NEGATIVE BIOMARKERS OF SPERM QUALITY AND MALE FERTILITY

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The undersigned, appointed by the dean of the Graduate School, have examined the thesis entitled

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and hereby certify that, in their opinion, it is worthy of acceptance.

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CHAPTER 1

INTRODUCTION

Semen evaluation via traditional light microscopy provides useful information about sperm motility and basic morphology. However, due to its subjective nature and inconsistency among technicians and laboratories, semen evaluation done in this manner has utility in predicting reproductive performance or fertilization ability in an assisted reproduction setting (Christensen et al., 2005). Furthermore, not all spermatozoa abnormalities are detectable with standard light microscopy. Semen samples with cryptic sperm defects and reduced fertilization ability may appear normal by conventional standards and be deemed suitable for insemination. In-depth analysis carried out quickly and with repeatable precision on a large number of sperm cells is of paramount importance to human assisted reproductive therapies (ART) and farm animal biotechnology. As a result, flow cytometric evaluation using biomarkers to detect specific spermatozoon characteristics is growing in popularity in both andrology laboratories and agricultural studs.

Fluorescently labeled biomarkers can be used to assess a variety of structural and functional properties, including sperm chromatin integrity, acrosomal status, mitochondrial membrane potential and cell viability. Antibodies are used to detect and quantify proteins that are up- or down-regulated in defective spermatozoa (Sutovsky and Lovercamp, 2010). The aim of my research is to validate several candidate biomarker

proteins or ligands associated with defective spermatozoa, even if defects are subtle or unnoticed during evaluation using standard light microscopy. The central hypothesis of this research is that these biomarkers, uniquely found in defective spermatozoa, are indicative of poor semen quality and decreased fertility. Thus such biomarkers are described as "negative" biomarkers of fertility.

The use of negative biomarkers to detect infertility, anticipate negative outcomes when using assisted reproductive techniques, or predict fertility parameters in maturing sire prospects could potentially save time and money. One such negative biomarker, ubiquitin, has already been validated and shown to correlate with various sperm quality parameters; the amount of ubiquitinated sperm in a sample has been positively correlated with poor quality or infertility in humans, stallions, bulls, and boars (Purdy, 2008). Furthermore, increased levels of sperm ubiquitination were found in samples from men with idiopathic infertility, which is an important finding considering that male factors represent roughly 40% of all infertility cases worldwide, with additional male infertility cases potentially misdiagnosed as idiopathic infertility (Ozanon et al., 2005). The sperm chromatin structure assay (SCSA) is able to predict even small changes in bull fertility (Bochenek et al., 2001), and sperm DNA damage as detected by SCSA has been implicated in having a role in offspring mortality (Ruiz-Lopez et al., 2010). The validation of additional negative biomarkers for detection of sperm quality and their relationship to fertility parameters is a necessary component in the improvement of semen analysis.

CHAPTER II

LITERATURE REVIEW

Flow cytometry

Flow cytometry is a process in which fluorescently labeled cells (in this instance spermatozoa) travel individually at high speed (hundreds or thousands per second) through a flow cell, where they are illuminated by one or more lasers. This causes light scattering and fluorescence excitation of markers located on specific parts of the sperm, which is then picked up by photo-detectors and sent to a computer program. The computer program presents the information in the form of relative fluorescent intensity units, which are typically displayed as either scatter plots or histograms (Martinez-Pastor et al., 2010); (**Figure 2.1**). The scatter plots and histograms can be analyzed and various sperm populations can be separated to produce information regarding fluorescence intensity, percentage of sperm population with certain fluorescence characteristics within a total sample, median fluorescence intensity, etc. One of the main concerns with analyzing spermatozoa by flow cytometry is the presence of other material in the sample such as immature forms of spermatogenic cells, bacteria, blood cells, tissue, and in the case of frozen-thawed or fresh-extended semen, extender contaminants such as egg yolk particles. During the data analysis, these contaminants can be eliminated from the evaluation by gating of the scatter diagram/histogram.

Biomarkers Used For Bovine Semen Analysis

Sub-fertile bulls can cost producers a significant amount of money – they may contribute to delayed conception, prolonged calving season, reduced calf weaning weight, and increase the number of female culls. When used in a multiple-sire or low breeding pressure situation, a sub-fertile bull may be less evident, but in a single sire, high breeding pressure, or artificial insemination service, the fertility of the bull is of extreme importance (Kastelic and Thundathil, 2008). Breeding soundness evaluation of yearling bulls may give some indication of fertility, but conventional semen analysis is less than ideal –it is time consuming; only a few hundred spermatozoa per sample are analyzed, and the agreement between microscopic assessments of sperm motility for different lab technicians can be low. The lack of precision in conventional semen analysis, coupled with the subjective nature of such an assessment, implies that some acceptable semen may be erroneously rejected, and at the same time semen of unacceptable quality may be used for inseminations (Christensen et al., 2005). A quick, precise, and accurate method for semen evaluation has been a goal of the breeding industry, and flow cytometry using fluorescent biomarkers may be the answer. Flow cytometry is fast, accurate, highly repeatable, and can analyze significantly more sperm per sample (up to 10,000) than standard semen analysis (Christensen et al., 2004). In addition to the speed, repeatability, and accuracy, flow cytometry allows close examination of numerous sperm characteristics, including sperm viability/membrane integrity (Evenson et al., 1982; Garner and Johnson, 1995; Garner et al., 1994), mitochondrial function and membrane potential (Evenson et al., 1982; Garner et al., 1997; Graham et al., 1990) chromatin structure (Bochenek et al., 2001; Evenson et al.,

1980), and acrosomal status (Graham et al., 1990; Nagy et al., 2003; Thomas et al., 1997).

Vital Dye Based Sperm Flow Cytometry

When coupled with specific fluorochromes conjugated to biomarker or reporter molecules, flow cytometry can be used to analyze a variety of structural and functional characteristics of spermatozoa, including plasma membrane integrity, mitochondrial activity/mitochondrial membrane potential, acrosome integrity, chromatin structure, changes in the sperm surface induced by sperm capacitation, and certain forms of morphological abnormalities present in a sperm sample (Bochenek et al., 2001). Some of these biomarkers are still in their infancy but are showing great promise and are correlating well with standard fertility parameters. Numerous biomarkers are available or are in development for use in sperm evaluation with flow cytometry. Some, such as ubiquitin and PNA, are present only in poor quality sperm and are generally referred to as negative biomarkers. Others, such as those used for mitochondrial membrane potential (Evenson et al., 1982; Graham et al., 1990; Thomas et al., 1997), viability (Evenson et al., 1982; Garner et al., 1994; Thomas et al., 1997), and PAWP (Wu et al., 2007a), are present in varying amounts depending on the quality of the spermatozoa.

The fluorescent probes JC-1 and MitoTracker are used for mitochondrial membrane potential and can reveal damage to the mitochondria that may occur after ejaculation or as a result of cryopreservation. The MitoTracker probe is taken up only by active mitochondria, and the reduced intensity of labeling in spermatozoa may also be reflective of malformations of the sperm tail midpiece/mitochondrial sheath. The JC-1

probe is transported into the interior of functioning mitochondria and senses the potential of the inner mitochondrial membrane.JC-1 emits green fluorescence when it exists as a monomer, but when the concentration of JC-1 inside the active mitochondria increases, the stain forms aggregates which fluoresce orange (Graham, 2001). Therefore, the mitochondria with high membrane potential fluoresce orange and those with medium to low membrane potential fluoresce green (Garner et al., 1997). Depolarization of this membrane impairs the electron transport chain, the proton gradient and aerobic ATP production in the midpiece of the spermatozoa, thereby rendering its motility characteristics inadequate for fertilization (Nagy et al., 2003). A correlation between mitochondrial fluorescence intensity and sperm motility has been established (Garner et al., 1997).

Viability is another parameter that can be measured by flow cytometry. Sperm viability of both raw and frozen-thawed bull semen correlates well with non-return rates (Christensen et al., 2005). Currently, one of the most commonly used viability stain combinations is SYBR-14 and Propidium Iodide (PI), sold commercially as LIVE/DEAD® Sperm Viability Kit. Both SYBR-14 and PI dyes target the same cellular component, DNA, eliminating the ambiguity that can arise when different components are targeted. With these biomarkers, the nuclei of live spermatozoa display green fluorescence due to the integration of SYBR-14, and dead/dying cells with compromised membrane integrity stain orange because of passive PI-uptake through damaged plasma membrane. By combining the viability stains with other biomarkers additional sperm functions such as acrosomal integrity and mitochondrial function can be assessed (Gillan et al., 2005). To date, the SYBR-14/PI combination has been used to effectively identify

live and dead sperm populations in bulls, boars, rams, rabbits, mice, rats, and men, though it has also been used to stain spermatozoa of more exotic species such as tigers and chinchillas (Garner and Johnson, 1995).

Sperm capacitation includes a process of plasma membrane destabilization which may lead to physiological acrosome reaction upon sperm-egg binding, or to sperm cell death in absence of sperm-zona interaction, both of which are dependent on an influx of calcium ions in the sperm interior (Maxwell and Johnson, 1997). Therefore, the capacitation status of spermatozoa can be detected using the fluorescent antibiotic chlortetracycline (CTC), which traverses the sperm plasma membrane and enters intracellular compartments containing free calcium. Once inside, the CTC becomes negatively charged and binds to the calcium, increasing the CTC fluorescence (Maxwell and Johnson, 1997). This CTC-calcium complex proceeds to bind to hydrophobic regions of plasma/acrosomal membranes and produces distinct staining patterns based on capacitation status: capacitated (B-pattern), non-capacitated (F-pattern), and acrosome reacted (AR-pattern) (Saling and Storey, 1979). The CTC staining has been routinely used with fluorescence microscopy to visualize capacitation and AR in several species, including mouse (Saling and Storey, 1979), bull (Fraser et al., 1995), boar (Wang et al., 1995), stallion (Bergqvist et al., 2011), and men (Li et al., 2009) and has been adapted for use in flow cytometry by Maxwell and Johnson (Maxwell and Johnson, 1997).

The collection of semen in livestock species is a less-than-sterile procedure. Despite rigorous attempts at cleanliness, bacterial contamination from the penis and prepuce, collection equipment, and handlers is possible (Yaniz et al., 2010). Consequently, bacteria may compromise semen quality and contaminate the receiving

female's reproductive tract. Though more frequently studied in humans, a variety of bacteria have been identified via culture of semen samples, and certain bacteria have been shown to have detrimental effects on semen quality in several domestic species (Akhter et al., 2008; Althouse et al., 2000; Aurich and Spergser, 2007). Fluorescent markers such as SYBR-Green 1 have been adapted to identify bacteria in semen, and the use of flow cytometry for bacterial counts is becoming more routine (Tripp, 2008). This enables the producer to inspect bacterial counts in collected semen prior to cryopreservation and to use an extender containing antibiotics, if necessary (Yaniz et al., 2010).

The sperm chromatin structure assay (SCSA) detects abnormal chromatin structure consisting of sperm DNA fragmentation index (%DFI) and abnormal nuclear proteins (%HDS).The DNA fragmentation index is the proportion of sperm containing fragmented DNA and is calculated from a histogram obtained from the ratio between red and total (red+green) fluorescence. High DNA stainability (% HDS) is calculated based on the percentage of spermatozoa with high levels of green fluorescence, representing immature spermatozoa with incomplete chromatin decondensation (Giwercman et al., 2010a). In a study by Bochenek et al., the sperm chromatin structure assay was performed on mature bulls considered qualified for AI. The results showed that ejaculates positively evaluated at the AI stud via microscope contained as much as 23.8% chromatin-defective spermatozoa (Bochenek et al., 2001). Furthermore, SCSA values were shown to correlate with fertility parameters and the percentage of spermatozoa with chromatin defects varied over several weeks, suggesting that defective chromatin structure could be a variable trait that can be affected by disturbances in the spermatogenetic process or external factors such as semen extender, heat stress, or chemicals (Bochenek et al., 2001).

Lectins

The visualization of acrosomal status could be an important factor to consider when evaluating semen quality, as some instances of male infertility could be the result of a lack of sperm with functional acrosomes at the time of ejaculation (Odhiambo, 2011). Acrosomal integrity of spermatozoa can be measured using fluorescently labeled plant lectins, proteins that recognize and bind glucosidic residues in different parts of the acrosomal membrane. *Pisum sativum* agglutinin (PSA) derived from the pea plant and *Arachis hypogaea* agglutinin (PNA) derived from the peanut plant are the most commonly used because of their specificity (Graham, 2001). However, PSA has a tendency to bind to the egg yolk particles in the extenders and has slightly less specific binding, so in agreement with other scientists (Gillan et al., 2005; Graham, 2001), the author's preference is to use peanut agglutinin (PNA). PNA shows a high affinity and strong specificity for disaccharides with terminal galactose, especially the D Gal α (1,3) D GalNac disaccharide, and binds to the outer acrosomal membrane, which becomes exposed during the acrosome reaction. Spermatozoa with reacted, damaged, or abnormally formed acrosomes acquire green fluorescence after PNA labeling, while intact, normal acrosomes have no fluorescence (Nagy et al., 2003). We have found that PNA values correlate with the conventionally established parameters of sperm morphology and sperm concentration, as well as with several industry-provided indexes reflective of bulls' reproductive performance in AI services (unpublished data/in preparation). A study by Thomas et al (1997) compared acrosomal integrity as determined by PNA via flow cytometry to the standard microscopic morphology assessments and found the percentage of spermatozoa with normal acrosomes, relative to

the values obtained using fluorometric methods, appeared to be understated in bulls producing semen of low quality and overstated in bulls producing semen of high quality, further illustrating the subjective nature of microscopic semen analysis and the tendency to bias visual examinations (Thomas et al., 1997). A third lectin, LCA from the lentil plant (*Lens culinaris*) has also been used to evaluate bull sperm quality. LCA shows a strong specificity to D-glucose and D-mannose residues and binds to the entire surface of defective spermatozoa, but only to the acrosomal surface in normal spermatozoa. Consequently, distinct histograms of LCA-induced fluorescence are seen in normal vs. defective spermatozoa. In our unpublished data, we have found a positive correlation between LCA and ubiquitin staining and a negative correlation of LCA with % normal sperm morphology. Thus, it appears LCA can be useful in the detection of abnormal spermatozoa via flow cytometry (unpublished data).

