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Sperm Genetic and Epigenetic Mechanisms Regulating Male Fertility

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Sperm genetic and epigenetic mechanisms regulating male fertility

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Male fertility, ability to fertilize and activate the egg and support early embryo development, is crucial for mammalian reproduction and development. Testis specific histone 2B (TH2B) of sperm, protamines (PRM1/2), and posttranslational modifications of histone 3 (H3K27me3 and H3k27ac) are involved in spermatogenesis and male fertility. However, molecular and cellular mechanisms by which TH2B regulates histone to protamine replacement is poorly defined. Immunocytochemistry, western blotting, flow cytometry, computer-assisted sperm analysis (CASA) and bioinformatic approaches were applied to analyze sperm from Holstein bulls with different *in vivo* fertility. Results from the immunocytochemistry experiments showed that while TH2B and H3K27me3 were localized predominantly at the equatorial and post acrosomal (localized as a crown around the sperm head) parts, respectively. The H3K27ac was also detectable in the bovine sperm head. Signal intensities of TH2B (mean ± SEM) were higher in sperm from the low fertility bulls (220.56 ± 9.20) as compared to those from the high fertility bulls (198.39 ± 10.0). Signal intensities of H3K27me3 (16.25 ± 1.69) were significantly different than those of H3K27ac (4.74 ± 0.88) in bull spermatozoa. Using the bioinformatic tools, including Clustal Omega, Cytoscape, Emboss Dotmatcher,

InterProScan, and STRING, we demonstrated that TH2B has the conserved histone H2B domain which has a strong association with proteins involved in chromosome organization and histone ubiquitination. Intensities of PRM1 and PRM2 were significantly associated with one another ($p < 0.0001$), but neither were significantly associated with fertility. Results from CASA revealed significant differences between high and low fertility bulls regarding average sperm pathway velocity, amplitude of lateral head displacement and straightness ($p < 0.05$). The interacting proteins of H3 are involved in subcellular processes such as regulation of H3K27 methylation, nucleosome assembly, regulation of DNA replication, and chromatin assembly. These results are significant because they help advance fundamental knowledge in sperm physiology involving epigenetic and genetic determinants. The new knowledge can be used to enhance reproductive biotechnology to improve fertility. In addition, the data generated using the unique bull model can be applied to study mammalian reproduction and development due to similarities in genetics and physiology between bovine and other mammals.

DEDICATION

Dedicated to all those who support the pursuit of making positive impacts on science and society on a global scale.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER I

INTRODUCTION

Bull fertility, ability of sperm to activate an egg and cause fertilization, is the essential factor in cattle reproduction. Important to this research is the observation that despite producing ample amounts of sperm with normal motility and morphology, fertility of some bulls remains subpar. We know that molecular, cellular, and physiological mechanisms, which occur because of sperm nuclear proteins can regulate sperm chromatin dynamics. However, we do not know specifically how testis specific histone (TH2B), post translational modifications (PTM) of Histone 3 (H3), protamine 1 (PRM1) as one of the predominant sperm nuclear proteins are able to regulate sperm chromatin dynamics and ultimately determine the health of sperm from bulls of differing fertility. These gaps in the scientific knowledge are important because they are limiting advances in animal science, chromatin biology, and agricultural biotechnology. This section will first review spermatogenesis followed by the fertilization process, sperm chromatin structure, testis specific histones, posttranslational modified histones, protamines and finally the bull a model for studying mammalian reproductive physiology and chromatin biology.

Spermatogenesis is the sequential, developmental process with continuous cell generation and differentiation of primordial germ cells into functional spermatozoa within the testis (Govindaraju et al., 2012). Primordial germ cells differentiate to

spermatogonia via mitosis to form primary spermatocytes that undergo meiosis producing haploid secondary spermatocytes, which then terminally differentiate into spermatozoa (Oliva, 2006). During spermatogenesis, sperm chromatin is tightly packed mostly with protamines (85–98%), forming toroids of DNA.

Initially in this intense compaction process called chromatin packaging, sperm histones with globular structure are first replaced by transition proteins and then by protamines, arginine-rich oligopeptides (Lewis et al., 2003) having α-helical structure with β-rings (Vilfan et al., 2004), or β-pleated structure (Biegeleisen, 2006). At this transition that occurs during spermatogenesis, DNA is compacted greater than 10 times more tightly than that in somatic cells which results in the arrest of DNA replication or RNA transcription in sperm (Shaman et al., 2007; Miller et al., 2010). However, the transition is not always complete as some histones are still partially retained as in mouse (1-4%) and human sperm (15–30%). The histone-bound DNA loci can encode transcription factors and indeed play important roles during post-fertilization events and early embryonic development (Hammoud et al., 2009). Errors in histone to protamine replacement and abnormal protamine ratios have been associated with sperm abnormalities, some causing male infertility (Balhorn et al., 1988; de Yebra et al., 1993). Instead of two protamines (PRM1 and PRM2) as in human and mouse sperms (Balhorn, 1989), the presence of only PRM1 in bull sperm raises an interesting question about the mechanism(s) regulating sperm chromatin structure.

Fertilization is a well-regulated process that occurs by the union of two gametes, each containing only one half of the diploid complement in order to ensure restoration of diploidy and subsequent embryonic development (Palermo et al., 1997). During

spermatogenesis, spermatozoa that are present in syncytium share mRNA and proteins through cytoplasmic bridges and are phenotypically diploid (Cho et al., 2003; Braun et al., 1989; Caldwell and Handel, 1991). Packing its DNA into a volume less than 5% that of somatic cells provides profound benefits to sperm, improves the hydrodynamics, and helps protect the sperm from endonucleases present in the female reproductive tract (Wyrobek et al., 1976). Sperm with their efficiently packaged chromatin due to replacement of histones with protamines are the sperm most likely to fertilize the oocyte and regulate egg activation and subsequent embryo development (Balhorn et al., 1988). During fertilization, sperm not only provides the protamines but also inherits retained histones and the sperm nuclear matrix attachment regions (MARs) to zygote, forming the paternal pronucleus (Ward, 2010). Epigenetic regulations involving posttranslational modifications (PTM) of chromatin remodeling proteins, such as histones, are thought to influence fertilization and early embryonic development (de Oliveira et al., 2013). Regions of sperm chromatin bound to methylated histones are mainly present in heterochromatin regions where the genes are occasionally silenced (Fischle et al., 2003). Acetylated histones, on the other hand, are associated with the euchromatin architecture, reducing histone–DNA links and facilitating both replacement of protamines and activation of transcription during spermatogenesis (Struhl, 1998). Researchers have recently demonstrated the presence of PTM in protamines (Brunner et al., 2014), and that PTM in histones may modulate protamine–DNA interactions (Balhorn, 2007).

Chromatin remodeling occurs during spermatogenesis where linker histones are gradually replaced by testis-specific variants, followed by the replacement of histones with transition proteins and then with protamines (Kimmins and Sassone-Corsi, 2005).

Testis-specific histone (TH2B) is the most commonly retained (Gatewood et al., 1990) and its increased levels in sperm lead to lower levels of protamines (Zhang et al., 2006). A decreased level of protamines in sperm due to an abnormality in retained histones is one of the major factors associated with infertility in humans (Blanchard et al., 1990). In placentals, the sperm chromatin has two different arginine and cysteine-rich protamines (PRM1 and PRM2) (Balhorn et al., 1977; Bélaïche et al., 1987; Bellvé et al., 1988). The PRM1 contains 50 amino acids with a highly conserved amino-terminal hexapeptide sequence and a central DNA binding region containing several arginine-rich anchoring domains (Bélaïche et al., 1987; Balhorn, 1989; Pirhonen et al., 1989). However, PRM2 is synthesized from a precursor protein molecule that is shortened by 40% and yields an arginine-rich and histidine-rich protein that is 63 amino acids in length (Yelick et al., 1987; Carré-Eusèbe et al., 1991; Debarle et al., 1995). Humans, some species of hamsters (Campbell, Syrian, Siberian, and Turkish), and stallions contain two protamines (PRM1 and PRM2) in the sperm chromatin (Balhorn, 1989; Pirhonen et al., 1989; Corzett et al., 1999), whereas bulls (Coelingh et al., 1972; Mazrimas et al., 1986) and, rams (Loir and Lanneau, 1980; Sautiere et al., 1984), and boars (Tobita et al., 1983) contain the single protamine, PRM1. A recent study revealed that, like histones, protamines are also subjected to PTM. Due to their positive charges, protamines are responsible for tight packaging of the sperm DNA, which is believed to later contribute to the transcriptional silencing in the mature sperm (Brunner et al., 2014). Following fertilization, protamines of the paternal genome are replaced by maternal histones, thus the possibility that the retained sperm histones with PTM could play a role in egg activation and embryo development. At this time, the functional significance of PTM of the protamine is

unknown. Abnormal protamine replacement is associated with increased histone retention (Zini et al., 2008), but the mechanisms regulating sperm viability in one versus two protamines are unknown. Furthermore, roles of protamine in protecting the sperm physiology from damaging agents are yet to be determined.

Sperm chromatin structure modulates sperm nuclear shape and function

Higher histone retention is a probable cause of infertility in males. Abnormal histone retention alters the protamine replacement causing inefficient chromatin condensation (Kazerooni et al., 2009). Abnormalities in chromatin condensation influences the size of the sperm at the time of the decondensation step (Kazerooni et al., 2009), which may prevent egg activation by the spermatozoa. Histone to protamine replacement occurs in two steps. First, the modified histones are replaced with the transition proteins 1 and 2. Then, transition nuclear proteins 1 and 2 are replaced with protamines (Aoki et al., 2006a). Variation in the types of protamines occurs among different animals. Humans and mice have PRM1 and PRM2, whereas bulls have PRM1 however the presence of PRM2 is still doubtful. Changes in the size and elongation of sperm head are associated with increased divergence of *PRM1* sequence in most mammals (Lüke et al., 2014). Replacement of histones with protamines plays an important role in crucial cellular processes such as changes in the sperm and temporary arrest of transcription in the haploid male genomes, which protects and stabilizes sperm DNA. For example, Lüke et al. (2014) demonstrated that abnormalities in replacement of histones with protamines can cause major defects in sperm morphology and physiology. Cho et al. (2003) demonstrated that defective histone replacement results in flagellum tightly wrapped around the head or elongated heads having reduced ventral flexure. As

such, proper nuclear architecture facilitating sperm hydrodynamics can offer an advantage for sperm to reach, fertilize, and activate the egg. Protamines in the sperm nucleus form a toroiodal structure that helps to attain ideal sperm nuclear shape and efficient compaction of DNA. The association of protamines with DNA brings about unique chromatin remodeling in spermatozoa and appears to facilitate the hydrodynamic shape of the sperm head (Brunner et al., 2014). Swimming velocity of spermatozoa depends on sperm nuclear shape and size, which in turn affects hydrodynamic efficiency of the sperm (Tourmente et al., 2011; Roldan et al., 1992). Smaller and more elongated heads with proper compaction have less resistive drag during swimming and allow higher sperm velocity (Humphries et al., 2008), enabling sperm to reach and fertilize the egg. Hydrodynamic spermatozoa become highly motile, and help protect the paternal genome from physical and chemical change and damage in the female reproductive tract (Rathke et al., 2014).

Although protamines are involved in sperm chromatin condensation and function, there is restrictive positive selection on only a few functional sites (Lüke et al., 2014). Abnormalities in associations between protamines and DNA lead to low sperm counts, loss of hydrodynamicity in spermatozoa, and increased damage to sperm chromatin and morphology. These abnormalities negatively affect ability of sperm to travel in the female reproductive tract and to fertilize the egg (Carrell et al., 2007; Aoki et al., 2005). Increased incorporation of protamines into sperm chromatin has been shown to block transcription of genes (Lee et al., 1995; Kierszenbaum, 2003).

Deficiencies in protamine levels are related to infertility in men (Balhorn et al., 1988; de Yebra et al., 1993; Belokopytova et al., 1993). Errors in sperm DNA

compaction due to improper protamine replacement is a major cause of sperm DNA damage. This is strongly correlated with failure of embryonic development (Morris et al., 2002).

