate # sperm/mL:  average no. sperm x DF x 0.05 = [sperm/mL x 10^6]
    Ex: 48.5 x 100 x 0.05 x 10^6 = 242.5x10^6 total sperm/mL

8. For fresh sperm, add 500,000 to 1 x 10^6 sperm/mL to each droplet.
   Ex: (concentration) (volume) = (concentration) (volume)
   (242.5 x 10^6 sp/mL) (x) = (1 x 10^6) (0.05mL) - i.e. we want 1x10^6 sp/mL in 50µl
   (50,000)/(242.5 x 10^6) = 0.00021mL of sperm or .21µl

   This volume of sperm is too small to measure accurately, so you need to dilute it.

   Ex: You need 0.21 µl of sperm:
   Make a 10X dilution: Add 5µl of sperm to 45µl of TALP NC for a 1:10 dilution

9. Add sperm to the droplet containing aggregates and bring the final volume of the droplet to 50µl using dmTALP C.  (i.e. 2.1µl sperm and 7.9µl of dmTALP C to the original 40ul droplet = 50µl final vol.)

10. Incubate sperm and aggregates at 39°C for 15 min.

11. During aggregation time, prepare glass slides to mount 30 aggregates per slide (3 3µL-droplets containing 10 aggregates each per slide).
- Clean slides with Sparkle and place a rectangular shape of petroleum jelly in preparation for a
  Draw three circles of a hydrophobic marker (PAP Pen). Remove aggregates from the incubator and place 10 aggregates in each circle for a total of 30 per slide. Place coverslip on top of petroleum jelly to create a seal.
  Document binding with Axioskop software or place in 5°C overnight if necessary.

12. Use an upright scope to capture images of aggregates with bound sperm using a bright field under 200 x magnifications. More than one picture many be necessary to acquire all sperm. Interchange with a fluorescent field when necessary.

Alternatively: count all bound sperm and record immediately. Depending on experimental design, you may find it easier to count and record, or take pictures and count at a later time as described above.
Zona Collection and Fixation Protocol

1. Aspirate 20 gilt ovaries using 12ml plastic syringe and 18 gauge needle. Yields ~ 400 oocytes.

2. Select oocytes in TALPNC and place in 200 µl holding drop.

3. Vortex at speed 7 using benchtop centrifuge for 8 min in ~ 500 µl TALPC to remove cumulus (tape to vortexer). May need to add hyaluronidase (100 µg/mL, 80-160 U/mL) if you cannot remove cumulus but decrease exposure time to ~ 90-120s.

4. Add 500 µl of TALPNC to centrifuge tube

5. Centrifuge at 10,000 x G for 15 sec, aspirate supernatant and using 100 µl tip, place oocytes in 100 µl holding drop of TALPNC.

6. Wash ~ 100 oocytes through three 100µl droplets of TALPCNC consecutively.

7. Fix oocytes in 100 µl of 0.5% Formaldehyde in Medium B for ~ 5 min

8. Remove oocytes and wash consecutively through five, 100 µl drops of TALPNC.

9. Place in 200 µl of TALPNC and cover in mineral oil. Store at 5°C for ~ 2 months
Zona Binding Protocol for Frozen Boar Sperm

1. Thaw sperm for 20 sec in 50°C water bath; hold on dry heat at 37°C for 7 min
2. Place ~0.8 ml of sperm on Sperm Prep using TALPC; use protocol in lab manual
3. Add ~ 1 ml TALPC to pellet and make 1:00 dilution in water
4. Extend $20 \times 10^6$ sperm/ml in 297 ul of TALPC and place in 1.5ml Eppendorf tube at 39°C for 1.5hr
5. Thaw fluorescent probes and make appropriate dilutions:
   a. Cell Trace Oregon Green, Invitrogen Cat No. C34555
      1) Prepare 2 mM working solution using DMSO and freeze
      2) Thaw working solution and dilute to 1 mM using TALPC
   b. Cell Tracker Orange CMTMR, Invitrogen Cat No. C2927
      1) Prepare 10 mM working solution using DMSO and freeze
      2) Thaw working solution and dilute to 50 µM using TALPC
6. Add 3 µl of probes to 297 µl of sperm and return to incubation for 30 min
   a. Oregon Green final concentration: 10 µM
   b. Cell Tracker Orange final concentration: 500 nM
7. In 1007 petri dish prepare three 100 µl wash drops and three 50 µl droplets for gamete co-incubation
   a. Make three 50µl drops and three 25µl drops using TALPC
   b. Cover with mineral oil
   c. Add 50µl to wash droplets and 25µl to gamete droplets
d. Remove fixed zona from refrigeration and wash consecutively through 3 wash 
droplets and place 10 oocytes into each of the three final droplets containing 50ul and 
place on heating stage at 37°C