Protein Biomarkers of Sperm Quality

Potential sperm quality/fertility biomarkers include proteins that are exclusively associated with certain types of sperm defects ("negative" fertility markers) and proteins more abundant in morphologically and functionally normal spermatozoa ("positive markers"). One of the protein biomarkers of bull sperm quality that has been studied in depth is ubiquitin. Abnormal spermatozoa are tagged by ubiquitination of the plasma membrane/sperm surface during epididymal passage (Baska et al., 2008). Though some of these ubiquitin tagged spermatozoa may disintegrate and be removed in the epididymis, many abnormal spermatozoa appear in the ejaculate, and their increased content is indicative of poor semen quality, or even infertility (Sutovsky et al., 2003).

Increased binding of fluorescently-labeled anti-ubiquitin antibodies to the sperm surface reflects the occurrence of sperm abnormalities, which is then detected by the flow cytometer as an increase in the relative fluorescence induced by the presence of ubiquitin on the sperm surface. Ubiquitin as a sperm biomarker has been assessed in numerous species including men (Sutovsky et al., 2001b), stallions (Sutovsky et al., 2003), bulls (Sutovsky et al., 2002), and boars (Kuster et al., 2004), and has been found to correlate with infertility and indications of poor sperm quality, including primary and total morphological defects (Purdy, 2008). Though not yet published, our data indicate ubiquitin may be correlated with non-return rate as well. In a human fertility trial, it was found that higher levels of ubiquitin in the sperm sample also correlated negatively with the percentage of cleaved embryos and the percentage of embryos with two pronuclei after infertility therapy by in vitro fertilization (IVF) or intracytoplasmic sperm injection (ICSI) (Ozanon et al., 2005). If the decreased embryo cleavage and pronuclei-formation rates also apply to cattle AI, it can be inferred that early embryo loss could account for an increased non-return rate.

Ubiquitin has also been used for validation of other candidate biomarkers of sperm quality, further illustrating the utility of multiple stains during flow cytometric analysis. A 2007 study by Sutovsky et al., examined the correlation between semen platelet activating factor-receptor (PAFr) and ubiquitin labeling, with interesting results (Sutovsky et al., 2007).

Platelet activating factor (PAF) is an important phospholipid mediator in reproduction and, its sperm plasma membrane receptor PAFr, has a positive association with sperm motility and high fertility history in boars (Roudebush and Diehl, 2001).

Sutovsky et al. examined the relationship between ubiquitin and PAFr via flow cytometry during breeding soundness evaluations in yearling bulls. Contrary to the association shown in swine, increased PAFr content was indicative of increased white blood cells (WBC) in bull semen samples, and PAFr-induced fluorescence correlated negatively with several BSE parameters (palpation, scrotal circumference, and satisfaction of evaluation). A positive correlation was found at the same time with semen ubiquitin content (Sutovsky et al., 2007). Without the additional parameter of ubiquitin expression, an increased PAFr expression could have resulted in an incorrect assumption of high semen quality.

One biomarker that correlates negatively with high levels of ubiquitin is PAWP, the post-acrosomal, ww-domain binding protein. PAWP is a novel protein found only in the post-acrosomal sheath (PAS) of the spermatid, within which it resides in the protective capsule enveloping the sperm nucleus, the perinuclear theca. PAWP first appears during spermatid elongation, coinciding with the time frame in which spermatids acquire their egg-activating ability (Wu et al., 2007a). Though the downstream elements of PAWP-dependent oocyte activation cascade are currently under investigation, PAWP triggers meiotic resumption and pronuclear development. A proper integration of PAWP in the sperm PAS is thought to be reflective of bulls' sperm quality/fertility. When flow cytometry is performed on anti-PAWP antibody labeled spermatozoa, a threshold of acceptable PAWP content can be established. Anything above or below the threshold amount would be considered abnormal. In normal spermatozoa, PAWP fluorescence formed a regular band around the proximal PAS. Defective spermatozoa displayed various anomalies of PAWP labeling including a jagged or abnormally wide band, an

irregular spot or completely absent labeling (Sutovsky and Oko, 2011). Depending on which population is analyzed, PAWP can have a positive or a negative correlation with secondary sperm morphology, conception rate, number of services, non-return rate and residual value reflective of bulls' performance in AI service, and has also been found to have a negative correlation with the ubiquitin biomarker (**Figure 2.2** and unpublished data/in preparation).

Heparin binding proteins (HBP) secreted by the seminal vesicles, prostate, and bulbourethral glands are present in the seminal fluid and bind to spermatozoa after ejaculation. The presence of a specific heparin binding protein, HBP-30, on the sperm plasma membrane has been correlated with increased fertility in bulls, inspiring a synonym the fertility-associated antigen (FAA) (Bellin et al., 1998). High semen content of FAA was predictive of high fertility in bulls of several breeds in trials grouping bulls based on the presence or absence of FAA (Bellin et al., 1994; Bellin et al., 1996; Bellin et al., 1998). Despite the fluctuation of absolute fertility values, groups of FAA-positive bulls were consistently more fertile (by 9 to 40 percentage points) than groups of FAA-negative bulls (Bellin et al., 1998). Furthermore, cows covered by FAA positive bulls were impregnated earlier in the breeding season, resulting in increased numbers of older and heavier calves at weaning (Bellin et al., 1998). Based on this information, an artificial insemination trial on beef cows inseminated using semen from FAA positive or FAA negative bulls was performed (Sprott et al., 2000). The results of this insemination trial support the usefulness of the fertility associated antigen as a biomarker of sperm quality: pregnancy rates in females inseminated with FAA-negative bull semen ranged from 2.8-69.6%, while females inseminated with FAA-positive bull semen had a

pregnancy rate of 22.1-91.3% (Sprott et al., 2000). The presence or absence of FAA can only be determined via biochemical analysis—it has no relation to breeding soundness or serving capacity (Sprott et al., 2000).

Clusterin, an acidic glycoprotein produced in the testis and epididymis, has been associated with sperm quality in several species, including ram (Tung and Fritz, 1985), rat (Sylvester et al., 1991; Sylvester et al., 1984), bull (Ibrahim et al., 1999), and men (O'Bryan et al., 1990). In human semen, one type of clusterin was detected only on abnormal spermatozoa, with another type only being detected on normal spermatozoa (O'Bryan et al., 1990). Bull and ram spermatozoa that exhibited morphological defects strongly reacted with anti-clusterin antibody (Ibrahim et al., 1999). Clusterin is associated with cell damage in several disease conditions (Ibrahim et al., 2000), but the reason for clusterin accumulation in abnormal spermatozoa is unknown. The result of a scrotal insulation trial in rams (Ibrahim et al., 1999) suggests that the accumulation of clusterin on abnormal spermatozoa may indicate unfavorable testicular conditions or individual germ cell aberrations (Ibrahim et al., 2000). The incidence of clusterin-positive bull spermatozoa, as determined via flow cytometry, negatively correlates with non-return rate and estimated relative conception rate (Ibrahim et al., 2000). In addition, an inverse relationship between clusterin-positive bull spermatozoa and pre/post-thaw motility has also been established, suggesting that clusterin is another potential biomarker of bull fertility.

Biomarkers for Human Semen Evaluation

When flow cytometric analysis is used on human spermatozoa, the focus is shifted from the identification of poor semen quality to the diagnosis of *why* the semen quality is poor in men, and what steps need to be taken in order for a successful pregnancy to occur. Fewer biomarkers are routinely used in human semen evaluation, but those in use focus on sperm chromatin (SCSA; aniline blue, chromomycin A3) and protein expression (SPTRX-3, clusterin, ubiquitin).

Sperm Chromatin Structure Evaluation

The sperm chromatin structure assay (SCSA) is used to detect spermatozoa with abnormal chromatin structure using the sperm DNA fragmentation index (%DFI) and high stainability due to abnormal nuclear proteins (%HDS). In this flow cytometric analysis, acridine orange stained spermatozoa fluoresce red or green, depending on their chromatin packaging. The ratio between red and total (red + green) fluorescence represents %DFI, and the percentage of spermatozoa with high levels of green fluorescence represents %HDS. SCSA parameters are more repeatable than conventional semen parameters such as sperm motility and can be indicative of subjects' exposure to industrial toxicants, smoking, or environmental pollutants (Virro et al., 2004). Numerous studies have evaluated the usefulness of SCSA in a human infertility clinic setting and have found that SCSA results can predict the degree of treatment success in assisted reproductive techniques such as IVF, IUI, and ICSI (Bungum et al., 2004). Furthermore, high levels of sperm DNA fragmentation (DFI $\geq$30%) may cause a significant decrease in blastocyst formation rates and an increase in spontaneous abortions (Virro et al., 2004).

Aniline blue (AB) staining is an indirect approach used to detect superfluous histones and thereby decreased amounts of protamines in the sperm nucleus (Hofmann and Hilscher, 1991). During spermiogenesis, protamines replace DNA histones to form a hypercondensed, tightly packed sperm chromatin structure. By detecting the presence of residual histones, the degree of histone-protamine replacement can be deduced. Normal spermatozoa have no staining or may appear grey, spermatozoa with a moderate amount of histones are stained light blue, and spermatozoa with increased amounts of histones are stained a bright blue color (Kazerooni et al., 2009). Though not as commonly used as SCSA, AB staining correlates with spontaneous recurrent abortion, progressive sperm motility, and abnormal morphology (Kazerooni et al., 2009).

Chromomycin A3 (CMA3) detects protamine deficiency in loosely packed chromatin by competing with protamines for binding to the minor groove of DNA (Kazerooni et al., 2009). Also correlated to DNA nicks, CMA3 staining brightness corresponds to the type of chromatin packaging: abnormally packaged chromatin causes the head of the spermatozoon to fluoresce bright green, while spermatozoa containing normally packaged chromatin faintly fluoresce dark green (Kazerooni et al., 2009). The number of brightly stained CMA3 labeled spermatozoa is increased in infertile patients. Patients with >30% CMA3 labeled spermatozoa and >10% DNA nicks had more than double the number of unfertilized oocytes containing spermatozoa that had remained condensed after ART (Sakkas et al., 1998). A study by Kazerooni *et al.* found an increased number of CMA3 and AB stained spermatozoa in men with recurrent spontaneous abortion, compared to fertile subjects (Kazerooni et al., 2009).

Biomarker Proteins

Spermatid-specific Thioredoxin-3 (SPTRX3 or TXNDC8) is exclusively expressed in the testis, localized to the Golgi apparatus in spermatocytes and spermatids (Jimenez et al., 2004). Thioredoxin systems exist in all organisms and perform a wide variety of functions, including DNA synthesis, regulation of apoptosis, antioxidant defense, and immune response. Abnormal expression of thioredoxin and thioredoxin reductase genes has been implicated in numerous diseases such as Alzheimer's and Parkinson's (Arner and Holmgren, 2000; Powis and Montfort, 2001). SPTRX3 is increased in abnormal spermatozoa and accumulates in superfluous cytoplasm, making it a good candidate for a marker of male infertility. Though few studies have been performed using SPTRX3 as a biomarker, preliminary results show elevated sperm SPTRX3 levels in male infertility patients and a strong positive correlation between SPTRX3 and a validated sperm biomarker, ubiquitin (Jimenez et al., 2004). In one study, men with >15% SPTRX-3 positive spermatozoa produced fewer pronuclear zygotes and had a significantly reduced chance of fathering children when using assisted reproductive techniques such as IVF or ICSI (Buckman et al., 2009).

Though not fully characterized in humans, clusterin has been associated with sperm quality in other species, as previously mentioned. In the human reproductive tract, clusterin is produced by the testis, epididymis, and seminal vesicles and has the ability to induce sperm aggregation (O'Bryan et al., 1990). Once thought to be a single protein, two biochemically distinct types of clusterin are found in human spermatozoa. Normal spermatozoa contain a form of clusterin immunoreactive with an anti-clusterin alpha-chain antibody, located within the acrosomal cap. Conversely, morphologically

abnormal spermatozoa have the conventional 80 kDa native clusterin on their entire surface (O'Bryan et al., 1990). However, this native form is undetectable on normal spermatozoa (O'Bryan et al., 1994). A pilot study with a small number of patients (n=25) undergoing IVF treatment for infertility revealed a significant relationship between seminal clusterin levels as determined by ELISA and fertilization rate; a larger study is necessary to confirm these findings (O'Bryan et al., 1990).