Efficient protamination is essential for chromatin integrity, function of sperm, and development of embryos (de Oliveira et al., 2013; Franken et al., 1999; Esterhuizen et al., 2000, 2002; Sakkas et al., 1996; Lolis et al., 1996; Nasr-Esfahani et al., 2001; Razavi et al., 2003). Improper retention of histones is a result of improper protamination that affects preferential activation of genes during early embryogenesis (Hammoud et al., 2009). Genes such as *igf1* and globin that are highly expressed during embryogenesis are enriched in histone binding (Gardiner-Garden et al., 1998; Wykes and Krawetz, 2003). The PTM such as methylation, acetylation, and phosphorylation of sperm histones are important epigenetic control mechanisms regulating the expression of the paternal genome by the maternal transcriptional factors during early embryonic development (Hammoud et al., 2009; Miller et al., 2010). Although the PTM of protamines has been demonstrated, precise mechanisms and specific causes and effects of these modifications on embryogenesis are still unclear (Brunner et al., 2014).

Chromatin integrity of sperm is important in successful fertilization, proper egg activation, and embryo development and ultimately in the survival of a species (Morris et al., 2002; Gawecka et al., 2013). The quality of sperm chromatin has such an important impact on the reproductive performance of the male, advanced methods are needed in the study of sperm chromatin health. These methods include sperm chromatin structure assays (SCSA), sperm chromatin dispersion (SCD), terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) or the Comet assay,

immunocytochemistry, and flow cytometry (de Oliveira et al., 2013; Noblanc et al., 2014; Dogan et al., 2015). Although these methods provide valuable insights into molecular integrity of the sperm chromatin, there is still lacking a technique to analyze (noninvasively) the chromatin integrity of a single spermatozoon that is completely ready to fertilize the egg.

Testis specific histone 2B (TH2B)

Testicular germ cells give rise to spermatogonia and finally spermatozoa, expressing histone variants which are distinct from somatic cells. The majority of histones and theses testis specific histone variants are evicted during histone to protamine transition (Balhorn et al., 1984). The replacement of canonical histones by testis specific histones, for example histone 2B (H2B) replaced by testis specific histone 2B (TH2B), causes relaxation of the histone-bound chromatin by eviction of canonical histones from sperm (Montellier et al., 2013). Homology between somatic histone (H2B) to testis specific (TH2B) in human sperm is 85% (Zalensky et al., 2002). Transition of H2B to TH2B sets nucleosome stability, ensuring a genome-wide change of nucleohistone to intermediate structural entities, which in turn are required for the assembly of TNPs (TNP1 & TNP2) and protamines (PRM1) (Montellier et al., 2013).

TH2B induces open chromatin structure and plays a role in inter-nuclear protein replacement in sperm chromatin (Shinagawa et al., 2015). Two chromatin remodelers, chromodomain helicase DNA binding protein 5 (CHD5), and bromodomain-containing protein 4 (BRD4) aid TH2B during its nuclear protein replacement of sperm chromatin. While CHD5 is a member of the chromodomain helicase DNA binding (CHD) family and regulates the sperm chromatin construction (Zhuang et al., 2014), BRD4 belongs to

the bromodomain-containing protein family that regulates transcription of genes in sperm by binding to the hyperacetylated genomic regions (Bryant et al., 2015).

The octamer-shaped nucleohistone complex consists of histones H2A, H2B, histone 3 (H3) and histone 4 (H4), all involved in sperm DNA packaging. Along with protamines, they form a tightly coiled and compacted nuclear structure of DNA in the sperm nucleus known as the toroidal model (Ward, 2010). Retained sperm histones are vital for the histone-bound genes activated before and/or after fertilization. Defects in sperm chromatin are linked to spontaneous abortion and failures of assisted reproductive techniques for humans (Boe-Hansen et al., 2006; Cebesoy et al., 2006). These defects include disrupted DNA integrity due to mutations, and apoptotic DNA fragmentation as a result of exposure to environmental agents and free radicals (Andrabi, 2007; Anifandis et al., 2015a). Such disruptions of chromatin proteins, transcriptional factors, and aberrant histone methylation are speculated to contribute to the decreased fertility in humans (Anifandis et al., 2015b; Belokopytova et al., 1993). Abnormal protamine *versus* histone ratios cause infertility (Aoki et al., 2006b). Therefore, proper TH2B *versus* protamine ratios are important for efficient packaging of sperm chromatin (Montellier et al., 2013). Functional significance of TH2B in the oocyte remains unclear, and the maternal TH2B might be involved in the release of protamines from sperm following fertilization of the egg, thereby facilitating incorporation of maternal histones into the chromatin of the developing embryo (Shinagawa et al., 2014; Shinagawa et al., 2015).

Histone three lysine 27 acetylation and tri-methylation

Retained histones in sperm undergo PTMs that regulate gene expression in a spatiotemporal manner (Kouzarides, 2007). Further, these histone modifications act as

epigenetic tags on paternal genes and are passed on to the offspring (Guerrero-Bosagna and Skinner, 2014). The influence of PTMs in sperm chromatin has a significant impact on correct chromatin conformation by which stability of histone octamers as well as interaction between histones and DNA are attained (Bao et al., 2013; Bao and Bedford, 2016; Bedford and Richard, 2005). Thus, these distinct and spatiotemporal PTMs of histone in conjunction with chromatin remodelers bromodomain containing protein 4 (BRD4) (Bryant et al., 2015), chromodomain helicase DNA binding protein 5 (CHD5) (Zhuang et al., 2014), testis specific histone 2B (TH2B) (Montellier et al., 2013; Shinagawa et al., 2015) as well as other effector modelers, play important roles in sperm chromatin remodeling, eviction of histones, and proper histone-protamine replacement. Additionally, it has been observed that increased H3 methylation in mature human sperm relates to poor sperm motility and functional quality (La Spina et al., 2014).

Proposed by Jenuwein and Allis in 2001, the "histone code" hypothesis states that histones and their post-translational modifications (PTM) regulate epigenetic information (Jenuwein and Allis, 2001). Since then, much has been discovered about the epigenetic function of histones and their associations with male fertility. Chow *et al*. demonstrated that histone 3.3, a histone variant that differs from canonical histone 3.1 by five amino acids, is enriched at the promoters of active genes (Chow et al., 2005). Histones regulate gene expression via PTM such as acetylation and methylation. Acetylation of histones are highly conserved across mammals (Grimes and Henderson, 1984; Meistrich et al., 1992; Oliva and Mezquita, 1982). Acetylation of lysine is known to affect chromatin by decreasing the positive charge of histones that causes a weak histone to negatively charged DNA interaction resulting in increased chromatin fluidity (Bao and Bedford,

2016). Methyltransferases reactions catalyze methylation of lysine on histone (Rivera et al., 2014). These posttranslational modifications of the core histones can also affect the male genome's accessibility to maternal transcription factors during embryo development (Miller et al., 2010). Recently, Verma *et al*. (Verma et al., 2015) demonstrated that trimethylated histone 3 lysine 27 (H3K27me3) differed at various genomic loci important for sperm function and embryonic development.

Protamines

Histones are highly basic proteins, whereas protamines are small arginine rich proteins and abundantly present in spermatozoa (Takeda et al., 2016). Nuclear remodeling and condensation during spermiogenesis result in sequential displacement of histones first by transition nuclear proteins and then primarily protamines, namely protamine 1 (PRM1) and protamine 2 (PRM2) (Balhorn et al., 1984; Kistler et al., 1996). The histone and protamine transition is essential for efficient chromatin compaction in order to silence sperm gene expression, protection and maintenance of sperm DNA integrity in the female reproductive tract, and for appropriate size of sperm head for better hydrodynamicity (Braun, 2001).

Mammalian sperm chromatin is not entirely packed with protamines, as 1-15% of mature sperm DNA is bound to histones (Erkek et al., 2013; Gatewood et al., 1987; Hammoud et al., 2009; Samans et al., 2014). Several of these histones are specific to spermatozoa such as TH2B, TH2A, H2AL1, H2AL2, H3.3A and H3.3B (Govin et al., 2007) and linker histones H1T2 and HILS1 (Martianov et al., 2005). Protamines are important for their efficient compression of sperm DNA into a 10-fold smaller sperm nuclear chromatin compaction configured as protamine toroids wherein DNA is attached

to the nuclear matrix (Ward, 2010). Protamines are positively charged which triggers increased condensation with the negatively charged paternal genomic DNA (Lewis et al., 2003; Oliva, 1995; Queralt and Oliva, 1995). Because of cysteine amino acids in protamines, tight disulphide bridges are created between the protamine molecules resulting in a stabilized nucleoprotamine complex (Lewis et al., 2003; Vilfan et al., 2004).

Most primates carry single a copy of PRM1 and PRM2 (Nelson and Krawetz, 1993; Nelson and Krawetz, 1994; Queralt and Oliva, 1993; Schlueter et al., 1996). The occurrence and function of PRM2 in bull sperm are not fully understood. The PRM1 and PRM2 genes are organized within the genome to create a loop domain in conjunction with the transition nuclear protein 2 gene (TNP2) plus a sequence called gene4 (Martins et al., 2004; Wykes and Krawetz, 2003). This three-dimensional spatial organization may facilitate a coordinated expression of these genes during spermatogenesis. While the protamines (PRM1 and PRM2) and transition protein (TNP2) genes are expressed at high levels and their functions have been extensively studied in human and mice, only limited information is available for bovines.

While we know that protamines play a crucial role in bovine sperm development and reproduction, the mechanisms regulating sperm physiology are unclear. Protamines are thought to protect the sperm genetic material and its delivery to the egg (Mengual et al., 2003; Oliva and Dixon, 1991). Damage of sperm by endogenous or exogenous agents, such as nucleases, free radicals or mutagens can occur because of the compromised protamination (Alvarez et al., 2002; Irvine et al., 2000). As determined by sperm chromatin structure assay (SCSA), abnormal protamination leads to DNA-

fragmentation causing and lower success in intracytoplasmic sperm injection (ICSI) and in *in vitro* fertilization (IVF) rates (Evenson et al., 1980; Evenson and Wixon, 2005). Levels of protamines in human sperm are directly related to sperm DNA integrity (Aoki et al., 2005). Developmental defects after fertilization have occurred with decreased DNA integrity in mice (Lewis and Aitken, 2005; Suganuma et al., 2005; Tesarik et al., 2004). The in depth studies on protamines could help increase understanding of epigenetic programming, and to develop novel approaches for early diagnosis and treatment of infertility (Bao and Bedford, 2016).

The bull as a suitable model for the study of male fertility in humans

There have been 3,799 published reported worldwide over the past 5 years concerning male infertility. While 2,740 of these were aimed at revealing male infertility in humans, the rest were animal based studies. We found only 57 published studies on sperm chromatin-related and male infertility studies. Sperm deficiency, including chromatin and DNA damage, is still considered to be the main cause of male infertility. As new and sophisticated technologies are discovered to analyze sperm DNA, it is not surprising to see that there has been a gradual increase in studies of sperm chromatin in both humans and livestock. Although tools are now available to analyze sperm DNA, for the case of humans, there is insufficient progeny data concerning human male fertility to draw decisive conclusions. Male infertility can be measured by determining the levels of hormones such as follicle-stimulating hormone (FSH), and testosterone (T), and to perform semen analysis measuring the sperm concentration, motility, and morphology.

There are significant similarities between the human and bovine genome (Elsik et al., 2009) and between their embryonic development. Contrary to human fertility research

relying on anecdotal records of one or a few infertility treatment cycles per couple, there is an abundance of field fertility data available in the agricultural sector, focused on improving livestock reproduction. Sperm samples from bulls with detailed breeding records for thousands of AI services exist along with their well-documented fertility phenotype records which enhance the background of knowledge from animal research data (Feugang et al., 2009). Bulls produce such vast numbers of spermatozoa that semen can be repeatedly collected from a single donor in large quantities, allowing IVF and embryo culture to be better developed, more accurate, and reliable.

Currently insufficient evidence is available on sperm genetic and epigenetic mechanisms regulating bull fertility. Similarly, the mechanisms by which retained testis specific histones, PTM histones and protamines are associated with sperm chromatin dynamics and bull fertility is poorly understood. From this, I establish my *central hypothesis* that sperm TH2B, PTM of H3 (H3K27me3 and H3K27ac) and PRM 1 regulate early mammalian fertility via epigenetic and genetic mechanisms with the following objectives:

1. Ascertain sperm histone dynamics linked with fertility using the following:
    a) Localization of testis specific histone 2B (TH2B) in sperm from various bulls with differing fertility.
    b) Measure expression ratios of TH2B with Protamine 1 (PRM1) in sperm from the same bulls.
    c) Measure post translational modifications of Histone H3 (H3K27me3 and H3K27ac) in sperm from the same bulls.