8. Remove sperm from incubation and centrifuge at 10,000 x G for 30 sec, remove supernatant 
and add 298 µl TALPC; resuspend sperm by flicking tube or vortexing for ~ 0.5 seconds 
(repeat if necessary)

9. Verify recovery rate: add 50ul sperm to 150ul of water; this yields $5 \times 10^6$ sperm/ml for 
counting and is a 4x dilution

10. Prepare a $6 \times 10^6$ sperm/ml dilution in 500ul:
    a. $(20 \times 10^6)(x) = (6 \times 10^6)(0.5\text{ml})$
    b. Add 75ul of sperm from Boar A and 75µl of sperm from Boar B to 350 ul of TALPC

11. Add 50ul of sperm for insemination to 50 µl droplets containing oocytes for a final 
    concentration of $3 \times 10^6$ sperm/ml

12. Return to incubation at 39°C for 15 min for gamete binding

13. Remove oocytes from droplets and place in previously used wash droplets

14. Rinse oocytes with 35µl of TALPC by dispensing short bursts of TALPC

15. Fix oocytes in 0.5% Formaldehyde for 2-5 min

16. Mount 10 oocytes onto glass slides
    a. Prepare slides by cleaning with Sparkle
    b. Draw three small circles on slide using a hydrophobic pen
    c. Dispense Vaseline in a rectangle around circles, place ~ 2 µl total of oocytes and 
       TALPC in respective circles and mount with coverslip

17. Count number of sperm bound to perimeter of oocytes using 20 x magnification and 
    appropriate filters for fluorescent dyes
APPENDIX C

PROTOTYPES AND IMAGES

Fig C.1. Ovi-Zon Fertility Assay Prototype
Fig C.2. Ovi-Zon Fertility assay prototype. Three wells of ~ 100 µl in volume were constructed using polydimethysiloxane (PDMS, Sylgard 185, Dow Corning Corp., Midland, MI). The wells are connected by a continuous channel.
Fig C.3. Fertility assay prototype (A). For estimating the fertility of frozen-thawed boar sperm by measuring motility, viability, acrosome integrity and sperm binding to a glycan matrix in a single unit (slide).
Fig C.4. Fertility assay prototype (B) with multiple chambers for simultaneous sampling. For estimating the fertility of frozen-thawed boar sperm by measuring motility, viability, acrosome integrity and sperm binding to a glycan matrix in a single unit (slide).
Fig C.5. PI + PNA kit to be used in conjunction with fertility assay prototype A or B.
Fig C.6 Pseudo-hermaphrodite (Pig)
Fig C.7. Candidate oviduct glycan receptors in stallion sperm.

Semen was collected from 1 stallion and cooled for 48 hr followed by protein extraction for glycan blotting. Protein (20 µg) were separated on a gel and probed for oviduct glycans (Bi-LN, sLe^x, LacNac and SiLN) known to bind porcine sperm.

* Possible receptor candidates
Fig C.8. Boar sperm were stained with a series of Syto dyes (16 - 64) to determine the compatibility of each dye with sperm competition experiments. Cooled, pooled semen from 4 boars was extended to a concentration of $35 \times 10^6$ total sperm/mL in a total volume of 2.5mL and incubated with fluorophores for 20 min at 37°C in Medium B. Final concentration of dyes tested was 200 nM in DMSO, (represented below) although some different concentrations were tested empirically in further experiments. The percent of the sperm population stained and relative intensity (subjective analysis) is provided for each dye. Dye color is indicated as red or green.

- Effect of dyes on sperm motility.

All stains appear to be toxic to sperm because motility was greatly reduced when measured by CASA. Syto64 bleaches quickly making it difficult to take photos. Tried Antiphade with glycerol but observed some cell shrinkage. For motility experiments, semen was centrifuged through a Percoll gradient containing Medium B and then resuspended in TALP and Syto64 was added at varying concentrations. Samples were centrifuged twice in TALP at speed 4 for 5 min prior to motility analyses. Serial dilutions of Syto64 from 1mM (1/2, 1/4, 1/8 and 1/16) concluded that ¼ or ~ 250 nM, was the ideal concentration for producing a signal. However, motility was still reduced compared to the control sample. TALP did not change dye intensity.

See pages 7 – 23 of Lab Notebook 2 if you don’t want to waste your time validating dyes for competitive assays. There are a number of experiments testing the compatibility of dyes with each other (including Mito Tracker) at various concentrations.
Fig C.9. Variability in embryo morphology from oocytes seminated with frozen boar sperm.
Fig C.10. Day 7 hatching blastocysts produced using frozen boar sperm.
Fig C.11. Variability in morphology and cell number of day 7 blastocysts generated using frozen-thawed boar sperm.

Fig C.12. Expanded, hatched blastocyst derived from frozen-thawed boar sperm.