A proven biomarker for sperm quality in both humans and animals, ubiquitin is a major protein in human spermatozoa (Ozanon et al., 2005; Sutovsky et al., 2004). Sperm ubiquitin levels directly correspond to semen quality parameters, as well as may reveal male infertility in couples initially diagnosed with idiopathic infertility. In one study, semen from 43 individuals was evaluated and sperm ubiquitin median values were found to negatively correlate with multiple conventional semen analysis parameters, including morphology, motility, and sperm count (Sutovsky et al., 2004). In another study of 27 individuals with idiopathic infertility, increased sperm ubiquitin levels were found in approximately half of the men and could possibly offer an explanation for the difficulty of these couples with conceiving (Ozanon et al., 2005). Additional studies have shown that the percentage of ubiquitinated spermatozoa negatively correlated with the success of assisted reproductive techniques such as IVF and ICSI (Ozanon et al., 2005; Rawe et al., 2002; Sutovsky et al., 2001b). Furthermore, increased levels of sperm ubiquitination may be reflected by increased number of abnormal spermatozoa, smoking, exposure to solvents, decreased sperm count, and reduced motility, which may assist clinicians in evaluating potential causes of male infertility (Sutovsky et al., 2004).

Sperm Proteomics: Searching for New Biomarkers

In the United States, roughly 70% of dairy cows are bred via artificial insemination (Killian, 1999). Of that 70%, only about 50% of inseminations result in a full term pregnancy, in part as a result of a lack of thorough understanding of the molecular events and mechanisms that determine the fertilizing potential of a semen sample (Peddinti et al., 2008). In order to develop new biomarkers to assess the quality of a semen sample, we must first develop an understanding of these mechanisms through proteomics. Proteomics refers to the qualitative and quantitative comparisons of proteomes to identify cellular mechanisms which are involved in biological processes. However, proteomics is not only about the identification of such mechanisms; it also involves the study of protein structure, localization, post-translational modifications, protein-protein interactions, biological activities, and function (Strzezek et al., 2005). Sperm proteomics can be used to discover new biomarkers of fertility, such as those described above. The proteomics of seminal plasma and epididymal fluid could be arguably just as important as sperm proteomics because spermatozoa acquire numerous sperm surface proteins that convey fertilization potential in the epididymis (Sutovsky, 2009). Comparison of bull sperm proteomes between fertile and sub/infertile bulls (Peddinti et al., 2008) as well as proteomic characterization of bovine seminal plasma (Kelly et al., 2006; Moura et al., 2007) have given some insight into which proteins at what levels are indicative of fertility or infertility.

New Instrumentation

As new technology is developed, instrumentation is updated or created to make use of the new biomarker-based methodology. The development of biomarkers is no exception, and new state of the art flow cytometers, including dedicated sperm flow cytometers have been introduced recently. These include the EasyCyte Plus 'bench-top' micro-capillary flow cytometer (IMV Technologies,L'Aigle, France) and the ImageStream high speed quantitative imaging cytometer (Amnis Corp., Seattle, WA)..

In a study performed by Odhiambo et al, the EasyCyte Plus flow cytometer was used to validate a dual ubiquitin-PNA based bull sperm assay. The EasyCyte Plus proved easy to use and can be operated by anyone who has completed the IMV Technologies web-based training program, which takes less than four hours—no expertise is needed. Setup is fast and the instrument can be fully operational in less than 15 minutes (Odhiambo et al., 2011). In addition to the quick setup, EasyCyte Plus has an automated self-cleaning cycle without the large-volume fluidics found in conventional flow cytometers. When the results of the ubiquitin-PNA trial were compared between the EasyCyte Plus and the conventional flow cytometer (Becton Dickinson FACScan), both instruments produced robust evaluations of the semen samples (Odhiambo et al., 2011). The relative affordability, compact 'bench-top' design, dedicated sperm analysis software and reliable results make the EasyCyte Plus an attractive choice for andrologists.

The ImageStream high speed quantitative imaging cytometer was evaluated in another sperm biomarker trial by Buckman et al. (2009). The ImageStream combines the standard fluorescence intensity measurements with bright field and epifluorescence imaging, enabling it to directly correlate biomarker intensity with individual cell

morphology, something standard flow cytometers cannot accomplish. This instrument captures up to six different images (four channels of fluorescence, side scatter, and bright field) of each passing cell at approx. 100 cells/second with a resolution comparable to that of a 40x microscope-lens magnification. It is equipped with image analysis tools that can measure parameters related to fluorescence intensity, cell size, shape, texture, and localization (Buckman et al., 2009). In addition, the ImageStream results correlate well with conventional flow cytometry results. However, the cost of this instrument makes it more suitable for core facilities shared by basic researchers rather than for andrology laboratories.

Concluding Remarks

The advent of sperm quality biomarkers and the implementation of flow cytometry should benefit the cattle industry greatly. Being able to identify markers of good fertility as well as poor fertility in a semen sample in a fast and objective manner could reduce the need for multiple inseminations and prevent expenses covering offspring testing of sub-fertile bulls producing poor pregnancy rates in AI service. While the idea of using flow cytometry is new, and some of the biomarkers are still being developed, an increase in speed, accuracy, and precision in the assessment of fertility in bulls should be welcomed by the cattle industry. Even if flow cytometry were only used to determine sperm viability and concentration, it could potentially still lead to more straws of semen being produced, and better quality control overall, which leads to an increase in profits. Finding the sub-fertile bulls that "fly under the radar" could potentially save producers considerable amounts of money, especially if sub-fertility

could be determined early on before the bulls reach breeding age. Furthermore, flow cytometry could be used to aid in the development of new cryopreservation techniques, as some scientists are testing the effect of cryopreservation on various sperm characteristics, such as organelle function and viability (Thomas et al., 1998), acrosomal integrity and viability (Thomas et al., 1997), and mitochondrial function and viability (Thomas et al., 1997).

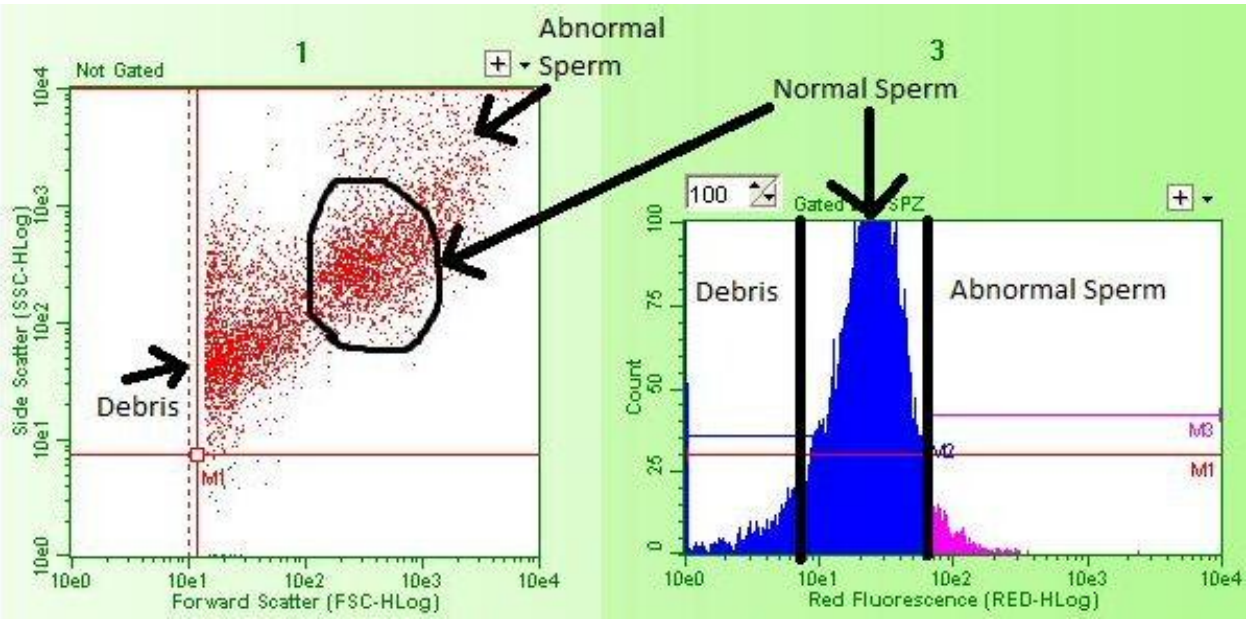

Figure 2.1: Typical scatter diagram of visible light (left) and a histogram of PAWP-induced fluorescence (right) yielded by flow cytometric measurement of a bull sperm sample. Normal spermatozoa are gated based on their size (forward and side scatter) in a scatter diagram, and based on their relative fluorescence in a histogram.

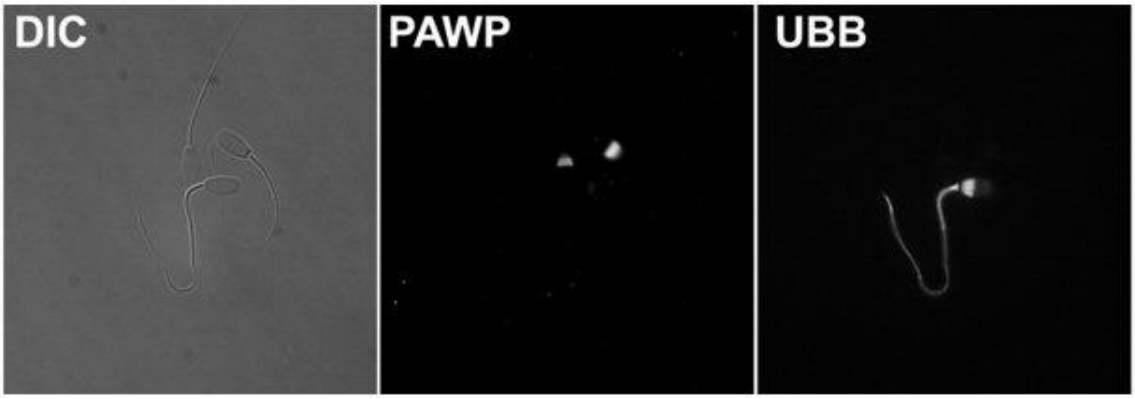

Figure 2.2: Dual immunofluorescence labeling of PAWP and ubiquitin (UBB) in bull spermatozoa. A spermatozoon displaying UBB labeling lacks PAWP on its postacrosomal sheath. Parfocal transmitted light image was acquired by using differential interference contrast (DIC) optics.

CHAPTER III

SPERM CHROMATIN STRUCTURE CORRELATES WITH SPONTANEOUS ABORTION RATES AND WITH THE INCIDENCE OF MULTIPLE PREGNANCIES IN ART COUPLES

ABSTRACT

The objective of this study was to determine if a relationship exists between sperm parameters measured by sperm chromatin structure assay (SCSA) and spontaneous abortion (SAB) and multiple births (MB) in couples treated by ART. Retrospective analysis of infertility treatment outcomes and occurrence of SAB & MB was conducted in 233 couples that underwent treatment by ICSI or IUI at the Sher Institute for Reproductive Medicine (SIRM), St, Louis, MO, between 2001 and 2004. Flow cytometric sperm chromatin structure assay (SCSA) analyses were performed for SIRM by a paid subcontractor. Sperm samples used for treatments were analyzed for **s**perm concentration, sperm motility and two different parameters of SCSA (%DFI/SCSI-1 & %HDS/SCSI-2). Pregnancy, SAB and multiple birth rates were recorded for all couples. A statistically significant correlation was observed between %DFI and SAB. However, the correlation between %HDS and SAB was not strong enough to be considered statistically significant. Significantly lower levels of %DFI were observed in men from couples having triplet pregnancies. It is concluded that the parameters of SCSA correlate

significantly with SAB and MB, and may provide guidance for clinical decision-making (number of embryos per transfer) and management of SAB-prone cases.

INTRODUCTION

Between 6.5 and 21% of pregnancies in the normal population end in spontaneous abortion (SAB), with a higher frequency of spontaneous abortions being observed in infertile couples (Hamamah et al., 1997). In addition, a higher prevalence of primary and secondary infertility has been seen among repeated spontaneous aborters. Reports show that despite a normal fertilization rate, a higher rate of early spontaneous abortions occurred in patients with <4% morphologically normal spermatozoa as assessed by Kruger strict criteria (Hamamah et al., 1997). This observation indicates that the main problem with morphologically abnormal spermatozoa was not an impaired fertilization, but rather that these spermatozoa may have resulted in a higher percentage of abnormal embryos which were aborted early in gestation.