2. Correlate the expression levels of PRM1 and PRM2 in sperm to determine their association with bull fertility.

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CHAPTER II

TESTIS SPECIFIC HISTONE 2B IS ASSOCIATED WITH SPERM CHROMATIN DYNAMICS AND BULL FERTILITY

Abstract

Bull fertility is the degree of sperm's ability to fertilize and activate the egg and support embryo development and this is critical for herd reproductive performance. We used the bull as a unique model organism for the study of male fertility because cattle genetics and physiology is similar to those of other mammals including humans. Moreover, reliable fertility data along with well-established *in vitro* systems are available for bovine. The objective of this original study was to ascertain evolutionary diversification and expression dynamics of Testis Specific Histone 2B (TH2B) in sperm from Holstein bulls with different fertility scores. The intensity of TH2B was determined by using flow cytometry in sperm from 13 high and 13 low fertility bulls. Expression levels of TH2B were measured using immunocytochemistry and Western blotting in sperm from five high and five low fertility bulls. Sequence identity, evolutionary distance and interactome of TH2B were evaluated by Emboss Dotmatcher, STRING and Cytoscape. Data were analyzed using linear mixed effects model and regression plots were drawn. The intensity of TH2B as measured by flow cytometry was significantly affected by an interaction between fertility group and fertility score (P= 0.0182). The intensity of TH2B in sperm from the high fertility group decreased (P= 0.0055) as

fertility increased. TH2B was consistently detectable in sperm and expression levels of TH2B decreased in relation to fertility in sperm from the high fertility group ($P= 0.018$). TH2B biological functions include male gamete generation, chromosome organization, DNA packaging, DNA conformation change, chromatin organization, nucleosome organization, chromatin disassembly, spermatid nucleus elongation, spermatid nucleus differentiation, sperm motility, chromatin organization, chromatin condensation, chromatin silencing, nucleus organization, and chromatin remodeling ($P< 0.05$). We determined the cellular localization and molecular physiology of TH2B using both computational and cell biology approaches. In addition to advancing the fundamental science of mammalian male gamete, the present findings can be potentially used to evaluate semen quality and predict male fertility in the future.

Introduction

Spermatogenesis is the development of mature male germ cells in the testes capable of forming highly differentiated sex cells called spermatozoa. During mammalian spermatogenesis, complex biochemical and morphological transformations occur in the sperm head. The nucleus of a sperm progressively differentiates, undergoing chromosomal condensation, synapsis, genetic recombination, and extensive chromatin reorganization (Bellve, 1979; Bellve and O'brien, 1983). During this chromatin reorganization, occurring in the testis, basic histones are replaced by transition nuclear proteins, (TNPs) and then by protamines (Balhorn et al., 1984). Such displacement of nuclear proteins causes transformation in the shape of sperm head (Cho et al., 2003), hydrodynamicity and chromatin compaction, both of which are important for sperm motility and fertilization (Oliva, 2006). Replacement of canonical histones by testis

specific histones, for example histone 2B (H2B), to testis specific histone 2B (TH2B) causes relaxation of the histone-bound chromatin by increased eviction of canonical histones from sperm (Montellier et al., 2013).

Transition of H2B to TH2B sets nucleosome stability, ensuring a genome-wide change of nucleohistone to intermediate struct ural entities, which in turn are required for the assembly of TNPs (TNP1 & TNP2) and protamines (PRM1) (Montellier et al., 2013). TH2B induces open chromatin structure and plays a role in inter-nuclear protein replacement in sperm chromatin (Shinagawa et al., 2015). Chromatin remodelers, chromodomain helicase DNA binding protein 5 (CHD5), and bromodomain-containing protein 4 (BRD4) aid TH2B during nuclear protein replacement in the sperm chromatin. While CHD5 is a member of the chromodomain helicase DNA binding (CHD) family and regulates sperm chromatin structure (Zhuang et al., 2014), BRD4 belongs to the bromodomain-containing protein family that regulates transcription of genes in sperm by binding to hyperacetylated genomic regions (Bryant et al., 2015).

The octamer-shaped nucleohistone complex consists of histones H2A, H2B, histone 3 (H3) and histone (H4), all involved in sperm DNA packaging. Along with protamines, they form a tightly coiled and compacted structure known as the toroidal model (Ward, 2010). Retained sperm histones are vital for the histone-bound genes activated before and/or after fertilization. Defects in sperm chromatin are linked to spontaneous abortion and failures of assisted reproductive techniques in human (Boe-Hansen et al., 2006; Cebesoy et al., 2006). These defects include disrupted DNA integrity due to mutations, and apoptotic DNA fragmentation as a result of exposure to environmental agents and free radicals (Andrabi, 2007; Anifandis et al., 2015a). Such

disruptions of chromatin proteins, transcriptional factors, and aberrant histone methylation have been speculated to contribute to the decreased fertility in human (Anifandis et al., 2015b; Belokopytova et al., 1993). Abnormal protamine to histone ratios cause infertility (Aoki et al., 2006). Therefore, proper TH2B to protamine ratios are important for efficient packaging of sperm chromatin (Montellier et al., 2013). Functional significance of TH2B in the oocyte remains unclear, and the maternal TH2B might be involved in the release of protamines from sperm following fertilization of the egg, thereby facilitating incorporation of maternal histones into the chromatin of developing embryo (Shinagawa et al., 2014; Shinagawa et al., 2015).

Abnormal retention of histones in the sperm is an indication of immature sperm, and abnormal ratios of protamine to histone cause infertility (Aoki et al., 2006). Therefore, TH2B is a potential biomolecular marker useful for analysis of semen quality, potentially predicting bull fertility and sire`s suitability for artificial insemination (AI). Better elucidation of the dynamic role of TH2B advances the understanding of its chromatin remodeling role in bull sperm. The present study was conducted to test the central hypothesis that different cellular intensities, expression levels, and localization of TH2B are associated with sperm chromatin dynamics and bull fertility. Bovine TH2B was localized in sperm head and its expression levels ($p = 0.0055$) and intensity ($p = 0.018$) were lower in sperm from the high fertility bulls as compared to low fertility ones. Protein sequence of TH2B is conserved among human, rat, mouse and bovine. Further, TH2B has significant gene ontology terms for molecular functions in chromatin and DNA binding and biological functions in sperm development differentiation and motility, nucleosome assembly, chromatin remodeling and spermatid nucleus differentiation (P<

0.05). Because of the genetic and physiological similarities among mammals (Feugang et al., 2010; Kutchy et al., 2017; Ménézo and Hérubel, 2002; Sirard, 2017), the findings of the present study advance fundamental science of mammalian development and reveal potential biomolecular markers for semen evaluation and fertility prediction.

Methods

Experimental Design

Cryopreserved semen straws from Holstein bulls (Alta Genetics, Inc., Watertown, WI, USA) were used in the present study. Straws from 26 bulls were used for the flow cytometry experiment (Table. 1) and 10 bulls were used for immunocytochemistry and Western blotting assays (Table. 2).

Table 1 Fertility phenotypes of the Holstein bulls used for flow cytometry analysis

High fertility (HF) bulls				**Low fertility (LF) bulls**		
Bull Number/Name	**Number of breeding's**	***In vivo* fertility score**		**Bull number/name**	**Number of breeding's**	***In vivo* fertility score**
11HO9467/Comish	705	3.9		11HO8060/Graham	974	-4.7
11HO9375/Discover	965	3.7		11HO7388/Kaleb	555	-4.2
11HO5570/Finley	2924	3.6		11HO8623/Damico	1083	-4.1
11HO9219/Alverez	737	3.5		11HO9455/Direct	793	-4.1
11HO6152/Bailiff	3137	3.4		11HO8524/Midnite	758	-4.1
11HO8245/Allegiance	859	3.4		11HO8128/Jetwave	862	-4.1
11HO8747/Rate	745	3.1		122HO2075/Mart	469	-4.1
11HO6671/Notion	2195	3		11HO8740/Navigator	892	-3.8
11HO8789/Formatic	1134	3		11HO4272/Dante	987	-3.7
11HO9269/Stateside	965	3		122HO1222/Champ	1105	-3.7
11HO4631/Oixie	682	2.9		11HO9492/Flyer	901	-3.6
11HO6440/Sylvester	3647	2.8		11HO8001/Cortland	927	-3.6
11HO5929/Ally	6051	2.6		11HO8105/Decker	1020	-3.6

Bulls 1–13 are designated as the HF group and bulls 14-26 are designated as the LF group. All bulls were individually represented with their *in vivo* fertility scores and the number of breeding's as well as no significant differences in their sperm parameters. Fertility scores are expressed as the percent deviation of each conception rate from the average conception rate of all the bulls during artificial insemination (AI) as previously described (de Oliveira et al., 2013).

Table 2 Fertility phenotypes of the Holstein bulls whose sperm were used for immunocytochemistry and Western blotting

High fertility (HF) bulls				**Low fertility (LF) bulls**		
Bull number/name	**Number of breeding's**	***In vivo* fertility score**		**Bull no**	**Number of breeding's**	**In vivo fertility score**
11HO9375/ Discover	965	3.7		11HO7969/ Aurora	867	-4.9
11HO8064/ Derrick	1026	3.7		11HO8060/ Graham	974	-4.7
11HO5770/ Finley	2924	3.6		11HO7388/ Kaleb	555	-4.2
11HO6152/ Bailiff	3137	3.4		11HO9455/ Direct	793	-4.1
11HO8245/ Allegiance	859	3.4		11HO8623/ Damico	1083	-4.1

Bulls 1–5 are designated as the high fertility (HF) group and bulls 6-10 are designated as the low fertility (LF) group. All bulls were individually represented with their *in vivo* fertility scores and the number of breeding's. Fertility scores are expressed as the percent deviation of each conception rate from the average conception rate of all the bulls as previously described (de Oliveira et al., 2013).

Bulls are considered fertile if they repeatedly mount to serve females and bring about 90% pregnancies in 50 females in a nine-week time (Murphy et al., 2013). Therefore, high fertility (HF) bulls have 80-90% pregnancies in 50 females in a nine-week time, whereas low fertility (LF) bulls have 70-80% pregnancies in 50 females in a nine-week time and less than 40% is sub fertile. In our study, the bulls were divided as HF and LF as reported in detail in our previous publications (de Oliveira et al., 2013;

Peddinti et al., 2008). Non-return rate (NRR-which is defined as portion of cows that are not subsequently re-bred within a specified period of time after an insemination) on day 40 after insemination were calculated for all bulls and the fertility score was ranked as the deviation of each NRR from the average. In the present study, we grouped the bulls with positive deviation from average NRR as HF and those animals with negative deviation from average as LF. Fertility data of the selected bulls are periodically updated with information from partnering herds (Peddinti et al., 2008). Then, we tested the hypothesis that different cellular intensities, expression levels, and localization of TH2B are associated with sperm chromatin dynamic and bull fertility using methods in immunocytochemistry, Western blotting, flow cytometry and bioinformatics.

Evaluation of TH2B expression in bull sperm by flow cytometry

Flow cytometry experiments were performed according to methods described by Dogan *et al.* (Dogan et al., 2015) and Odhiambo *et al.* (Odhiambo et al., 2011), with modifications. Flow cytometry was used to quantify the TH2B cell population by passing individual sperm through a laser beam of the appropriate wavelength. The following settings for BD-FACSCalibur flow cytometer (BD Bioscience San Jose, CA 95131-1807, USA), were used; laser line 488 nm, emission filters 530/30 nm and fluorochrome 542 nm. TH2B molecules were conjugated to fluorescent antibodies to detect and quantify the presence of this protein. Semen straws from 26 Holstein bulls, 13 high fertility (HF) and 13 low fertility (LF), were removed from liquid nitrogen and thawed at 37°C for 30 sec. The extenders were then separated from the cells by centrifugation at 2,000 x *g* at 4°C for 5 min. and pellets were washed twice in washing buffer [WB: Phosphate buffer saline (PBS) with 0.1% Bovine Serum Albumin (BSA)] and again centrifuged at 2,000 x *g* at

4°C for 5 min. The pellets were then fixed in 1 ml of 4% formaldehyde at room temperature (RT) for 1 hr in separate centrifuge tubes. The samples were then centrifuged at 3,000 x *g* at 4°C for 5 min and pellets were resuspended in 250 µl of PBS and immediately permeabilized in 250 µl of 0.1% Triton X-100 in 0.1% sodium citrate in PBS on ice for 2 min. The pellets were resuspended in 500 µl of PBS, filtered through a flow cytometric tube using a cell strainer cap (Becton Dickinson Labware; catalogue no. 352235), and then incubated with the primary antibody at 4°C overnight. Primary antibody was TH2B (Rabbit polyclonal to Testes Specific Histone H2B; Abcam, Cambridge, MA, USA; catalog # 23913; 1/250 dilution). Next day, samples were centrifuged at 3,000 x *g* at 4°C for 5 min, washed once in 500 µl of washing buffer, centrifuged at 3,000 x *g* at 4°C for 5 min and incubated with secondary antibodies for two hours at RT. The secondary antibody was donkey anti-rabbit IgG-FITC (Santa Cruz, Dallas, Texas, USA; catalog # 2090; 1/250 dilution). Following incubation, the samples were washed twice in WB (3,000 *g* at 4°C for 5 min). Sperm samples were then analyzed using the BD-FACSCalibur flow cytometer (BD Bioscience San Jose, CA 95131-1807 USA).