The sperm chromatin structure assay (SCSA) detects spermatozoa with abnormal sperm chromatin structure contributed by abnormal nuclear proteins (%HDS) and sperm DNA fragmentation (%DFI). Both of these features are dependent on the highly precise interaction of acridine orange (AO) with histone /protamine complexed DNA, and with single and double stranded DNA. The DNA fragmentation index (%DFI) is the proportion of sperm containing fragmented DNA and is calculated from the DFI frequency histogram obtained from the ratio between the red and total (red + green) fluorescence intensity. High DNA stainability (% HDS or SCSI-2) is calculated based on

the percentage of spermatozoa with high levels of green fluorescence, which are thought to represent immature spermatozoa with incomplete chromatin condensation (Giwercman et al., 2010b; Giwercman et al., 2003). The %DFI and %HDS parameters are more repeatable than conventional, subjectively evaluated semen parameters such as sperm count, morphology, and motility (Evenson et al., 2002; Evenson and Wixon, 2006; Virro et al., 2004).

Relatively few papers examined the correlation between spontaneous abortions and sperm chromatin quality, mostly pointing to male factor being the single most common cause of infertility, with sperm defects responsible for between 30 and 50% of infertility cases (Boe-Hansen et al., 2006; Larson et al., 2000). A %DFI above 27%, 30%, and 40% was related to male sub- or infertility, respectively, and a %DFI level >27%, while compatible with ongoing pregnancy and delivery after IUI, IVF, or ICSI, has a lower probability of live birth (Boe-Hansen et al., 2006).

Studies show that neither %DFI nor %HDS correlate strongly with conventional semen parameters (Giwercman et al., 2003). However, these parameters may prove to be robust indicators of the potential for spontaneous abortions when attempting to conceive via ART (Larson-Cook et al., 2003). These studies are in disagreement regarding the %DFI threshold at which spontaneous abortions increase. In a recent study, couples where men had %HDS levels of >15% experienced significantly higher SAB rates; authors suggested that ICSI may be indicated for men with %HDS >15% (Lin et al., 2008; Ming-Huei Lin, 2008). While %DFI did not have a statistically significant trend toward increased abortion rate in the high %DFI group (>27%) in this particular study, a study by Virro et al.., reported that 146 couples failed to have sustained pregnancies post

ART when the %DFI was >27% in the raw ejaculate (Virro et al., 2004). A study by Evenson and Wixon (2006) indicated a trend towards increased spontaneous abortions when the %DFI was ≥30%; a ≥30% DFI score was associated with increased miscarriage rates and a higher rate of SAB at 12 weeks of gestation ($P<0.01$) in comparison to the <30% group (Evenson and Wixon, 2006). This same study suggested that 39% of miscarriages were related to a %DFI >30%, and a meta-analysis indicated that couples were 7.1x more likely to achieve a term pregnancy if the %DFI was <30% ($P=.0001$) (Evenson and Wixon, 2006).

Altogether, the above studies indicate a relationship between SCSA parameters and the incidence of SAB. However, none of the above studies examined the effect of sperm chromatin quality on the occurrence of multiple births after ART. The present study, for the first time, examines the possibility for a dual relationship between sperm chromatin structure and the incidence of SAB and MB in consenting ART couples.

MATERIALS & METHODS

Patients and Data Collection

Sperm samples and clinical data were provided by 233 consenting couples with varied etiologies of male/female infertility, with male partners aged 23 to 48 years at the time of the study. All samples were labeled with an identifier number and the treatment applied after which they were frozen by immediately submerging in liquid Nitrogen. All samples were subjected to DNA Fragmentation Assay for HDS and DFI via SCSA diagnostics as

described previously (Evenson and Jost, 2000). Couples in this study conceived predominantly via IVF/ICSI, with very few patients conceiving via IUI. All couples were treated with gonadotropins and closely monitored via ultrasound to maximize follicular response to treatment. In IUI patients, ultrasound was also used to optimize timing of the HCG trigger administration with subsequent IUI.

Statistical Analysis

Statistical analysis, supervised by a professional statistician, was performed using Microsoft Excel and SAS to test for statistical significance. In addition a t-test was performed to check for differences between groups. Means with at least one letter in common are not different ($p \leq .05$); (Table 3.1).

RESULTS

A retrospective analysis was performed on the data from 233 consenting ART couples (**TABLE 3.1**), and of all categories analyzed, the SAB group had the highest value for %DFI (24.80%±3.2), highest value for %HDS (8.50% ±0.015), and lowest sperm concentration (55.85 ±12.25). In comparison, sperm motility within the SAB group was moderately reduced at 67.86 ±10.58 % motile spermatozoa.

It is concluded that %DFI is the only parameter that showed statistical significance with regard to SAB; all others were determined to be equal. Judging from

the t-groupings, it can be inferred that the spontaneous abortion group differs from couples having twins, triplets, and no embryo transfer due to lack of suitable embryos.

A correlation analysis (**TABLE 3.2**) was performed on the groups deemed pregnant (chemical pregnancy, singleton, twins, triplets) and the groups deemed non-pregnant (no ET, spontaneous abortion, negative). Significant correlation was found between %DFI and %HDS for both the pregnant and non-pregnant groups. Concentration and motility were also correlated ($r=.41$; $p\leq.01$).

For analysis using SAS, treatments were grouped together in terms of spontaneous abortion vs. live births (singleton, twins, triplets). The correlation was significant at 95% probability for %DFI, but none of the other parameters showed significance (**TABLE 3.1**). In addition, pregnant vs. non-pregnant couples were compared, the pregnant group including singletons, twins, and triplets, while the non-pregnant group contained individuals from the chemical pregnancy, no ET, negative, and SAB group (**TABLE 3.1**).

DISCUSSION

According to our findings, % DFI was the best predictor of pregnancy outcome. However, when interpreting the above data, it is important to consider the potential impact of both %DFI and %HDS parameters on fertility. SCSI-1 (%DFI) is measured using the DNA fragmentation index; it is an expression of how many cells contain denatured DNA (Virro et al., 2004). The high degree of DNA fragmentation may not

necessarily affect fertilization rates, but the consequences of damaged paternal DNA could manifest themselves once the embryonic genome is activated, potentially triggering apoptosis and causing an abortion early after implantation. The %DFI value for SAB group seems to support this hypothesis, as it was significantly higher than the other groups (24.8% DFI compared to 18.8% DFI average for all pregnant couples, an increase of ~32%). The SCSI-2 (%HDS) index reflects the number of spermatozoa with abnormal chromatin structure, which has a negative effect on pregnancy rates, proposed to be due to intact DNA not being fully protaminated (85% for humans), or an altered ratio of protamine forms (Virro et al., 2004). Again, this hypothesis appears supported by our data. The %HDS value was highest again in the SAB group, 10.6% higher than the next highest value of 7.6% in the singleton group. Due to the impact that both %DFI and %HDS have on fertility and the viability of resulting embryos, it can be deduced that poor sperm quality, as indicated by SCSA, increases the risk of spontaneous abortions.

When the %DFI, %HDS, sperm concentration, and motility values are compared between the SAB group and triplets group, another observation can be made regarding the importance of sperm quality and pregnancy. In all parameters, the SAB and triplet groups were on opposite ends of the spectrum. Regarding %DFI and %HDS, the SAB group had the highest values for both (24.80% and 8.50%) while the triplet group displayed the lowest values (13.70% and 6.00%, respectively). The SAB group had the lowest sperm concentration at 55.85 million/ml while the triplet group had a sperm concentration nearly one and a half times higher, at 83.11 million/ml. The difference in motility, however, was not as obvious as the previous parameters. The triplet group featured the highest motility at 73.56%, but the SAB group's motility (67.86%) was only

moderately reduced, and even higher than both the singleton and twin groups. A few possible explanations for this observation exist. First and foremost, motility is a subjective measurement and can be affected by sample processing and intra/inter-laboratory differences in semen evaluation (Drobnis, 1992; Jorgensen et al., 1997). Motility begins to decline immediately after the sperm sample is harvested, and if any differences in handling occurred between the groups, the motility data could be affected. In addition, motility does not necessarily guarantee healthy spermatozoa, and decreased motility does not guarantee an infertile male (Drobnis, 1992).

The differences in sperm quality parameters between the SAB group and the triplet group have practical implications in an ART setting. By performing sperm chromatin structure assays in addition to the routine concentration and motility parameters, modifications to the ART cycle can be made to increase the likelihood of a successful pregnancy by selecting for additional oocytes to be fertilized. In some cases, ICSI may be preferentially used over standard IVF. With the introduction of higher resolution ICSI systems (IMSI as described by Bartoov et al.), this may be the optimal method of sperm selection over traditional IVF treatment (Bartoov et al., 2003). In the instance of low SCSI levels and high concentration and motility levels, fewer embryos could be transferred to decrease the likelihood of unwanted multiple births.

A relatively small number of papers have been published comparing the incidence of spontaneous abortions and sperm chromatin structure assays, with the majority of them being in agreement with our findings. Virro et al., state that the SCSA test is a valuable noninvasive assay with adequate sensitivity and repeatability to identify men at increased risk of poor blastocyst rates and negative pregnancy outcomes in a clinical setting (Virro

et al., 2004). Lin et al., showed a relationship between the %HDS (SCSI-2) and spontaneous abortion rate in IVF (Lin et al., 2008). In other papers, low sperm chromatin quality was one of the common etiologies of spontaneous recurrent abortion following IVF or ICSI (Hamamah et al., 1997; Kazerooni et al., 2009). To the best of our knowledge there are no publications to date examining the influence of SCSA parameters, sperm concentration or motility on the incidence of multiple births after ART.

CONCLUSION

From our findings, we can conclude that poor sperm quality as determined by the sperm chromatin structure assay indexes %DFI and %HDS coincides with an increased risk of spontaneous abortions in ART couples. Conversely, good sperm quality as determined by SCSA can be indicative of the potential for multiple births after ART. Decision making in ART could be adjusted based on SCSA and/or other objective sperm quality analysis decrease pregnancy loss and decrease unwanted multiple births.

TABLE 3.2: Statistically Significant Correlation Coefficients of Pregnant and Non-pregnant Couples for SCSI-1, SCSI-2, Sperm Concentration and Sperm Motility

	SCSI-1	SCSI-2	Concentration	Motility
SCSI-1	N/A	**.42****	**-.27***	**-.22***
SCSI-2	*.47***	N/A	**-.29***	**-.32****
Concentration	*-.15 NS*	*-.23***	N/A	**.41****
Motility	*-.32***	*-.33***	*.59***	N/A

* p value ≤.05
**p value ≤.01
NS = not statistically significant
Bold values = pregnant group
Italicized values = non-pregnant group

CHAPTER IV

PROTEIN EXPRESSION PATTERN OF PAWP REFLECTS BULL SPERM QUALITY AND FERTILITY IN ARTIFICIAL INSEMINATION SERVICE

ABSTRACT

The post-acrosomal WW-domain binding protein (PAWP) is a signaling protein located in the post-acrosomal sheath (PAS) of mammalian spermatozoa. We examined the hypothesis that a proper integration of PAWP in the sperm PAS is reflective of bulls' sperm quality and fertility. Two sets of semen samples from a total of 298 sires of acceptable but varied fertility in artificial insemination (AI) services were analyzed using immunofluorescence and flow cytometry after labeling with an anti-PAWP antibody. In normal spermatozoa, PAWP fluorescence formed a regular band around the proximal PAS. Anomalies of PAWP labeling included a jagged or abnormally wide PAS band, an irregular spot or completely absent labeling in defective spermatozoa. This variability was reflected by varied intensities of PAWP-induced fluorescence in flow cytometry. Based on morphometry and flow cytometry, total sperm sample population was divided into morphologically normal spermatozoa with median levels of PAWP, grossly defective spermatozoa (typically with sperm tail defects and/or crested heads) with low/no PAWP and defective spermatozoa (typically with macrocephalic, tapered or

pyriform heads) with extremely high PAWP. Flow cytometric measurements yielded statistically significant correlations between PAWP intensities and several semen quality/fertility parameters used by the AI industry, including secondary sperm morphology (SSP), conception rate (CR), number of services (NS), non-return rate (NR) and residual value (RV) reflecting sires' fertility in AI service. Depending on which subpopulation of spermatozoa was gated, both positive and negative correlations were found. Percentage of spermatozoa with normal PAWP-fluorescence levels correlated negatively with SSP (percentage of secondary sperm defects)(r=-0.448, p=0.05) and NR (r=-0.400, p=0.02), and positively with CR (r=0.399, p=0.007), NS (r=0.589, p=0.0001), and RV(r=0.445, p=0.0001). The percentage of sperm with abnormally high PAWP fluorescence correlated negatively with conception rates CR (r=-0.389; p=008). We conclude that PAWP correlates with semen/fertility parameters used in the cattle AI industry, making PAWP a potential candidate biomarker of bull fertility.

INTRODUCTION

The post-acrosomal WW-domain binding protein (PAWP) is an evolutionarily conserved protein, in mammals found exclusively in the perinuclear theca (PT) of sperm head post acrosomal sheath (PAS). PAWP first appears in the spermatid during elongation, coinciding with the time frame in which spermatids acquire their oocyte-activating ability(Oko and Sutovsky, 2009; Wu et al., 2007b). The PAWP protein is released from the PT into the ooplasm during fertilization and the porcine oocytes

microinjected with anti-PAWP antibodies or competitive inhibitory peptides derived from PAWP-signaling domain are unable to undergo oocyte activation, completion of the second meiotic division and pronuclei formation after in vitro fertilization (IVF) or intracytoplasmic sperm injection (ICSI)(Wu et al., 2007a). Under similar conditions, frog eggs also fail to undergo fertilization/oocyte activation-associated calcium release from their internal Ca^{2+} stores (Aarabi et al.). Upon sperm-oolemma fusion, the PAWP protein is released from the PAS and spreads across the ooplasm. Some PAWP molecules also enter the interior of the sperm nucleus and migrate towards its anterior pole during the initial swelling of the sperm head, marking the onset of sperm nucleus decondensation and male pronuclear development (Wu et al., 2007a). The downstream signaling pathway by which PAWP triggers meiotic resumption and pronuclear formation is under investigation, but several explanations are possible. Additionally, the PAWP-mediated signaling pathway could directly act upstream of phospholipase-induced calcium signaling during fertilization, or be relatively independent of it. Regardless of the mode of action, blocking PAWP signaling prevents the metaphase-anaphase transition of oocyte chromosomes and male pronuclear formation, which in turn leads to developmental arrest (Wu et al., 2007a).

Steady progress has been made in the improvement of reproductive performance of cattle through management of female fertility. Major advances include estrus synchronization and introduction of timed AI (Perry et al., 2002). Genetic, but not reproductive performance progress, has been achieved on male side, where the fertility of AI sires is still being evaluated by outdated, light microscopy based methods (Garner, 1997). Conventional semen analysis is subjective, time consuming, and variable. Lab

technicians only analyze 100-200 spermatozoa per sample, and inter-laboratory as well as intra-laboratory differences exist in the evaluation of sperm motility and morphology. Compared to conventional semen analysis, biomarker based analysis using flow cytometry is a highly repeatable, quantitative, fast way to measure thousands of spermatozoa per sample (Christensen et al., 2004). A variety of flow cytometric markers are available for use in detailed examination of multiple sperm characteristics, including viability (Garner et al., 1994), membrane integrity, mitochondrial membrane potential (Evenson et al., 1982; Garner et al., 1997; Graham et al., 1990), acrosome integrity (Graham et al., 1990), chromatin structure (Bochenek et al., 2001; Evenson et al., 1980) and the presence of proteins associated with certain types of sperm defects (Sutovsky and Lovercamp, ; Sutovsky et al., 2003). Increasing acceptance of biomarker based flow cytometric semen analysis by the cattle AI industry brings about the need for new biomarkers of bull sperm quality, reflective not only of sperm/semen traits, but also of sires' fertility/reproductive performance in AI services (Gillan et al., 2005; Sutovsky and Lovercamp).

Due to its proposed role in fertilization and its well defined localization pattern in fully differentiated spermatozoa, PAWP was investigated in the present study as a potential biomarker of bull sperm quality/fertility. To our present knowledge this is the first instance in which a WW-domain binding protein has been used as a biomarker to evaluate sperm quality for practical application in the cattle AI industry.

MATERIALS AND METHODS

Sample Preparation and Labeling

Semen samples from 298 AI bulls with good or acceptable fertility and detailed records of AI services were obtained from Genex Cooperative, Inc., Shawano, WI as part of a collaborative effort. Samples were washed by centrifugation in Tyrode's lactate-HEPES medium (TLHEPES) and fixed in 2% formaldehyde for 40 minutes at room temperature. Starting volume for antibody labeling was 85uL, with a concentration of 20-50 million sperm/mL. In preparation for flow cytometry, the samples were permeabilized by adding 2% stock solution of Triton X-100 (TX-100) to each sample tube (final concentration in tube 0.1%) and incubating for 40 minutes at room temperature. The primary antibody, the affinity purified rabbit anti-PAWP antiserum, characterized in detail in our previous studies (Aarabi et al., ; Wu et al., 2007a; Wu et al., 2007b), was pre-diluted 1:10 in PBS buffer supplemented with 0.1% TX-100 and 1% normal goat serum (NGS; Sigma), added to sample tubes for a final dilution of 1/200 and incubated overnight at +4˚C. The following morning, goat anti-rabbit secondary antibody conjugated to red (TRITC; Zymed Laboratories, San Francisco, CA, USA) or green (FITC; Zymed Laboratories, San Francisco, CA, USA) dye was added at a final 1:200 dilution in PBS + 0.1% TX-100 + 1% NGS and allowed to incubate for 40 min. at room temperature. Prior to performing flow cytometry, the sample aliquots were checked for fluorescent labeling under a Nikon Eclipse 800 epifluorescence microscope (Nikon Instruments, Melville, NY, USA). After positive labeling was confirmed, filtered PBS was added to each sample to reach the

approximate concentration of 500,000 sperm/well of a 96 well plate used to perform flow cytometry. Samples were filtered through a cell strainer cap (Becton Dickinson Labware, Franklin Lakes, NJ, USA; catalog #352235) prior to being loaded into a 96-well plate (Becton Dickinson Labware, Franklin Lakes, NJ, USA; catalog #353915).

Flow Cytometry

Flow cytometric analysis was performed using a dedicated sperm flow cytometer EasyCyte Plus (IMV Technologies, L'Aigle, France)(Odhiambo et al., 2011). The voltage settings were as follows: SSC 500, Green 413, Yellow 403, and Red 701. The level of PAWP-induced fluorescence was measured for 5,000 cells per sample and was recorded in the form of a scatter diagram and a histogram for each sample. After all of the samples were measured, a standardized histogram was produced and markers were set accordingly to allow the fluorescence of specific sperm populations to be measured. Standardized gating was used to interrogate specific sperm sub-populations on the scatter diagrams. The resulting flow cytometry data was analyzed by computing the relative fluorescence and percentage of the sperm cells within individual marker areas of the histogram (**Fig. 4.2**). Two flow cytometric trials with two different sample sets were performed. In the first sample set of 162 bulls, the markers were set to reflect specific populations of spermatozoa labeled with TRITC-conjugated secondary antibody. In this analysis, marker area M1 reflected the entire sperm population, M2 was representative of normal spermatozoa with near-average levels of PAWP, and spermatozoa in M3 had high PAWP-induced fluorescence and were considered abnormal (**Fig 4.2**). Sample processing and analysis for the second trial was adjusted based on the analysis of data from the first trial. In the second trial, with a sample set of 136 bulls, the markers were

set to reflect three specific populations of spermatozoa labeled with FITC-conjugated secondary antibody: marker area M1 contained flow cytometric events with low relative fluorescence, including PAWP-free cellular debris and spermatozoa with low or no PAWP labeling; M2 represented spermatozoa with a normal, near-average range of PAWP-induced fluorescence; the M3 population had increased fluorescence intensity. A fourth marker (M4) was set, essentially a narrowed version of marker M2, containing spermatozoa with a perceived optimal level of fluorescence intensity, based on the fluorescence intensity of motile spermatozoa separated by swim-up technique. In order to isolate a specific sperm population on a scatter plot or histogram, CytoSoft software of the EasyCyte instrument (IMV Technologies) was used to set markers (M1-4 on the histogram, **Figure 4.2B**) and gates (G1-4 on the scatter plot in **Figure 4.2B**). Based on these markers and gates, the CytoSoft program generated data such as median fluorescence induced by excitation of sperm-bound, fluorescently labeled anti-PAWP antibody (further PAWP-median), percentage of spermatozoa within the marker and/or gate, and other values based on the spermatozoa within the marker (M1 % Total, M2 % Gated, etc) and also the entire population (P3.All % Total, % Gated, X-Median). For example, the histogram M1 % Total referred to the percentage of all flow cytometric events that were within in the M1 marker. The histogram M1 % Gated would refer to the percentage of flow cytometric events within the M1 gate on the scatter plot, a more refined sperm population. The median fluorescence of a sample was represented by X-median. The field data including fertility records, semen parameters, and semen collection data and the results of the flow cytometric analysis were compared with conventional sperm parameters listed for the bulls by using Pearson's correlation

analysis. The cohort sizes were 162 and 136 bulls for first and second trial, respectively. However, the field data were incomplete for several sires; therefore, only those sires with available values, for the parameter being compared, were included in analysis.

Additional Biomarkers for Flow Cytometric Analysis

In order to further validate the use of PAWP as a biomarker of bull fertility, we compared the flow cytometric results for PAWP to flow cytometric results from the established sperm quality biomarkers ubiquitin (UBB) (Odhiambo et al., 2011; Sutovsky et al., 2002), peanut agglutinin (PNA) (Graham, 2001; Nagy et al., 2003; Thomas et al., 1997), and lentil lectin from *Lens culinaris* (LCA) (Baska and Sutovsky, 2005). The PAWP data was also compared with a fourth potential biomarker, the ProteoStat Aggresome Detection Kit (AGG) (Enzo Life Sciences International, Inc., Plymouth Meeting, PA, USA). The AGG kit contains a 488 nm excitable molecular rotor dye that binds to denatured proteins within aggresomes and aggresome-like inclusion bodies, as elaborated in manufacturer's data sheet. After finding encouraging correlations between their flow cytometric parameters, we chose to double-label spermatozoa with PAWP and UBB for a cell imaging study. For this purpose, non-specific antibody-sperm binding was blocked by adding 5uL of 2% TX-100 with 20% normal goat serum (NGS) to each sample with a 40 minute incubation period at room temperature. A 10 uL volume of prediluted mouse anti-UBB and rabbit anti-PAWP primary antibodies were added to each 85uL sample (concentration of 20-50 million sperm/mL) in a 1.5mL Eppendorf tube and allowed to incubate overnight at +4°C. After incubation, 10uL of 1:40 pre-diluted GAM IGG-FITC-conjugated secondary antibody (goat anti-mouse IgG FITC; Zymed Laboratories, San Francisco, CA, USA) and GAR IGG TRITC secondary antibody (goat anti-rabbit IgG

TRITC; Zymed Laboratories, San Francisco, CA, USA) were added to each sample and incubated for 40 min. at room temperature.

Statistical Analysis

The % total, % gated, and x-median values were exported into Microsoft Excel spreadsheet which contained field fertility data and semen parameters for each bull in the analysis, including but not limited to conception rate, non-return rate, the number of services, sperm morphology data, and residual values. The median value for each parameter was obtained and the bulls were sorted into groups based on above or below median values. After sorting, Pearson's correlation analysis was conducted between the parameter in question and the flow cytometry results using Microsoft Excel Data Analysis Add-On. R values ≥ 0.33 were tested and the findings considered statistically significant if $P \leq 0.05$. Statistically significant findings were grouped into a table and trends were noted.

Immunofluorecence and Cell Imaging

Prior to performing flow cytometry on samples, spermatozoa were examined for proper labeling using a Nikon Eclipse 800 microscope (Nikon Inc., Melville, NY, USA), equipped with epifluorescence, differential-interference contrast (DIC), and infinity corrected lenses (primary lens used was infinity corrected oil immersion lens, 100x magnification). Cell imaging equipment consisted of a CoolSnap CCD camera (Princeton Instruments Trenton, NJ, USA) and MetaMorph Imaging Software (Molecular Devices Inc., Sunnyvale, CA, USA). Adobe Photoshop CS3 Extended software (Adobe Systems Inc., San Jose, CA, USA) was used for all image processing and editing. After

image acquisition of fluorescently labeled spermatozoa using both epifluorescence and differential interference contrast optics, color channels were combined and brightness/contrast levels adjusted to produce the final images faithful of the patterns and intensities observed through the microscope eyepiece. For FITC or TRITC PAWP-labeled spermatozoa this process consisted of one fluorescence image and one transmitted light image; for the UBI-PAWP double labeling two fluorescence images (one FITC for UBI labeling and one TRITC for PAWP labeling) and one transmitted light image was acquired and color channels were subsequently combined. Where applicable, pixel intensities of PAWP labeling in individual sperm measured (**Figure 4.2 C, D**) using morphometric function of the MetaMorph software, and expressed as average pixel intensities (no units).

RESULTS

Differential Labeling Patterns of PAWP in Normal and Defective Spermatozoa

In normal spermatozoa, PAWP labeling manifested itself as a narrow band of uniform width around the proximal PAS (**Figure 4.1 A**). Defective spermatozoa displayed various anomalies of PAWP labeling including a jagged or abnormally wide PAS band, an irregular spot of PAWP on PAS, ectopic labeling extending to sperm head acrosomal region or sperm tail midpiece, or completely absent labeling (**Figure 4.1 B-O**). In the samples double-labeled for PAWP and UBB, the morphologically normal spermatozoa lacked UBB labeling. In abnormal spermatozoa, UBB labeling patterns ranged from a

wide PAS band to the entire spermatozoon being fluorescently labeled (**Figure 4.1 D, K, N)**. In double-labeled spermatozoa, UBB and PAWP labeling occasionally overlapped in the PAS region of the sperm head, resulting in a yellow appearance of the fluorescent labeling in color channel-combined images (**Figure 4.1K, N**).

Macrocephalic spermatozoa and those with pyriform heads often displayed increased PAWP labeling (**Figure 4.1O;** see also **Figure 4.2 C1, D4**). In morphometric analysis, the pixel intensity of PAWP labeling of these spermatozoa was 1.5-2x higher than in spermatozoa with normal morphology, possibly due to abnormal chromatin packaging or additional DNA (aneuploidy) resulting in increased diameter of sperm nucleus and PAS. Some of the microcephalic spermatozoa also displayed strong PAWP labeling in form of a wide band expanding around the entire PAS (**Figure 4.1B**).