Statistical methods used for flow cytometry

The effect of *in vivo* fertility on TH2B intensity as measured by flow cytometry was assessed using mixed model analysis with PROC MIXED with SAS for Windows 9.4. One straw per bull was used, and experiment was repeated three times. *In vivo* fertility group was the fixed effect and bull was included as a random effect. The relationship of fertility score and intensity was assessed through mixed model linear regression using PROC MIXED with SAS for Windows 9.4. In an initial model, fertility

score, fertility group, and their interaction term were included as fixed effects. The random statement included bull as a random effect. Fitting this model indicated the interaction between fertility score and fertility group was highly significant. Separate models with fertility score as the fixed effect were fit for both the high and low fertility groups to better understand the relationship between fertility score and intensity. The distribution of the conditional residuals was assessed to ensure the assumptions of the statistical models had been met. An alpha level of 0.05 was used to determine statistical significance.

Visualization of Sperm TH2B using Immunocytochemistry

Immunocytochemistry was performed according to the methods described by Li *et al.* (Li et al., 2008) and de Oliveira *et al.* (de Oliveira et al., 2013), with modifications. Briefly, cryopreserved semen straws from five high fertility and five low fertility bulls were thawed in a water bath at 37ºC for 30 seconds (sec). Sperm samples were washed with PBS containing protease inhibitors (cOmplete; Roche, Indianapolis, IN, USA; catalog # 04693116001), and 10 mM ethylenediaminetetraacetic acid (EDTA). Then, the solution was centrifuged at 2,000 × *g* at RT for 5 min. In addition, the sperm pellets were incubated with 20 mM CHAPS at RT for 20 min. Sperm chromatin was then decondensed in 10 mM DTT and 1 mg/ml of heparin at RT for 30 min (Motoishi et al., 1996). Moreover, sperm were fixed in 4% paraformaldehyde at 4ºC for 10 min. Following fixation, cells were permeabilized with 0.2% Triton X-100 and 0.1% bovine serum albumin (BSA) in PBS at RT for 15 min. Sperm were then washed in 50%, 70%, 95% and 100% ethanol at RT for 1 min each. The excess ethanol was removed by quick decanting followed by an additional step of fixation using 100% methanol at -20ºC for 20

min. Excess methanol was removed using washing buffer (WB: PBS containing 0.1% Triton X-100; please note that the WB were different for flow cytometry and immunocytochemistry) and the sample was blocked with 1% BSA in the WB at RT for 1 hour (h). Sperm were probed with primary antibodies against TH2B (Rabbit polyclonal to Testes Specific Histone H2B; Abcam, Cambridge, MA, USA; catalog # 23913; 1/200 dilution) at 4^{o}C overnight followed by a washing step and probing with secondary antibody of donkey anti-rabbit IgG-FITC against TH2B (Santa Cruz, Dallas, Texas, USA; catalog # 2090; 1/5,000 dilution) at RT for 1 h, and then with 2.5 mg/ml of DAPI at RT for 10 minutes. Coverslips were placed onto the slides using a drop of an antifade mounting medium (VECTAshield, H-1000) and sealed using a nail polish border. The samples were examined under a confocal fluorescence microscope (Zeiss LSM 510) under 40X and 63X magnifications using immersion oil. The experiments were repeated three times and the data were statistically analyzed.

Statistical methods used for immunocytochemistry

The association of *in vivo* fertility scores with sperm TH2B intensity detected by immunocytochemistry was assessed using mixed model analysis with PROC MIXED with SAS for Windows 9.4 (SAS Institute, Inc., Cary, NC). One straw was distributed into three replicates, three straws per bull were used and experiment was repeated three times. *In vivo* fertility group was the fixed effect in the model. The random statement included replicate within repeat, repeat within bull, and bull as random effects. The relationship of fertility score and intensity was assessed through mixed model linear regression using PROC MIXED with SAS for Windows 9.4. In an initial model, fertility

score, fertility group, and their interaction term were included as fixed effects. The random statement included replicate within repeat, repeat within bull, and bull as random effects. Fitting this model indicated a no significant interaction between fertility score and fertility group but the fertility scores were widely separated between the two groups. Accordingly, separate models with fertility score as the fixed effect were fit for both the high and low fertility groups to better understand the relationship between fertility and TH2B intensity. Regression line plots were made using SGPLOT with SAS for Windows 9.4. The distribution of the conditional residuals was assessed to ensure the assumptions of the statistical models had been met. An alpha level of 0.05 was used to determine statistical significance.

Extraction of nuclear sperm protein for Western blotting

Sperm TH2B was extracted according to the methods of Aoki *et al.* (Aoki et al., 2005) and de Oliveria *et al.* (de Oliveira et al., 2013), with some modifications. Briefly, cryopreserved semen straws from five high fertility and five low fertility bulls were thawed at 37°C for 30 sec and washed twice in PBS with protease inhibitor, centrifuging each time at 700 ×*g* at 4°C for 5 min. An aliquot containing 25-40 x 10^6 sperm was used to extract the nuclear proteins. In order to lyse the cells, sperm samples were washed twice with 400 µL of 1 mM Phenyl methylsulfonyl fluoride (PMSF) in ddH_2O, centrifuged each time at 700 ×*g* at 4°C for 5 min. Then, 100 µL of 20 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 100 mM Tris (pH 8.0) were added to the pellets followed by the addition of 100 µL of 6 M guanidine hydrochloride, 575 mM dithiothreitol (DTT), and 200 µL of 552 mM iodoacetamide. The samples were protected from light and incubated at 20°C for 30 min. In addition, the samples were supplemented

with 1 mL of cold ethanol (-20ºC), and each sample was then incubated at -20ºC for 1 min and centrifuged at 12,000 ×*g* at 4ºC for 10 min. The ethanol wash was repeated once more and the pellet was resuspended in 1 mL of 0.5 M HCl and incubated at 37ºC for 15 min and centrifuged at 10,000 ×*g* at 25ºC for 10 min. The supernatant was kept and the nuclear proteins were precipitated by the addition of 300 µL of 100% trichloroacetic acid (TCA) to a final concentration of 20% TCA. The solution was incubated at 4ºC for 5 min and centrifuged at 12,000×*g* for 10 min. The pellet was washed twice in 500 µL of 1% 2-mercaptoethanol in acetone. The final pellet was dried out and stored at -30ºC.

Western blotting analyses of sperm nuclear proteins

Protein concentration in samples containing sperm protein extracts was determined in triplicates using Quick Start™ Bradford Protein Assay Kit 2 (Bio Rad®, Hercules, CA, USA; catalogue # 5000202). In addition, sperm nuclear proteins were precipitated with cold acetone at -20°C for 3h, followed by centrifugation at 10,000 ×*g* at 4°C for 10 min. The supernatants were discarded and the pellets were resuspended in 50 µL of 1× Laemmli sample buffer (Bio Rad®, Hercules, CA, USA) with 5% 2-mercaptoethanol, vortexed (10 sec), boiled for 10 min, and stored -30°C. An aliquot of sperm nuclear proteins (10 µg) was reduced, denatured and separated with a vertical polyacrylamide gel electrophoresis (4-20% SDS-PAGE; Mini-Protean TGX™ gel). Protein bands were transferred from the gels to an Immobilon®-P polyvinylidene difluoride (PVDF) membrane using HEP-1 semi-dry electro blotting (Thermo-Scientific Inc®; Waltham, MA USA) set at 46mA for 2.5h. Binding sites were blocked with 5% BSA in PBS-0.1% Tween 20 (PBS-T) at RT under mild agitation for 1 h, followed by an incubation with primary antibodies against bovine TH2B (Abcam®, Cambridge, MA,

USA; 1/1,000 dilution; rabbit polyclonal IgG; catalog #23913). Lamin B1 was used as internal loading control (Abcam®, Cambridge, MA, USA 1: 2,000, rabbit polyclonal IgG; catalog #16048; Abcam) in PBS-T with 1% of BSA at RT for 1 h. Because, the expression levels of Lamin B1 were not stable because we recorded some variation in its band among high and low fertility bulls, therefore another unknown protein band (~ 25 KDa) served as second internal control. Membranes were then washed three times for 10 min in PBS-T and incubated with secondary antibodies (1:10,000; donkey ant-rabbit IgG-HRP; sc-2313; Santa Cruz Biotechnology®) and Precision Protein™ StrepTactin-HRP Conjugate (Bio Rad®, Hercules, CA, USA; 1/10,000; catalog # #1610380) in PBS-T with 1% of BSA at RT for 1 h. Membranes were washed with PBS-T three times for 5 min each. The bands were revealed using a chemoluminescence reagent (Clarity™ Western ECL Substrate, Bio-rad®, Hercules, CA, USA) and Image Laboratory software (Bio-Rad®) for 30 sec. The band intensity of TH2B histone protein was analyzed using ImageJ 1.x (Schneider et al., 2012).

Statistical methods used for Western blotting

Association of *in vivo* fertility with sperm TH2B concentration was assessed using linear mixed model analysis with PROC MIXED with SAS for Windows 9.4. Three straws per bull were used and nuclear protein was extracted and blotted, same experiment was used three times (Table. 3). *In vivo* fertility, group of animals and their interaction were included as fixed effects in the model. The random statement included bull as a random effect. Separate models with fertility scores as the fixed effect were fit for both the high and low fertility groups to better understand the relationship between

fertility scores and the TH2B levels. The relationship between fertility scores and levels of TH2B was assessed through the mixed model linear regression using PROC MIXED with SAS for Windows 9.4. The distribution of the conditional residuals was assessed to ensure that the assumptions for the statistical models had been met. An alpha level of 0.05 was used to determine statistical significance.

Table 3 Statistical model for flow cytometry, immunocytochemistry and Western blotting

Class	Levels	Values
Group	2	1 2
Bull/ Bull names	10	Allegiance Aurora Bailiff Damico Derrick Direct Discover Finley Graham Kaleb
Repeat	3	1 2 3
Replicate	3	1 2 3

Ten Holstein bulls were used for ICC and above model was followed. For WB protein was extracted from three straws together, whereas 26 animals were used for flow cytometry and above model was followed.

Analyses of sequences and interactions of TH2B using bioinformatics

Emboss dotmatcher was used to compare protein sequences of predicted bovine TH2B (predicted sequence was obtained from protein-NCBI; https://www.ncbi.nlm.nih.gov/protein/741900513) with human TH2B, bovine H2B with human H2B, and bovine TH2B with bovine H2B (http://www.bioinformatics.nl/cgi-bin/emboss) to ensure the sequence similarity of bovine TH2B for downstream computational biology analysis. Protein sequences of predicted TH2B and H2B of bovine were compared using computational tools. Protein sequences of TH2B and H2B were

first aligned using Clustal Omega method and the percent identity matrixes between the sequences were obtained (http://www.ebi.ac.uk/Tools/msa/clustalo/).

Predicted protein-protein interactions among TH2B, H2B, H2A, H3, H4 TNP1, TNP2, PRM1, BRD4 and CHD5 of bovine, human and mouse were obtained using STRING database (Search Tool for the Retrieval of Inter-Acting Proteins; http://string-db.org/) (Szklarczyk et al., 2014). The testis specific histone 2B (TH2B) in mature sperm interacts with chromatin proteins H2A, H3, H4, H1, TNP1, TNP2, PRM1, BRD4 and CHD5. Thus, the interactome of *TH2B* using the biological networks gene ontology tool (BiNGO) within Cytoscape 3.3 (http://www.cytoscape.org) was elucidated as well (Cline et al., 2007). To analyze the interactome of *HIST1H2B (TH2B)*, a merged-network was generated in Cytoscape by entering the query keywords "*HIST1H2B* for *TH2B*", "*H2A*", "*H3*", "*H4*", "*TNP1*", "*TNP2*", "*PRM1*", "*PRM2*", "*H2B*", "*BRD4*" and "*CHD5*" into the search bar and results were retrieved from the BiNGO plugin to assess overrepresentation of GO categories.

Results

Expression dynamics of TH2B in bull sperm using flow cytometry

A total of 150,000 sperm per bull were analyzed to investigate the expression of testis specific histone 2B (TH2B) from 13 high fertility and 13 low fertility bulls (Table.1). Flow cytometric measurements of sperm TH2B showed different histogram profiles between HF and LF bulls (Table. 4; Fig. 1).

Table 4 TH2B flow cytometry analysis between high and low fertility bulls.

Group	Number of Observation	Mean	Std. Dev	Std. Error	Minimum	Maximum
High Fertility	39	16.94	9.85	1.58	3.36	48.60
Low Fertility	39	19.98	9.07	1.45	9.29	45.60

High fertility bulls flow cytometry was repeated three times and the mean, standard deviation and standard error of analysis are reported in above table.

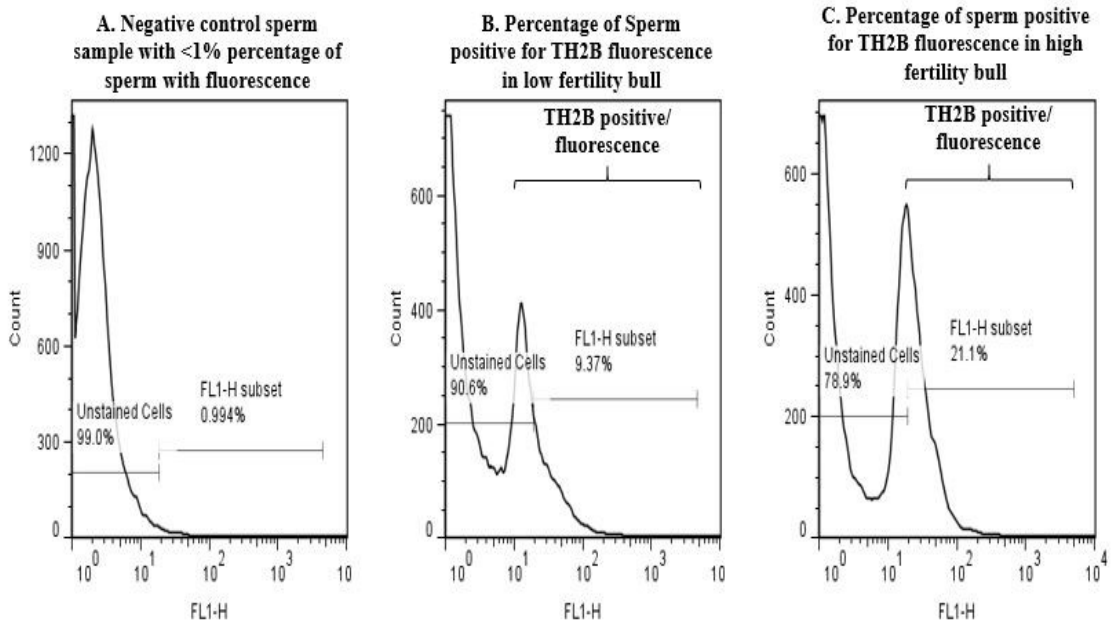

Figure 1 Differential expression of TH2B in sperm from bulls with different fertility scores.

The negative control showing the absence of TH2B fluorescence in the unstained sperm (A). Percentage of sperm expressing TH2B and unstained in high fertility bulls (B). The percentage of sperm in low fertility bulls expressing TH2B positive and unstained cells (C). The percentage of sperm in FL1-H subset indicates expression of TH2B.

As evaluated by regression analysis, the interactions between TH2B intensity (which are percentage of sperm positive for TH2B and emitting fluorescence) and fertility scores of bulls were significant (P= 0.0182). The intensities levels of TH2B was

highly significant compared to fertility score in the high fertility group (P= 0.0055; y = -12.3828x + 56.9464) but no significance was found in the low fertility group (Fig. 2; y = 2.0450x + 28.0499).

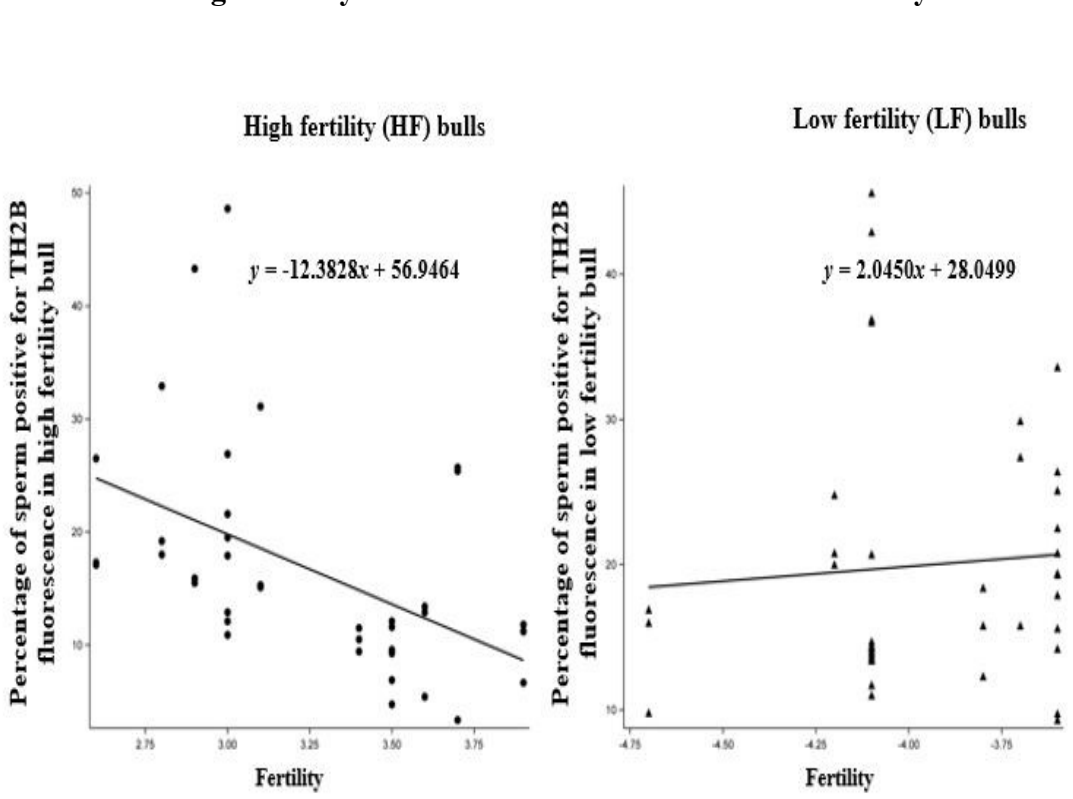

Figure 2 Regression models showing in the percentage of sperm positive for TH2B fluorescence (TH2B-intensity) as related to fertility scores in bulls

The regression line was determined using the model predicted intensity (which was determined as percentage of sperm positive for TH2B fluorescence) values for each value of fertility score using the mixed effects model. A scatter plot of unadjusted data points was superimposed on the regression line plot. Regression equations are shown for high fertility (y = -12.3828x + 56.9464) and low fertility (y = 2.0450x + 28.0499) bulls.

Cellular localization of TH2B in bull sperm using immunocytochemistry

We determined the cellular localization of TH2B in sperm of low *versus* high fertile bulls using immunocytochemistry and confocal microscopy.TH2B signal was

detectable in sperm head, concentrated over the equatorial and subacrosomal area of sperm. The signal was visualized as a band just beneath the acrosome, with a clear space between the base of the band and the proximal end of tail of sperm (Fig. 3).

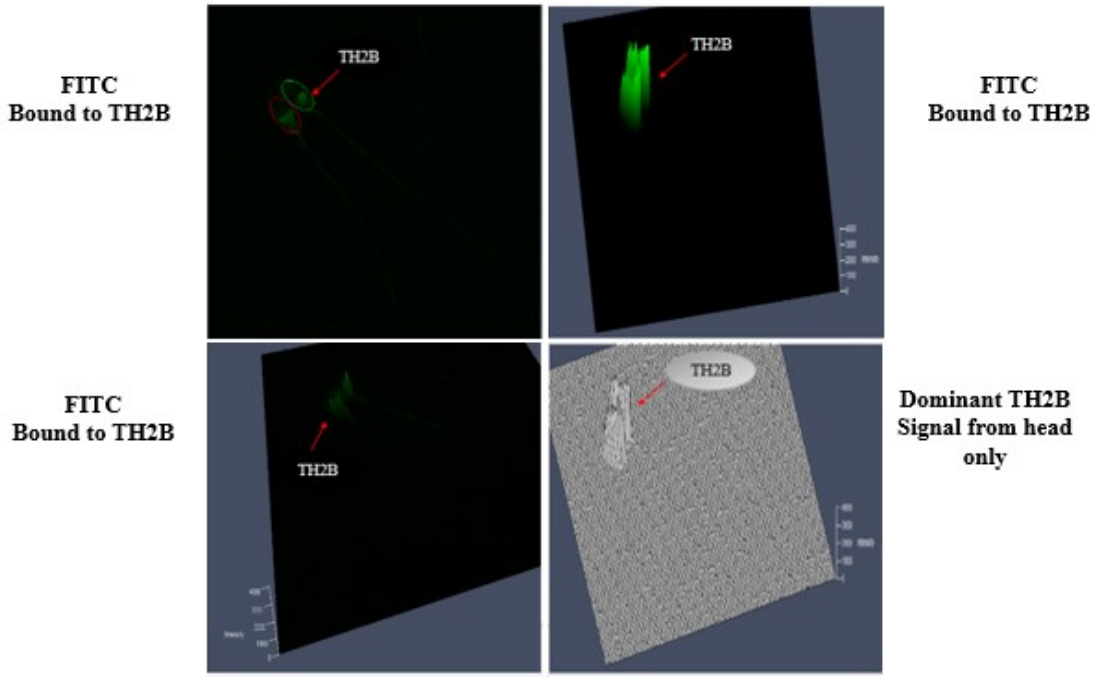

Figure 3 Cellular localization of TH2B in bull spermatozoa

The intensity of FITC bound to TH2B protein. Dominant signal of TH2B coming from sperm head, depict merged images of two sperm using confocal microscope, showing expression dynamics and localization of TH2B in head of bovine spermatozoa.

The immunocytochemistry signal for TH2B was brighter in sperm of the low fertile than that of the high fertile bulls (Fig. 4 and Fig. 5; Table. 2). As evaluated by regression analysis, association between TH2B and bull fertility was not different. The fact that the signals measured by immunocytochemistry were obtained from 45 spermatozoa per bull could be the reason for the non-significance.