PAWP and Sperm Morphology

Conventional sperm morphology parameters in both trials included primary, secondary, tertiary, and average total morphology. The primary morphology refers to sperm head defects (shape, large vacuoles, crested and pyriform heads, acrosomal defects, etc.); secondary morphology describes retained cytoplasmic droplets, both proximal and distal; tertiary morphology refers to sperm tail defects (coiled, folded, broken, missing, etc.). These three morphology parameters are added up as average total morphology (i.e. percentage of morphologically aberrant spermatozoa). While PAWP did not have statistically significant correlations with primary, tertiary, or total morphology in the first trial, a relationship between PAWP and secondary morphology was observed in a subset of samples. A statistically significant negative correlation ($r=-0.441$, $n=14$, $p=0.05$) was

found between the PAWP content and secondary morphological defects such as retained cytoplasmic droplets This could have been caused by PAWP-accumulation in the retained cytoplasmic droplets. Though not statistically significant, a positive correlation(r=0.291) was also noted between PAWP and primary morphological defects in the first group of 162 bulls. In the second group of 136 bulls, statistically significant correlations between primary, secondary, and total morphology were present. The median fluorescence within the M2 marker correlated negatively with primary morphology (sperm head) defects (r=-0.3449, n=38, p=0.03). The median fluorescence of the entire sample correlated positively with secondary morphological defects, suggesting PAWP accumulation in the cytoplasmic droplets may cause increased median fluorescence (r=0.417, n=27, p=0.027). The marker containing spermatozoa with the perceived optimal level of fluorescence intensity, M4, correlated negatively with the average total morphology, or percentage of morphologically aberrant spermatozoa (r=-0.323, n=39, p=0.042).

PAWP and Conception Rate

Flow cytometric measurements of sperm PAWP content showed statistically significant correlations with conception rate in both trials, even though the division of histograms was changed in the second trial. In the first trial, a statistically significant positive correlation was found between the % spermatozoa in marker area M2 (% sperm with normal/average PAWP fluorescence) and total CR (r=0.3706; n=38; p =0.0073, **Figure 4.3A**). A statistically significant positive correlation was also found between %M3 (sperm with increased PAWP levels) and CR(r=0.3992; n=38; p=.0072). In the second trial, CR had a negative correlation with the median fluorescence level in M3 marker area

(sperm with elevated PAWP) (r=-0.3598; n = 38; p=.0248) and a positive correlation with the percentage of spermatozoa within the M2 marker, representative of the spermatozoa with normal PAWP levels (0.37135, n=38, p=.0204).

PAWP and Non-Return Rate

The non-return rate, or the number of cows that do not return to estrus after being inseminated, had a statistically significant negative correlation with the median PAWP-induced fluorescence of an entire sample in the first trial (r=-0.4004; n=29; p = 0.0168). Most likely, abnormal spermatozoa with increased levels of the PAWP protein caused this parameter of sperm PAWP to behave as a negative biomarker. This observation inspired us to divide the subpopulation of PAWP-positive spermatozoa into two subpopulations (normal and elevated/high PAWP) in the second trial. Consequently, the revised %M2 parameter (% sperm with normal PAWP level) correlated positively (r=0.52054, n=14, p=0.0417) and %M3 (high-PAWP sperm) correlates negatively (-0.53178, n=14, p=0.05) with NR, further supporting the hypothesis that spermatozoa with enlarged nuclei contain more PAWP than morphologically normal spermatozoa.

PAWP and Residual Value

The RV is a statistical parameter used to account for variation of AI-conception rates due to factors that may influence bull fertility for a particular breeding period, such as bull age, age and parity of the cows bred, technicians, etc. A high positive residual value (e.g. r=0.99) represents a bull with uncharacteristically high fertility for a particular breeding season, while a high negative residual value (e.g. r=-0.99) represents a bull with uncharacteristically low fertility. An ideal residual value is zero, which would imply that

there are no extraneous variables that cannot be corrected or compensated for. Flow cytometric PAWP intensities (%M2) had a moderate positive correlation with RV in the first trial (r=0.445; n=18; p=.0001) and a medium-level positive correlation between %M2 and RV (0.56289, n=14, p=0.0314, **Figure 4.3B**). Since residual value is calculated as an attempt to account for previously unaccountable variation between fertility rates per breeding season and PAWP correlates with residual value, it can be inferred that sperm PAWP content contributes to high bull fertility, and may in part account for the variation between breeding seasons.

PAWP and Number of Services

Number of services (NS) refers to a sum of semen collections in sire's reproductive lifespan. It is assumed that sires with average/above-average AI-fertility have higher NS than sires with below-average fertility. In both trials, PAWP had a positive correlation with number of services (1st trial, %M2: r=0.589; n=41; p=.0001) (second trial, %M4: r=0.683; n=44; p=.0035, **Figure 4.3C**), possibly because bulls with a higher number of services were older and may have had better sperm quality than young bulls whose sperm production has not yet peaked.

Relationship between Sperm PAWP and Other Biomarkers of Bull Sperm Quality

As mentioned previously, the flow cytometric data from PAWP labeled spermatozoa were compared with those from the pre-established biomarkers UBB and PNA, and also with two putative novel biomarkers, LCA and AGG. Pearson's correlation analyses revealed that various populations of PAWP-labeled spermatozoa correlated positively or negatively with corresponding populations of UBB, PNA, LCA, or AGG-labeled

spermatozoa. The spermatozoa exhibiting normal PAWP labeling (%M2) had a negative correlation with spermatozoa exhibiting abnormal UBB labeling (%M3) (r=-0.599; n=126; p<0.0001) (**Figure 4.3D)** and a negative correlation with spermatozoa exhibiting AGG labeling in the second trial (AGG labeling was not performed in Trial 1) (r=-0.538; n=136; p<0.0001) (**Figure 4.3E)**. The spermatozoa exhibiting abnormally high levels of PAWP labeling (%M3) had a positive correlation with spermatozoa expressing high levels of PNA (%M6; r=0.648; n=126; p<0.0001) (**Figure 4.3F)** and high levels of LCA (%M7; r=0.667; n=126; p<0.0001) (**Figure 4.3G)**.

DISCUSSION

Trials summarized here provide insight into a relationship of sperm PAWP content and fertility of bulls in AI-service. In the first trial, a simple division was applied to flow cytometric histograms of PAWP-induced fluorescence, separating spermatozoa with below-and above-average levels of PAWP. After analyzing this data set, consideration was given to the localization and accumulation patterns of PAWP in different sperm populations within a semen sample. Because PAWP is inserted in the PAS during spermatid elongation (Wu et al., 2007b), immature sperm forms arising from that phase of spermiogenesis may either lack PAWP or retain it at a superfluous level. Abnormal spermatozoa with primary, sperm head defects, and particularly those with enlarged or pyriform heads indeed show increased PAWP, possibly arising from abnormalities in chromatin packaging or ploidy. Consistent with the

immunocytochemical localization and pixel intensity of PAWP labeling in macrocephalic and pyriform spermatozoa, the increased amount of PAWP protein measured in morphologically defective spermatozoa (the M3 population in Trial 2) by flow cytometry correlated with fertility parameters, such as CR, NR and RV. Since sperm quality eventually declines with bull's age (Fuerst-Waltl et al., 2006), it can be assumed that PAWP could also have a negative correlation with number of services when compared to old bulls (e.g. 5+ years old) with deteriorating sperm quality. None of the bulls in this study were old enough to experience decreased sperm quality due to age as the average age at collection for this group of bulls was 443 days. As a result this hypothesis remains untested.

Flow cytometric PAWP parameters were compared to data from UBB flow cytometry in order to validate PAWP as a biomarker of bull fertility and sperm quality. Abnormal spermatozoa are tagged via ubiquitination of the plasma membrane in the epididymis. Defective spermatozoa become ubiquitinated during their passage through the epididymis. Some spermatozoa tagged with ubiquitin may disintegrate, but many are still found in the ejaculate that can be quantified to provide an indication of sperm quality or fertility (Baska et al., 2008; Sutovsky et al., 2001a; Sutovsky et al., 2007). Increased binding of anti-ubiquitin antibodies to the surface of defective spermatozoa reflects the occurrence of sperm abnormalities, that is conveniently evaluated by flow cytometry (Sutovsky and Lovercamp, 2010; Sutovsky and Lovercamp). The use of ubiquitin as a biomarker of sperm quality has been tested in several species including stallions, bulls, boars, and humans, where high levels of sperm ubiquitin correlate with infertility and poor sperm quality measured by primary and secondary morphology (Lovercamp et al.,

2007; Odhiambo et al., 2011; Ozanon et al., 2005; Purdy, 2008; Sutovsky and Lovercamp, 2010; Sutovsky et al., 2007; Sutovsky et al., 2003). In the first trial of the present study, the percentage of spermatozoa with a normal PAWP content correlated negatively with sperm content of ubiquitin ($r=-0.599$, $n=126$, $p<0.0001$). In agreement with flow cytometry, double-labeling of spermatozoa with anti-PAWP and anti-UBB antibodies showed aberrant PAWP labeling, or a complete lack of PAWP in the spermatozoa with high levels of UBB on their surface.

An additional potential biomarker reflective of poor sperm quality, AGG, also showed a negative correlation with PAWP in Trial 2. Aggresomes are proteinaceous inclusion bodies that form when the ubiquitin-proteosome machinery is overloaded as a result of cellular stresses such as hyperthermia or exposure to reactive oxygen species (Amijee et al., 2009). While aggresomes are most frequently associated with diseases such as Alzheimer's or Parkinson's, the use of AGG to detect misfolded or partially denatured proteins in spermatozoa has proven useful in boar (in preparation) and bull semen (this study), as reflected by a negative correlation of PAWP with all AGG-labeled spermatozoa ($r=-0.538$; $n=136$; $p<0.0001$). This suggests that the PAWP-labeled spermatozoa in the M2 population were uncompromised, healthy spermatozoa that did not contain any inclusion bodies resulting from cellular stresses, hence the negative correlation (**Figure 4.3B**).

Visualization of acrosomal status via flow cytometry is an important tool for evaluating sperm quality. Acrosomal integrity of spermatozoa can be measured by using fluorescently labeled plant lectins such as PNA and LCA that bind to specific sperm surface glycans. The PNA shows a high affinity and strong specificity for D Gal α (1,3)

D GalNAc disaccharide as well as other disaccharides with a terminal galactose; it binds to the outer acrosomal membrane exposed during sperm capacitation, acrosomal exocytosis or acrosomal damage (Graham, 2001). The LCA shows a strong affinity to D-glucose and D-mannose residues and binds to the entire surface of defective spermatozoa but only the acrosomal surface in normal spermatozoa (Apichela et al., 2010; Baska and Sutovsky, 2005; Graham, 2001). While PNA is more commonly used, both PNA and LCA have been validated using several species, including boar (Thurston et al., 2003) and bull (Odhiambo et al., 2011; Thomas et al., 1997). The positive correlation found between these two lectins and PAWP further solidifies PAWP's usefulness as a biomarker to indicate bull sperm quality.

A single collection taken at a random point in a bull's reproductive lifespan may not reflect his lifelong AI-fertility, especially when consideration is given to the variables impacting semen quality, not only on the day of collection (temperature, handler, season) but also during spermatogenesis (overall health/body condition, diet, illness). Therefore, it is encouraging to see correlations between sperm biomarker intensities in individual collections and lifelong records of sires' fertility in AI program. Evaluation via flow cytometry using biomarkers such as PAWP may assist in eliminating animals with threshold- acceptable semen quality from breeding pool. Bull age negatively impacts viability and motility of spermatozoa (Fuerst-Waltl et al., 2006). Consequently, flow cytometric analysis may assist in identifying bulls that are past their reproductive prime and may need increased time for sexual preparation if collections are to maintain acceptable quality levels (Fuerst-Waltl et al., 2006). Flow cytometric analysis may also aid in identifying young bulls with the potential to be excellent candidates for AI services

or in contrast may allow producers to cull young bulls with predicted inferior sperm quality.

Differences in secondary antibodies (FITC vs. TRITC-conjugated secondary antibodies) as well as in division of flow cytometric histograms are likely sources of variation in the correlation coefficients between Trial 1 and 2. Furthermore, laser used in the EasyCyte instrument appears to be better suited for excitation of dyes with excitation optimum around 488 nm (in this case, FITC). Indeed, optimal excitation was recorded in Trial 2, and closer correlations between PAWP-induced fluorescence and field semen/fertility parameters were observed in Trial 2. Inherent to FITC, some non-specific background fluorescence is observed; this could be eliminated by using secondary antibodies and lectins conjugated with phycoerythrin (PE) derivatives, such as PE-Cy5 (excitation peak 498 nm; emission peak 667 nm), which are very efficiently excited by 488 nm laser and detected by EasyCyte Plus at a wavelength of >600 nm, reducing the intensity of non-specific fluorescence.