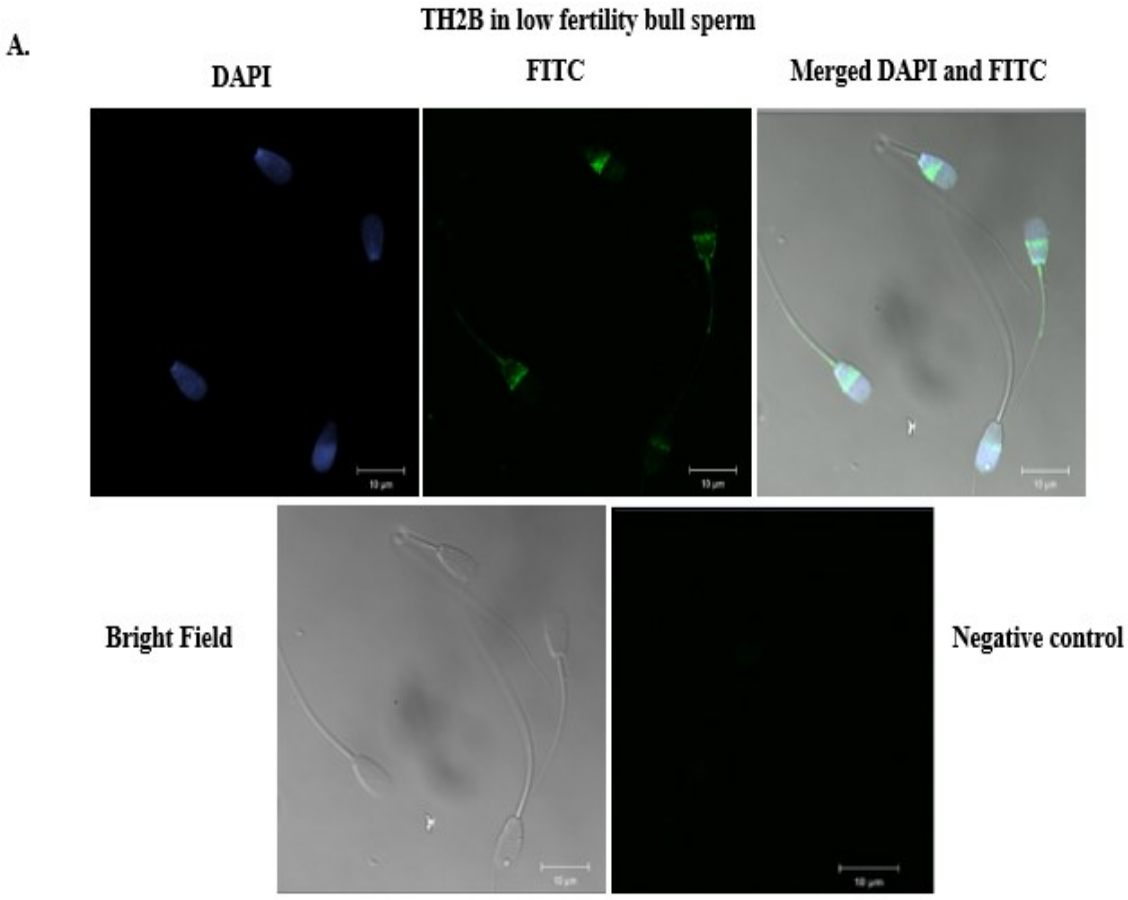

Figure 4 Immunostaining of TH2B in low and high fertility bull spermatozoa

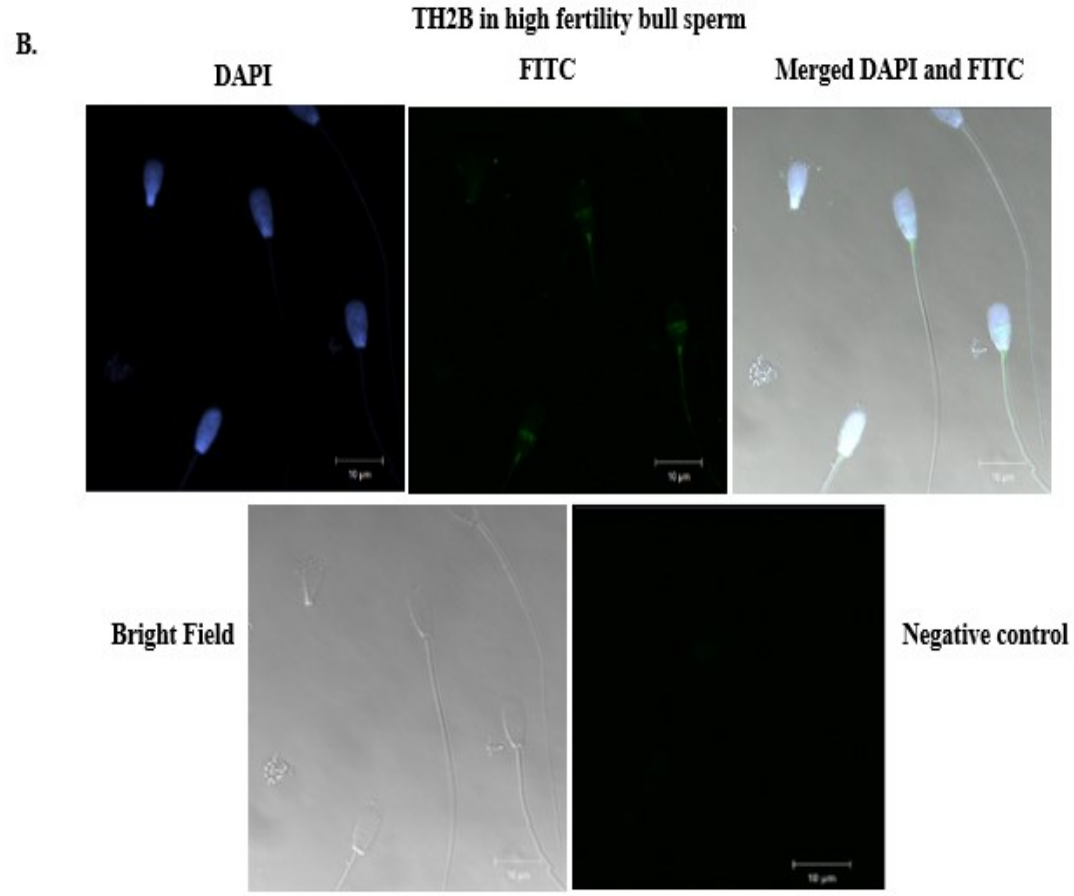

Figure 5 Immunostaining of TH2B in low and high fertility bull spermatozoa

Intensity of TH2B in sperm from low (A) and high fertile bulls (B) using confocal microscopy. DNA stained with DAPI (blue) in a bull sperm; TH2B histone linked to FITC-conjugated secondary antibody (green) in bull sperm; merged images of DAPI and FITC; bright field images; and negative controls.

Expression of TH2B in bull sperm using Western blotting

An immunoblotting approach was used to detect the expression of TH2B in the sperm from low *versus.* high fertility bulls. TH2B protein band intensity was analyzed using ImageJ (Schneider et al., 2012). Our results showed that the TH2B was consistently detectable in sperm from all of bulls (Fig. 6).

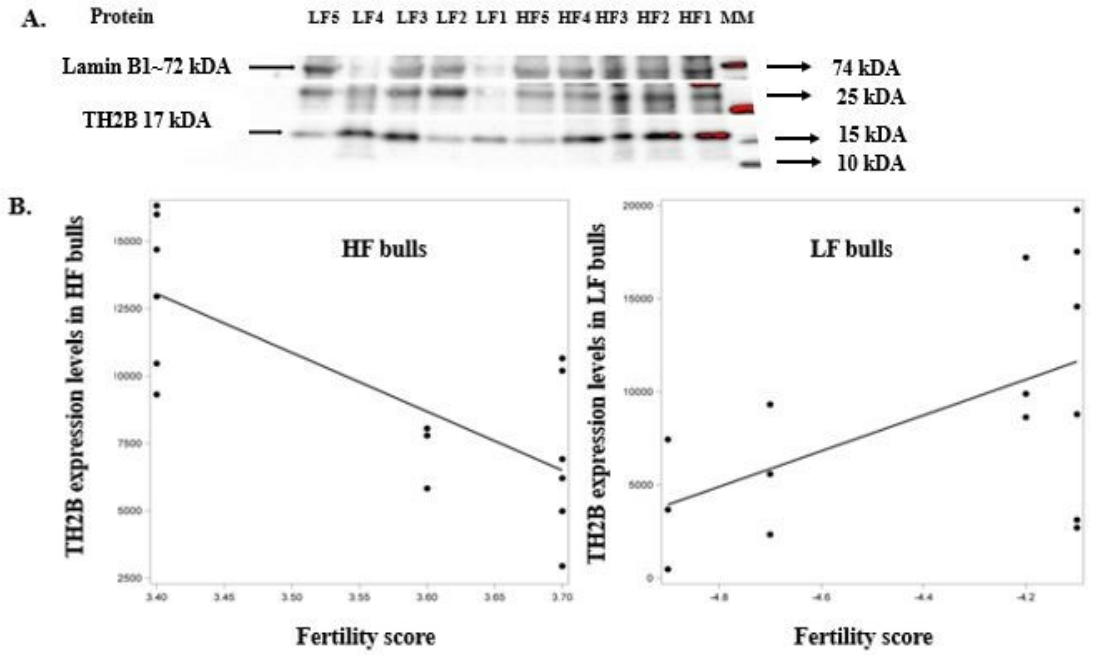

Figure 6 Detection of TH2B protein using Western blotting and regression models showing variation in the expression of TH2B in sperm as related to fertility scores in Holstein bulls

(A) MM stands for molecular markers; LF1-5 and HF1-5 refer to samples from low versus. high fertility bulls, respectively. The same amount of nuclear proteins were loaded into each lane. As TH2B is a nuclear protein and the expression levels of our internal control (Lamin B1) were not stable, another protein band (~ 25 KDa) served as the internal control. (B) The regression line was determined using the model predicted intensity values for each fertility score using the mixed effects model. A scatter plot of unadjusted data points was superimposed on the regression line plot. Regression equations are shown for high fertility (y = -22315x + 89049) and low fertility (y = 9603x + 50993) bulls.

The expression levels of TH2B were significantly affected by an interaction between fertility score and fertility group (P= 0.0058). In high fertility bulls, as fertility decreased, TH2B expression increased (P= 0.0182; y = -22315x + 89049) signifying that the bulls with the highest fertility had the least TH2B retention in their spermatozoa. In

low fertility bulls, as fertility decreased, TH2B expression decreased (P= 0.0737; $y = 9603x + 50993$; Fig. 6) indicating that the bulls with the lowest fertility had the highest of retained TH2B in their spermatozoa.

Bioinformatic analyses of TH2B and H2B

Histone variant of TH2B was identified as the testis specific H2B (Shires et al., 1976; Trostle-Weige et al., 1982). TH2B is encoded by the gene *HIST1H2B* (Marzluff et al., 2002). Mouse *TH2B* gene as well as TH2B protein have been sequenced (Shinagawa et al., 2014), and a predicted sequence of bovine *TH2B* gene *HIST1H2BA* (http://www.ncbi.nlm.nih.gov/nuccore/XM_010825421.2) has been used in our study, because bovine *TH2B* gene as well as TH2B protein have not been sequenced yet. The predicted gene *HIST1H2BA (TH2B)* had 85.7% similarity with known bovine *H2B,* using multiple sequence alignment method Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/) (Sievers et al., 2011). Comparisons among predicted TH2B protein sequences in bovine and human revealed higher similarity patterns in the form of diagonal lines (Fig. 7).

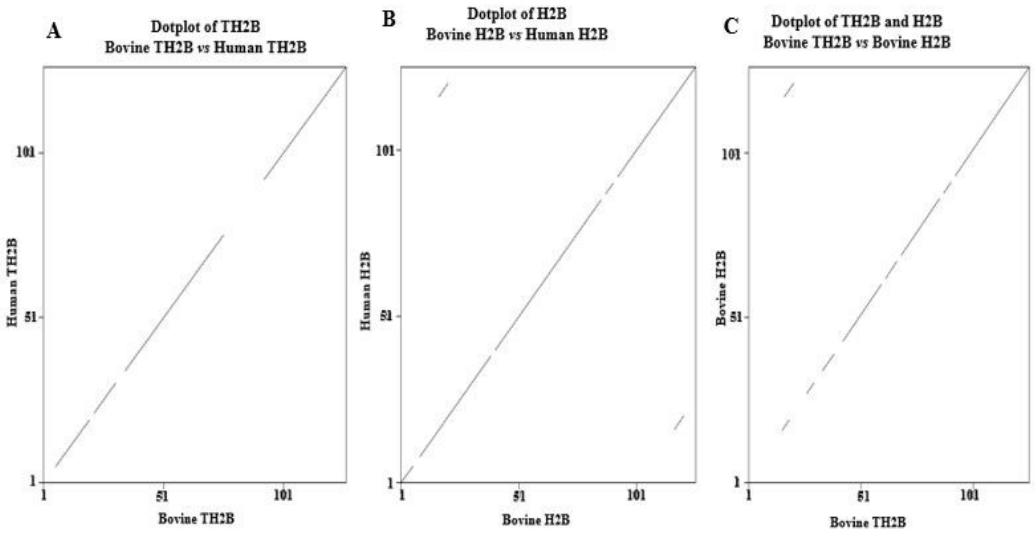

Figure 7 Threshold dotplot of TH2B and H2B amino acids between bovine and human

The emboss dotmatcher of predicted TH2B of bull versus TH2B of human is a perfect diagonal line with two frame shift mutation sites (A). The emboss dotmatcher of H2B of bull versus. H2B of human revealed a low complexity region between bovine H2B and human H2B (B). Dotmatcher of TH2B of bull versus. H2B of bull showed two areas of different amino acids (C).

The comparison of predicted bovine TH2B sequence with human TH2B showed that the match was a perfect diagonal line with three frame shift mutation sites, which might have resulted in change in sequence of amino acids (A). Similarly, comparison between bovine H2B and human H2B showed the match was a perfect diagonal line having four frame shift mutation sites, resulted change in sequence of amino acids (B). The sequence comparison of bovine TH2B with bovine H2B showed two areas of different amino acids, due to frame shift mutations resulting in different amino acid sequences (C) (Zimin et al., 2009).

Interactions among sperm nuclear proteins from bovine, human and mouse

We used the STRING database to depict the interactions of sperm proteins in human, mice, and bovines respectively. Analyses of the interactions of nuclear proteins of developed using STRING for human, mouse, and bovine (Fig. 8) showed the missing link as TH2B; which functions in mediating the transition of nuclear proteins from histone to protamine in bovine spermatozoa. In humans and mice, TH2B is known to interact with chromatin remodelers (BRD4 and CHD5) and is involved in replacement of histones with protamines but function(s) of bovine TH2B has yet to be discovered. However, we found the high prediction of likeliness that bovine TH2B is also involved in eviction of retained histones and replacement of those with highly basic protamines, while assisted by two chromatin remodelers BRD4 and CHD5 (Fig. 8).

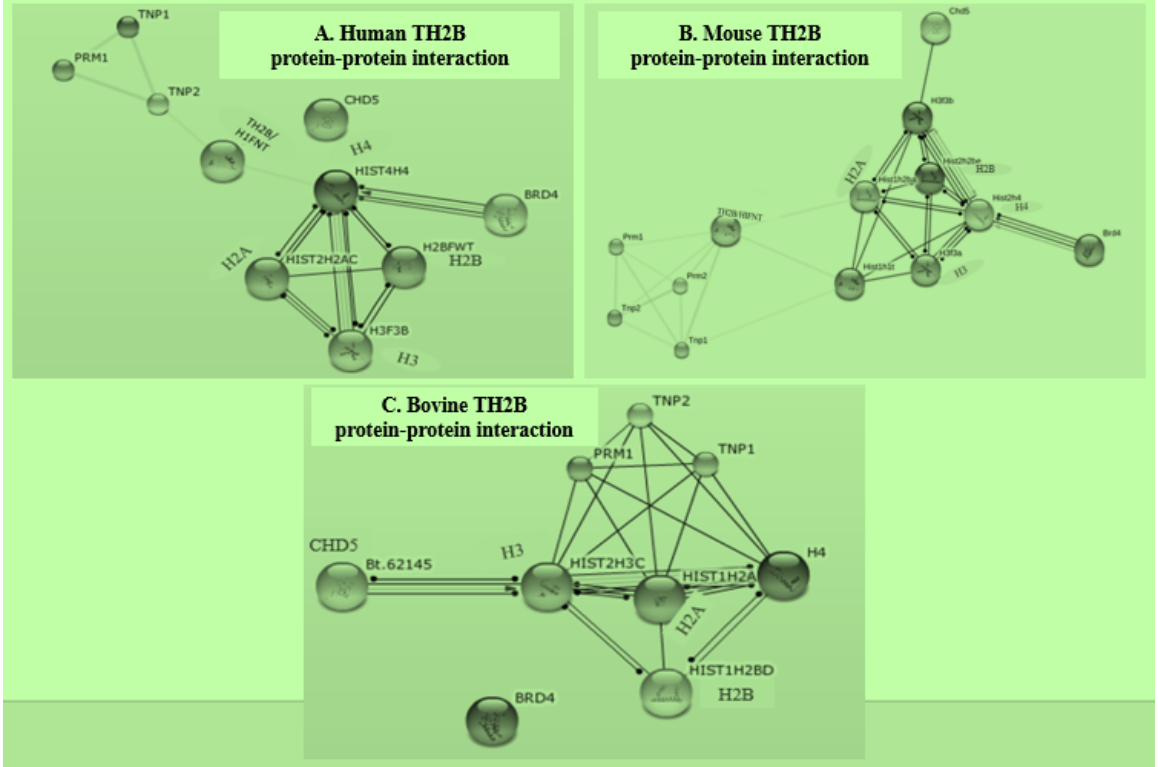

Figure 8 Protein-protein interactions involving TH2B

TH2B/H1FNT involved in replacement of TNPs by protamines in human and mouse are depicted in (A; B) and the missing link with possibly same role in bovines (C)

Interactome of testis specific histone 2B (TH2B)

The interactome networking of bovine TH2B with nuclear proteins (H2B, H2A, H3, H4 TNP1, TNP2, PRM1, BRD4 and CHD5) showed the significant gene ontology overexpression terms ($P < 0.05$; Table. 5).

Table 5 Gene Ontology (GO) overexpression terms for *TH2B* in bull.

GO-ID	Description	E-value	P-value
48232	Male gamete generation	1.16E-05	<0.0001
51276	Chromosome Organization	7.92E-07	<0.0001
7283	Spermatogenesis	1.16E-05	<0.0001
7276	Gamete generation	2.21E-05	<0.0001
6323	DNA packaging	2.43E-05	<0.0001
71103	DNA conformation change	2.43E-05	<0.0001
6333	Chromatin assembly or disassembly	2.43E-05	<0.0001
6325	Chromatin organization	1.35E-04	<0.0001
22414	Reproductive process	1.35E-04	<0.0001
30261	Chromatin condensation	1.59E-04	<0.0001
34728	Nucleosome organization	6.08E-04	<0.0001
31498	Chromatin disassembly	4.12E-03	0.00412
7290	Spermatid nucleus elongation	4.12E-03	0.00412
6337	Nucleosome assembly	4.12E-03	0.00412
32986	Protein-DNA complex disassembly	4.12E-03	0.00412
12	Single strand break repair	1.36E-02	0.0136
7289	Spermatid nucleus differentiation	1.36E-02	0.0136
6334	Nucleosome assembly	1.39E-02	0.0139

Table 5 (Continued)

31497	Chromatin assembly	1.39E-02	0.0139
30317	Sperm motility	2.13E-02	0.0213
6338	Chromatin remodeling	2.78E-02	0.0278
6342	Chromatin silencing	2.78E-02	0.0278
6997	Nucleus organization	2.88E-02	0.0288
7286	Spermatid development	3.50E-02	0.0350
48515	Spermatid differentiation	3.63E-02	0.0363

The interactome of *TH2B* with *H2B, H2A, H3, H4 TNP1, TNP2, PRM1, BRD4* and *CHD5* was generated using BiNGO of Cytoscape 3.3.

The interactome showed that TH2B is involved in male gamete generation, chromosome organization, spermatogenesis, gamete generation, DNA packaging, DNA conformation change, chromatin organization, reproductive process, nucleosome organization, chromatin disassembly, spermatid nucleus elongation, nucleosome assembly, protein-DNA complex disassembly, spermatid nucleus differentiation, sperm motility, chromatin organization, chromatin condensation, single strand break repair, chromatin assembly, chromatin silencing, nucleus organization, spermatid development, spermatid differentiation, and chromatin remodeling (Fig. 9).

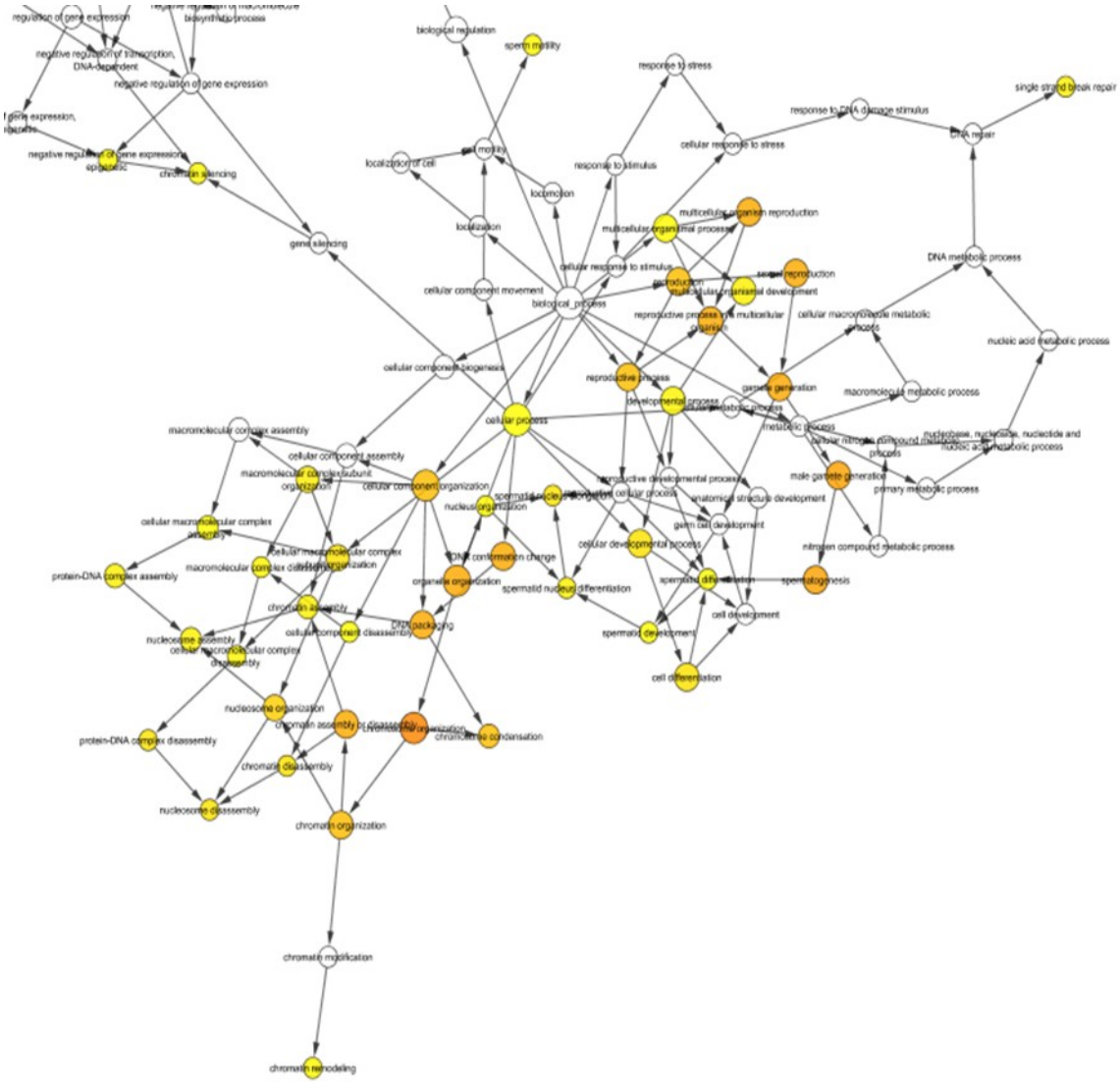

Figure 9 The interactome of TH2B protein with chromatin remodeling proteins.

Cytoscape was used in conjunction with large protein- protein databases, understanding protein-DNA and genetic interactions. The circles represent the genes, links represent the interactions, edges depict protein-protein interactions. The bigger circle indicates the significance of expression of that gene for the given process involved. The colors represent the involvement in same function or process

Discussion

We conducted the present study to test the hypothesis that different cellular intensities, expression levels, and localization of TH2B are associated with sperm

chromatin dynamics and bull fertility. To test such hypothesis, we used immunocytochemistry, Western blotting, flow cytometry and bioinformatic approaches.

Our study presents evidence that bovine TH2B was localized in the sperm head. The intensity of TH2B in sperm from high fertility bulls decreased as fertility increased ($P = 0.018$). The expression levels of TH2B decreased in sperm from high fertility bulls as fertility score increased ($P = 0.0055$) and increased in low fertility bulls but was not significant ($P= 0.0737$). Bovine TH2B and H2B sequences were highly similar (85.71%), as revealed by percent identity matrix (PIM) score. The H2B Emboss dotmatcher showed that the predicted bovine TH2B sequence is similar to TH2B of human, which has few possible frame shift mutation sites. Bovine H2B and human H2B have a remarkably perfect diagonal line match, revealing that H2B is highly conserved. STRING database (Szklarczyk et al., 2014)) was employed to provide a critical assessment, specific protein-protein interactions of bovine TH2B and sperm nuclear proteins, and comparisons with human and mouse proteins.