CONCLUSION

Correlations between sperm PAWP levels and conventional semen and fertility parameters indicate that it will be possible to standardize the PAWP-based assay and to establish a threshold and a range for acceptable PAWP content—anything above or below this level would be considered abnormal and have decreased fertilization potential. PAWP's negative correlation with the established 'negative" biomarker of sperm quality,

ubiquitin, supports this conclusion. Thus, PAWP is a suitable candidate-biomarker of sperm quality and fertility due to its correlations with standard fertility parameters in the artificial insemination industry. Furthermore, PAWP's correlation with ambiguous variables affecting cattle fertility in AI programs, as captured by the parameter of residual value, predisposes PAWP for identification of non-obvious factors that could negatively impact sperm quality and fertility.

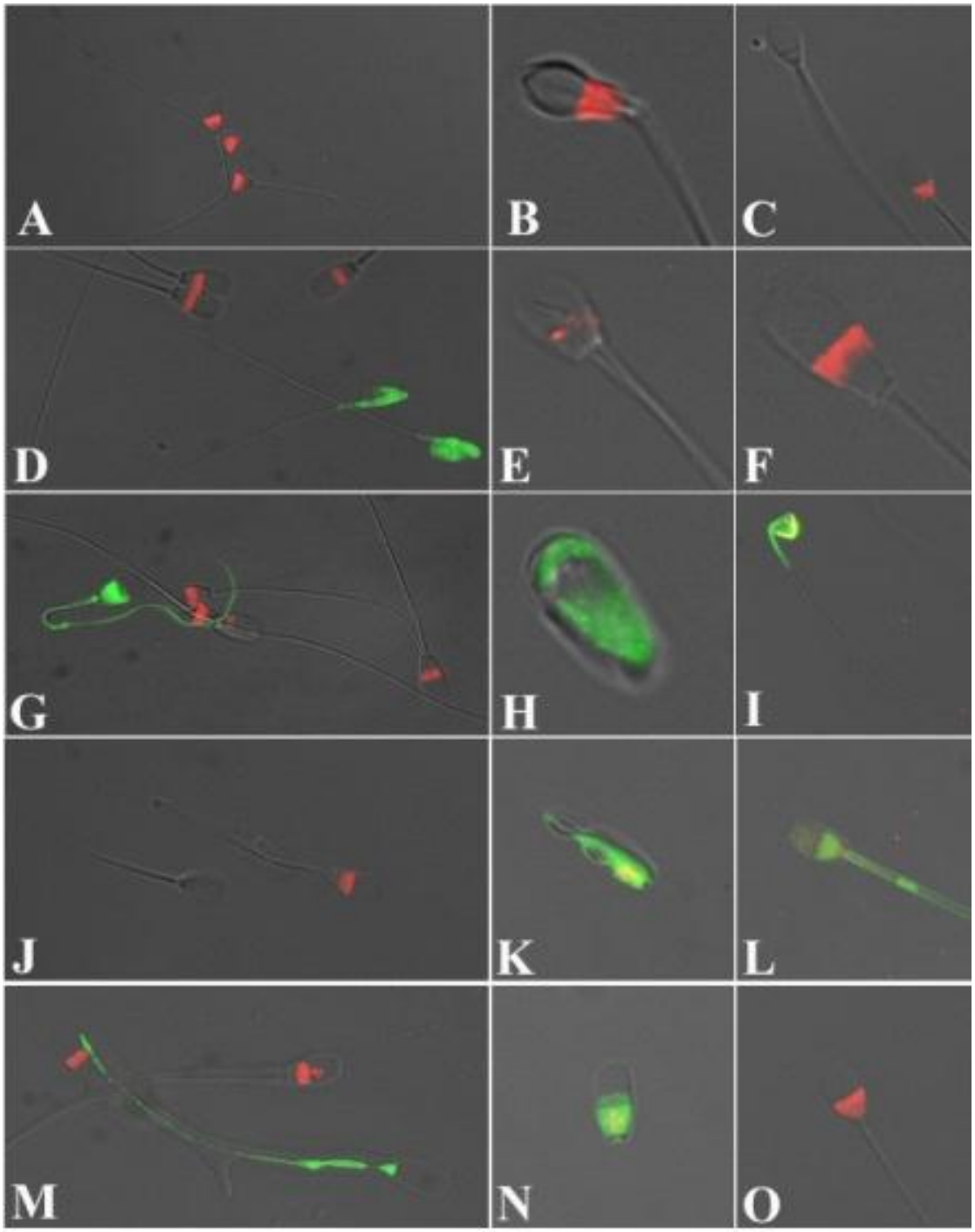
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Figure 4.1: Immunofluorescence of PAWP (red) and ubiquitin (UBI; biomarker detecting altered surface in defective spermatozoa; green in D, G-N) in bull spermatozoa. In normal spermatozoa, PAWP forms a band of regular width, enveloping the PAS. In defective, ubiquitin-tagged spermatozoa, a variety of PAWP aberrations is observed including a wider band spanning the entire PAS (e.g. B, O), irregular, spotted labeling (e.g. E, F), or complete absence of labeling. Yellow color reveals overlap of PAWP and UBI. Sperm morphologies are as follows:

A – Normal spermatozoa

B – Microcephalic, tapered sperm head

C – Left spermatozoon; pyriform sperm head with abnormal PAS/equatorial segment

D – Connecting piece defect & deformed sperm heads

E – Macrocephalic/twin spermatozoa

F – Elongated head

G –Distal cytoplasmic reflex (green, UBI-tagged spermatozoon)

H – "Mummy" sperm with a tail coiled around the head

I – Misshapen head

J –Macrocephalic sperm (left) lacks PAWP labeling (no UBI labeling was performed)

K – Tail tangled around elongated head

L – UBI and PAWP labeled abnormal sperm

M –Abnormal sperm head, abnormal sperm with UBI labeling

N – Detached head with spotted PAWP labeling (yellow UBI-PAWP overlap in PAS)

O – Pyriform/tapered head

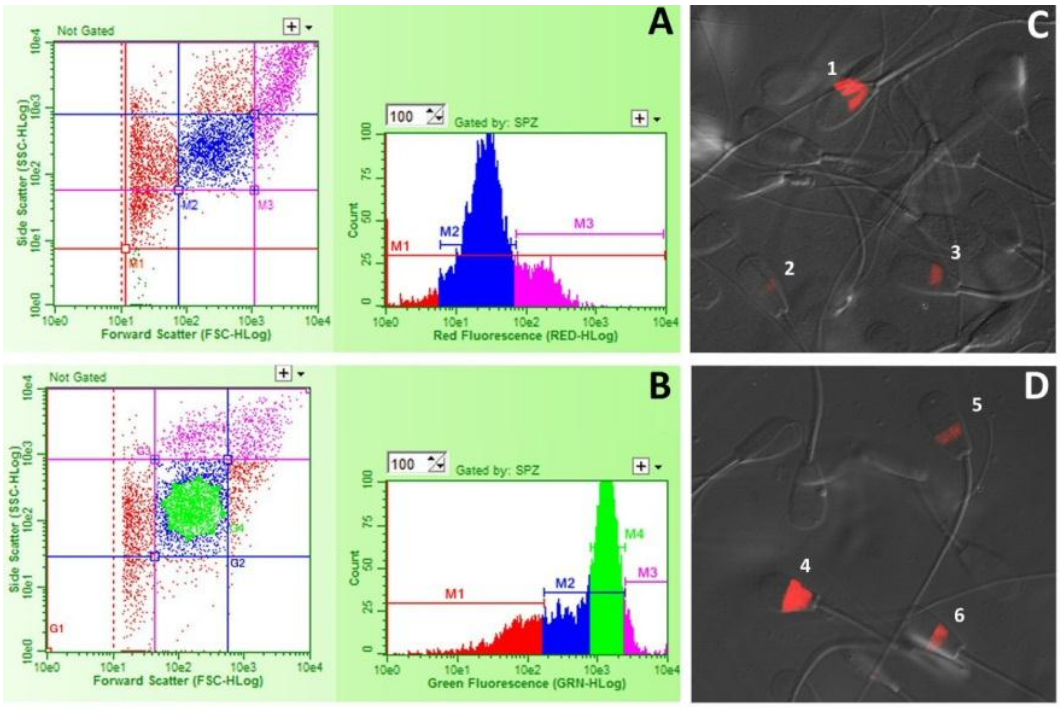

Figure 4.2–Typical flow cytometric scatter diagrams (left) and histograms (right) of the PAWP-induced fluorescence in both trials, representing 5,000 bull spermatozoa/sample; PAWP labeling and pixel intensities of normal and abnormal spermatozoa.

A - Trial 1 with TRITC labeling; markers shown on histogram, with corresponding color coding on scatter plot. M1 comprises the entire sperm population (red); normal spermatozoa are in M2; abnormal spermatozoa with increased fluorescence are in M3.

B - Trial 2 with FITC labeling; markers shown on histogram, with corresponding color coding on scatter plot. M1 represents amorphous debris and sperm fragments. Spermatozoa with normal PAWP content are in M2; M3 contains cells/events with abnormally high fluorescence; the M4 marker contains normal spermatozoa with a specific level of fluorescence hypothesized to be spermatozoa with ideal PAWP content, based on swim-up studies and measurements of pixel intensities, examples of which are shown in panels C &D. C, D - PAWP labeling of normal and abnormal spermatozoa, showing different fluorescence intensities.

1 - macrocephalic spermatozoon displaying a jagged band with increased PAWP labeling: average pixel intensity of 928.85

2,3 - normal spermatozoa with a normal band of acceptable PAWP labeling: average pixel intensities of 448.57 and 506.07, respectively.

4 - pyriform spermatozoon exhibiting a wide PAWP band with increased fluorescence intensity: average pixel intensity of 876.43

5,6 - normal spermatozoa displaying normal PAWP labeling around the PAS. Average pixel intensities of 428.36 and 550.83, respectively.

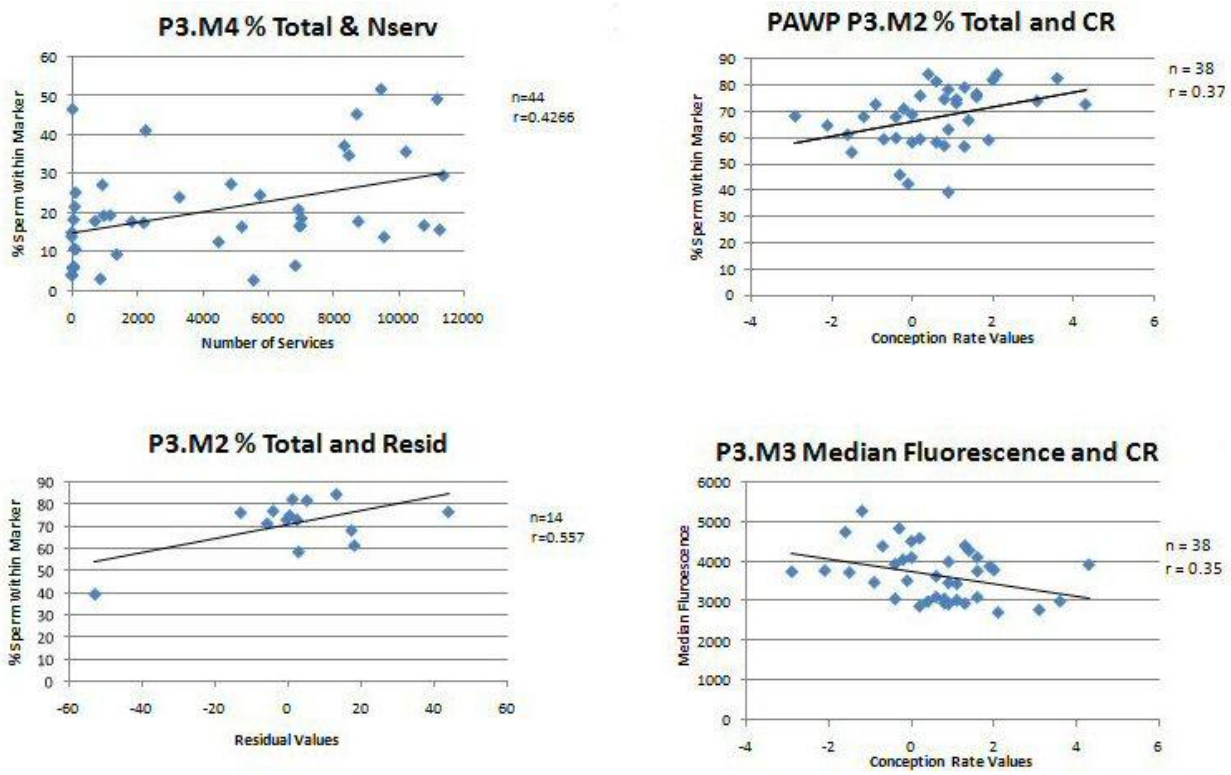

Figure 4.3: Scatter plots reflecting statistically significant correlations between fertility parameters and PAWP-induce fluorescence measured by flow cytometry.

A – Positive correlation between %M2PAWP (normal spermatozoa) and conception rate.

B – Positive correlation between %M2PAWP (normal spermatozoa) and residual value.

C – Positive correlation between % M4PAWP (best spermatozoa in the sample as defined by swim-up and light microscopic measurements of pixel intensities) and number of services.

D – Negative correlation between PAWP and ubiquitin

E – Negative correlation between PAWP and aggresome

F – Positive correlation between PAWP and lectin PNA

G – Positive correlation between t PAWP and lectin LCA

CHAPTER V

CONCLUSIONS, IMPLICATIONS, AND FUTURE DIRECTIONS

With the aid of flow cytometric analysis, multiple negative biomarkers have been validated for use in semen evaluation and prediction of successful fertilizing ability. The different relationships among these biomarkers allow for a simple yet precise analysis of sperm status, such as PNA's ability to determine acrosomal integrity, or a more in depth evaluation of the suitability of a sire for use in a bull stud, such as PAWP's ability to determine previously unaccountable variations in AI conception rates. The discovery and validation of biomarkers in conjunction with flow cytometry allows laboratories to better evaluate sperm attributes on a large scale. Hopefully, the biomarker based fertility testing will make expensive, time consuming preliminary test matings and offspring evaluations unnecessary.

As with any new technology, additional research and development is necessary in order to facilitate ease of use and implementation into a clinical/production setting. Validation of additional biomarkers that correlate with more semen parameters will be an asset to semen evaluation, as well utilizing the diagnostic capability of current biomarkers in research to improve cryopreservation techniques and semen extenders. Additional trials with SPTRX3 and ubiquitin in a clinical setting to examine their correlation with poor ART outcomes could assist clinicians in understanding causes and devising treatments for male infertility. Furthermore, developing the technology to

remove poor quality spermatozoa from a semen sample using nanoparticles coated with antibodies against defective sperm surface biomarkers would be a tremendous asset to reproductive success from an agricultural and andrology standpoint.

APPENDIX

SPERM GIRK2-CONTAINING K+ INWARD RECTIFYING CHANNELS PARTICIPATE IN SPERM CAPACITATION AND FERTILIZATION

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The GIRK2-containing inward-rectifying K^+ ion channels have been implicated in mammalian spermatogenesis. While the *Girk2* null mice are fertile, the male *weaver* transgenic mice carrying a gain-of-function mutation in the *Girk2* gene are infertile. To establish the exact period of spermatogenesis affected by this mutation, we performed StaPut isolation and morphological characterization of the germ cells present in the *weaver* testis. Germ cells representing all periods of spermatogenesis were identified. However, no spermatozoa were present, suggesting that this mutation only affected the haploid phase of spermatogenesis. Real-time PCR studies performed on StaPut purified germ cells from wild-type mice indicated that the *Girk2* transcripts were exclusively expressed in spermatids. Immunofluorscence studies of mouse and boar spermatids/spermatozoa localized the GIRK2 K^+ containing channels to the acrosomal region of the sperm plasma membrane. During porcine *in vitro* fertilization (IVF), GIRK2-containing channels remained associated with the acrosomal shroud following

zona-induced acrosome reaction. Fertilization was blocked by tertiapin-Q (TQ), a specific inhibitor of GIRK channels, and by anti-GIRK2 antibodies. Altogether, studies in two different mammalian species point to a conserved mechanism by which the GIRK2 inward-rectifying K^+ ion channels support sperm function during fertilization.

As a supplemental study flow cytometry was performed using FITC-PNA lectin to investigate the capacitation of boar semen and Ca-ionophore induced acrosome reaction in the presence of the GIRK channel inhibitor TQ. The experiment was performed twice using different boars, in three replicates. A total of ten treatments were examined: non-capacitated, capacitated + TQ, capacitated + inactivated TQ, capacitated + distilled water (the vehicle for TQ), capacitated/acrosome reacted, capacitated/acrosome reacted + TQ, capacitated/acrosome reacted + inactive TQ, capacitated/acrosome reacted + distilled water, and capacitated/acrosome reacted + DMSO (the vehicle for the Ca-ionophore inducing the acrosome reaction). Each of these treatments was incubated with FITC-PNA lectin for 30 minutes and flow cytometry was performed on 5,000 events per sample using predetermined settings. Four markers were set to distinguish between different spermatozoa populations and % total, % gated, and median fluorescence was evaluated for each marker. The GIRK channel inhibitor TQ did not inhibit sperm capacitation as measured by flow cytometry and had a minor effect on Ca-ionophore induced acrosome reaction in boar spermatozoa labeled with PNA lectin. Data for this experiment can be seen in Supplemental Figure 2 of the above manuscript.

IDENTIFICATION OF THE INORGANIC PYROPHOSPHATE METABOLIZING, ATP SUBSTITUTING PATHWAY IN MAMMALIAN SPERMATOZOA

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Inorganic pyrophosphate (PPi) is generated by ATP hydrolysis in the cells and also present in extracellular matrix, cartilage and bodily fluids. Fueling an alternative pathway for energy production in cells, PPi is hydrolyzed by inorganic pyrophosphate (PPA1) in a highly exergonic reaction that can under certain conditions substitute for ATP-derived energy. Recombinant PPA1 is used for energy-regeneration in the cell-free systems used to study the zymology of ATP-dependent ubiquitin-proteasome system, including the role of sperm-borne proteasomes in mammalian fertilization. Inspired by an observation of reduced *in vitro* fertilization (IVF) rates in the presence of external, recombinant PPA1, this study reveals, for the first time, the presence of PPi, PPA1 and PPi transporter, progressive ankylosis protein ANKH in mammalian spermatozoa. Addition of PPi during porcine IVF increased fertilization rates significantly ($p<0.05$) and in a dose-dependent manner. Fluorometric assay detected high levels of PPi in porcine seminal plasma, oviductal fluid and spermatozoa. Immunofluorescence detected PPA1 in the postacrosomal sheath (PAS) and connecting piece of boar spermatozoa; ANKH was present in the sperm head PAS and equatorial segment. Both ANKH and PPA1 were also detected in human and mouse spermatozoa, and in porcine spermatids. Higher

proteasomal-proteolytic activity, indispensable for fertilization, was measured in spermatozoa preserved with PPi. The identification of an alternative, PPi dependent pathway for ATP production is spermatozoa elevates our understanding of sperm physiology and sets the stage for the improvement of semen extenders, storage media and IVF media for animal biotechnology and human assisted reproductive therapies.

In order to better evaluate the use of PPi as an alternative energy source for spermatozoa during long term storage, flow cytometry was performed on semen samples stored for up to 10 days in the presence/absence of 10 μM PPi. Assessment of sperm viability using SYBR-14/PI and mitochondrial membrane potential using JC-1 occurred on days 3 and 10 of storage. Staining protocols were performed in accordance with the manufacturer's recommendations and flow cytometric analysis of 5,000 events per sample occurred using the standard boar spermatozoa viability and mitopotential settings in the Guava EasyCyte Plus CytoSoft software. Using the standard gating and markers in the CytoSoft software, scatter plots were analyzed using % total, % gated, and median fluorescence intensities to isolate specific sperm populations. As expected, the percentage of live spermatozoa detected using SYBR-14/PI was higher on day 3 than on day 10 of storage ($p<0.05$). There was no statistically significant difference between the control samples and those supplemented with 10 μM PPi. However, PPi supplementation increased the number of metabolically active spermatozoa as detected by JC-1 on day 3 and day 10 of storage. Based on these results, it was concluded that PPi may be a beneficial addition to semen extenders and storage media as a method to improve overall sperm viability.

UBIQUITIN-ACTIVATING ENZYME (UBA1) IS REQUIRED FOR SPERM CAPACITATION, ACROSOMAL EXOCYTOSIS AND SPERM-EGG COAT PENETRATION DURING PORCINE FERTILIZATION

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Protein ubiquitination is a stable, covalent post-translational modification that alters protein activity and/or targets proteins for proteolysis by the 26S proteasome. The E1-type ubiquitin-activating enzyme (UBA1) is responsible for ubiquitin activation, the initial step of ubiquitin-protein ligation. Proteasomal proteolysis of ubiquitinated spermatozoa and oocyte proteins occurs during mammalian fertilization, particularly at the site of sperm acrosome contact with oocyte zona pellucida. However, it is not clear whether the substrates are solely proteins ubiquitinated during gametogenesis or if de novo ubiquitination also occurs during fertilization supported by ubiquitin-activating and -conjugating enzymes present in the sperm acrosome. Along this line of inquiry, UBA1 was detected in boar sperm-acrosomal extracts by Western blotting (WB). Immunofluorescence revealed accumulation of UBA1 in the nuclei of spermatogonia,

spermatocytes and spermatids, and in the acrosomal caps of round and elongating spermatids. Thiol ester assays utilizing biotinylated ubiquitin and isolated sperm acrosomes confirmed the enzymatic activity of the resident UBA1. A specific UBA1 inhibitor, PYR-41, altered the remodelling of the outer acrosomal membrane (OAM) during sperm capacitation, monitored using flow cytometry of fluorescein isothiocyanate-conjugated peanut agglutinin (FITC-PNA). Although viable and motile, the spermatozoa capacitated in the presence of PYR-41, showed significantly reduced fertilization rates during in vitro fertilization (IVF; $p<0.05$). Similarly, the fertilization rate was lowered by the addition of PYR-41 directly into fertilization medium during IVF. In WB, high Mrbands, suggestive of protein ubiquitination, were detected in non-capacitated spermatozoa by antibodies against ubiquitin; WB with anti-phosphotyrosine antibodies and antibodies against acrosomal proteins SPINK2 (acrosin inhibitor) and AQN1 (spermadhesin) revealed that the capacitation-induced modification of those proteins was altered by PYR-41. In summary, it appears that de novo protein ubiquitination involving UBA1 contributes to sperm capacitation and acrosomal function during fertilization.

Flow cytometry was used to examine acrosomal integrity and sperm viability using PNA-FITC/PI or SYBR-14/PI dyes, respectively. Prior to evaluation of acrosomal status and viability, capacitation was induced in sperm samples with/without UBA1 inhibitor (10 μM or 100 μM), MG132 (proteasomal inhibitor control, 10 μM or 100 μM), DMSO (vehicle for UBA1 inhibitor), or ethanol (MG132 vehicle). Upon completion of the assays results were examined using appropriate markers and gatings. PNA-FITC histogram markers were set to distinguish between acrosome reacted, non-capacitated, and capacitating/acrosome reacting spermatozoa populations. Viability markers and

gatings were positioned using standard Guava EasyCyte Plus boar viability settings. For both assays, % total, % gated, and median fluorescence intensity values were evaluated for each marker, revealing that the UBA1 inhibitor PYR-14 successfully blocked acrosome remodeling during capacitation, as indicated by decreased fluorescence intensity. Furthermore, no differences in sperm viability between the control groups and inhibitor-treated spermatozoa were discovered, suggesting that the inhibitor has no effect on sperm survival during incubation.

ACRONYMS USED WITHIN THESIS

AB	Aniline blue
AGG	Proteostat Aggresome Detection Kit
AI	Artificial insemination
ART	Assisted reproductive therapies
BSE	Breeding soundness evaluation
CMA3	Chromomycin A3
CR	Conception rate
CTC	Chlortetracycline
DFI	DNA fragmentation index
ET	Embryo transfer
FAA	Fertility-associated antigen
FITC	Fluorescein isothiocyanate
HBP	Heparin binding proteins
HDS	High DNA stainability
ICSI	Intra-cytoplasmic sperm injection
IUI	Intra-uterine insemination
IVF	In vitro fertilization
JC-1	5,5',6,6'-tetrachloro-1,1',3,3' tetraethylbenzimidazolylcarbocyanine iodide
LCA	*Lens culinaris* agglutinin
MB	Multiple births
NR	Non-return rate
NS	Number of services
PAFr	Platelet activating factor receptor
PAS	Post-acrosomal sheath
PAWP	Post-acrosomal ww-domain binding protein
PI	Propidium Iodide
PNA	*Arachis hypogaea* agglutinin
PSA	*Pisum sativum* agglutinin
PT	Perinuclear theca
RV	Residual value
SAB	Spontaneous abortion
SCSA	Sperm chromatin structure assay
SPTRX3	Spermatid-specific Thioredoxin-3
SSP	Secondary sperm morphology
SYBR-14	Membrane-permanent nucleic acid stain
TRITC	Tetramethyl Rhodamine Isothiocyanate
UBB/UBI	Ubiquitin

BIOMARKER SUMMARY

Biomarker	What it detects or binds to	Correlates with
PAWP	Post-acrosomal ww-domain binding protein content within the post-acrosomal sheath	Secondary sperm morphology, conception rate, number of services, non-return rate, ubiquitin
PNA	Disaccharides with terminal galactose, especially the D Gal α (1,3)D GalNac, outer acrosomal membrane No labeling in normal spermatozoa, acrosomal labeling in spermatozoa with abnormal or reacted acrosomes	Sperm morphology Sperm Concentration AI industry fertility parameters
LCA	D-glucose and D-mannose residues Binds to acrosome in normal spermatozoa, entire surface in abnormal spermatozoa	Sperm morphology Ubiquitin labeled spermatozoa
AGG (in development)	Binds to proteinaceous inclusion bodies formed when ubiquitin-proteasome machinery is overloaded Detects misfolded or partially denatured proteins	Ubiquitin, PAWP, morphology parameters
SCSA	Abnormal chromatin structure	Fertility parameters, fertilization success/failure
UBI	Abnormal spermatozoa with surface ubiquitination	Infertility, primary and total morphological defects, percentage of cleaved embryos

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