Since TH2B plays an important role in eviction of canonical histones and replacement of TNPs with protamines in the mouse (Montellier et al., 2013), disruption of *th2b* gene in the mouse resulted in absence of TH2B protein, causing sterility in mouse (Shinagawa et al., 2014). However, it was unclear to assign which testis specific histone is responsible for this paradigm shift. Our results suggest that, in the bovine, such histone is TH2B. The interactome of TH2B revealed that TH2B influences male gamete generation, chromosome organization, spermatogenesis, gamete generation, DNA packaging, DNA conformation change, chromatin organization, reproductive process, nucleosome organization, chromatin disassembly, spermatid nucleus elongation,

nucleosome assembly, protein-DNA complex disassembly, spermatid nucleus differentiation, sperm motility, chromatin organization, chromatin condensation, single strand break repair, chromatin assembly, chromatin silencing, nucleus organization, spermatid development, spermatid differentiation, and chromatin remodeling with significant GO terms. All of these functions had not been known for bovine TH2B and hence we generated new knowledge on understanding chromatin dynamics and functions regulated by TH2B.

Our results shed light on the role of TH2B in shaping bovine sperm chromatin and interacting with chromatin remodeling proteins BRD4 and CHD5. The intensity as well as expression levels of TH2B were significantly lower in sperm from high fertility group, paving the way for increased incorporation of protamines into the matured sperm chromatin resulting into increased number of healthy sperm. However, this trend was completely reversed in low fertility group; higher TH2B retention resulting in less protamine incorporation into chromatin suggesting loosening of sperm chromatin and higher numbers of immature sperm. Another possibility is that eviction of the canonical histones by TH2B might be hindering the protamine incorporation to a certain level because of abnormal retention TH2B. This is consistent with the findings of Dogan *et al.* (2015) [27], who showed that sperm from high fertile bulls have higher protamine levels than those from low fertile bulls resulted in less amounts of TH2B in HF and increased retention of TH2B in LF bulls.

The TH2B fluorescence signal was faint in the sperm tail, probably because TH2B was not involved with sperm chromatin (de Oliveira et al., 2013). In this study, we used predicted gene and protein sequences for bovine TH2B

(http://www.ncbi.nlm.nih.gov/nuccore/XM_010825421.2) because the actual bovine sequences have not yet been reported to NCBI. However, an 85% homology between human sperm TH2B and somatic H2B has been clearly demonstrated through laboratory experiments (Zalensky et al., 2002). Moreover, the homology between predicted bovine TH2B and H2B used in our computational analysis was 85.7%, signifying the similar proportion of changes in H2B to TH2B and possibly similar kind of roles in bovines.

In the bull, sperm chromatin dynamics was thought to be controlled by replacement of histones by protamines. This is a multi-step process involving chromatin remodelers BRD4, CHD5 and TH2B with a scope of many additional nuclear chromatin associated proteins. Therefore, the mechanisms of histone to protamine replacement are multi-cascade reactions, in which different chromatin remodelers such as BRD and CHD family proteins are involved in loosening the DNA nuclear protein binding in sperm chromatin. The results presented in our study advance the understanding of the role of TH2B in remodeling sperm chromatin dynamics.

Conclusions

Presence of TH2B variant in bull sperm initiates replacement of histones by TNPs and then by protamines, all of which are in accordance with the prior findings about the function of TH2B in the mouse (Montellier et al., 2013; Shinagawa et al., 2014; Shinagawa et al., 2015). In theory, the role of TH2B is in facilitating the histone to protamine transformation in bovine. Recently, researchers have shown that chromatin remodelers BRD4 and CHD5 regulate histone to protamine transition in sperm chromatin in the mouse (Montellier et al., 2013; Shinagawa et al., 2015). Our study about bovine functional genome also suggests that the chromatin compaction and eviction of histones

is dependent on all three proteins together, BRD4, CHD5 and TH2B, where TH2B may be involved in catalyzing that transformation. We analyzed TH2B networks in the chromatin of bovine sperm to depict the cellular location of TH2B variant, and to detect the TH2B protein. The results are significant because they help us better understand the mechanisms of chromatin compaction in bovine sperm and can be applicable in reproductive biotechnology in mammals including humans.

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CHAPTER III

ACETYLATION AND METHYLATION OF SPERM HISTONE 3 LYSINE 27 (H3K27AC AND H3K27ME3) ARE ASSOCIATED WITH BULL FERTILITY

Abstract

Epigenetic modifications in histones are crucial for proper sperm physiology, egg activation and reproductive development of males. The objectives of this study were to determine the conservation and interactomes of histone three (H3) and ascertain the expression levels of acetylated and methylated H3 lysine 27 (H3K27ac and H3K27me3) in sperm from Holstein bulls with different fertility. Methods in immunocytochemistry and flow cytometry were used to evaluate the expression dynamics of H3K27ac and H3K27me3 in sperm from 10 bulls with different *in vivo* fertility. Computational biology methods including Clustal Omega and Cytoscape were performed to determine the evolutionary conservation and interactome of H3. The posttranslational modifications (PTM) of H3 (H3K27ac and H3K27me3) had different localizations in the sperm head. Intensities of methylation were higher than those of acetylation and inversely correlated between the two fertility groups ($p = 0.0032$). The interacting proteins of H3 are involved in critical subcellular processes such as regulation of methylation, nucleosome assembly, regulation of DNA replication, and chromatin assembly. In conclusion, the present results are significant because they help advance fundamental science and biotechnology of mammalian reproduction using the bull which is a suitable model for the study of

mammalian reproduction and development due to its close similarities in genetics and physiology to other mammals and humans.

Introduction

The ability of sperm to fertilize and activate the egg and to support embryonic development is critical for bovine reproduction, development and cattle production. During spermiogensis, most of the histone nuclear proteins are replaced with protamines, thus causing much greater chromatin condensation (Balhorn, 2007; Ward and Coffey, 1991). This process is crucial because inadequate histone replacement correlates with bull subfertility (Dogan et al., 2015). The remaining histones are not randomly dispersed as would be expected from inefficient replacement, but they are instead enriched at loci important for embryonic development (Arpanahi et al., 2009; Brunner et al., 2014; Hammoud et al., 2009). In addition, histone retention in sperm occurs over repeat elements such as long and short interspersed nuclear elements (LINE and SINE) (Carone et al., 2014; Pittoggi et al., 1999; Samans et al., 2014), pericentric repeats (Carone et al., 2014; Govin et al., 2007; Samans et al., 2014) , and at sperm chromocenter (Carone et al., 2014; Govin et al., 2007; Samans et al., 2014; Van der Heijden et al., 2006).

Retained histones in sperm undergo PTMs that regulate gene expression in a spatiotemporal manner (Kouzarides, 2007). Further, these histone modifications act as epigenetic tags on paternal genes and are passed to the offspring (Guerrero-Bosagna and Skinner, 2014). The influence of the PTMs in sperm chromatin has a significant impact on correct chromatin conformation by which stability of histone octamer as well as interaction between histones and DNA are attained (Bao et al., 2013; Bao and Bedford, 2016; Bedford and Richard, 2005). Therefore, these distinct and localized PTMs of

histone in conjunction with chromatin remodelers bromodomain containing protein 4 (BRD4; (Bryant et al., 2015), chromodomain helicase DNA binding protein 5 (CHD5; (Zhuang et al., 2014), testis specific histone 2B (TH2B;(Montellier et al., 2013; Shinagawa et al., 2015) as well as other effector modelers, play important roles in sperm chromatin remodeling, eviction of histones, and proper histone-protamine replacement. Additionally, it has been observed that increased H3 methylation in mature human sperm relates to poor sperm motility and functional quality (La Spina et al., 2014).

Proposed by Jenuwein and Allis in 2001, the "histone code" hypothesis states that histones and their posttranslational modifications regulate epigenetic information (Jenuwein and Allis, 2001). Since then, much has been discovered about the epigenetic function of histones and their associations with male fertility. Chow *et al*. found that histone 3.3, a histone variant that differs from canonical histone 3.1 by five amino acids, is enriched at the promoters of active genes (Chow et al., 2005). Histones regulate gene expression via PTMs such as acetylation and methylation. Acetylation of histones are highly conserved across mammals (Grimes and Henderson, 1984; Meistrich et al., 1992; Oliva and Mezquita, 1982). Acetylation of lysine is known to affect chromatin by decreasing the positive charge of histones that causes a weak histone to negatively charged DNA interaction resulting in increased chromatin fluidity (Bao and Bedford, 2016). Methyltransferases catalyze methylation of lysine in the histone (Rivera et al., 2014). These posttranslational modifications of the core histones can also affect the male genome's accessibility to maternal transcription factors during embryo development (Miller et al., 2010). Recently, Verma *et al*. (Verma et al., 2015) demonstrated that

trimethylated histone 3 lysine 27 (H3K27me3) differed at various genomic loci important for sperm function and embryonic development in *Bubalus bubalis*.

Noncompensable infertility, a class of low fertility status that is not improved by increasing the number of sperm, is likely caused by genetic and molecular defects in the sperm (Blaschek et al., 2011). Bovine sperm share many physiological and genetic similarities with that of other mammals, and reliable fertility phenotypic data are available for bulls. Also, there are interactions among the expression dynamics of the post-translationally modified sperm H3, its evolutionary conservation as well as the biological networks. It is known that acetylated and methylated H3K27 affect gene expression through activation (Heintzman et al., 2009; Pradeepa, 2017; Tie et al., 2009) and repression (Filion et al., 2010; Kharchenko et al., 2011; Li et al., 2014; Tie et al., 2009), respectively. According to Hammoud et al (2009), mouse spermatozoa carry histones and their posttranslational modifications (PTM) which are enriched at loci with developmental importance including clusters of imprinted genes, miRNAs, HOX genes, and the promoters of stand-alone developmental transcription and signaling factors. We intended to compare the PTM of histone 3 lysine 27 in sperm from bulls with different fertility to ascertain to what extent the levels of histone 3 acetylation/methylation are associated with bull fertility. Thus, increased knowledge about H3 may provide solutions for male noncompensable infertility. In addition, because fertility is critical for cattle agriculture, molecular markers and mechanisms can be used to improve efficiency of cattle reproduction (Sagirkaya et al., 2006). The objectives of this study were to determine the conservation and interactomes of H3 and ascertain the expression

dynamics of acetylated and methylated H3K27 in sperm from Holstein bulls with different fertility.

Materials and methods

Experimental design

Cryopreserved semen straws from five high fertility (HF) and five low fertility (LF) Holstein bulls were used (Table. 6).

Table 6 Fertility phenotypes of Holstein bulls whose sperm were used for flow cytometry analysis.

Bull number	Number of breeding's		*In vivo* fertility score	
High fertility (HF) bulls				
1	737		3.5	
2	965		3.0	
3	682		2.9	
4	3647		2.8	
5	6051		2.6	
Low fertility (LF) bulls		**P= 0.2001**		**P< 0.0001**
6	758		-4.1	
7	862		-4.1	
8	892		-3.8	
9	1105		-3.7	
10	1020		-3.6	

Bulls 1–5 are designated as the high HF group and bulls 6-10 are designated as the LF group. All bulls were individually represented with their *in vivo* fertility scores ($p < 0.0001$) and the number of breeding's ($p = 0.2001$). Fertility scores are expressed as the

percent deviation of each conception rate from the average conception rate of all the bulls as previously described de Oliveira *et al*. (2013).

Determination of bull fertility was described previously (Peddinti et al., 2008), and the fertility phenotypes were distributed into HF and LF groups (de Oliveira et al., 2013). Although the number of breedings between the HF and LF bulls was not significant ($p = 0.2001$), fertility scores between HF and LF bulls were different ($p < 0.0001$) (Table. 5), although semen parameters between groups were not different. Immunocytochemistry and flow cytometry experiments were performed using sperm samples from 10 bulls to determine the expression dynamics of H3K27ac and H3K27me3 as related to bull fertility. Computational biology methods and software, including Clustal Omega (Goujon et al., 2010; Sievers et al., 2011) and Ctyoscape (Shannon et al., 2003), were applied to determine the conservation and interactome of bovine histone H3.

Immunolocalization of H3K27ac and H3K27me3 in bull sperm

Immunocytochemistry was performed to ascertain the localization of acetylated and trimethylated histone 3 lysine 27 (H3K27) in bovine sperm. First, cover slips were cleaned using 70% ethanol, then air-dried and kept at 37°C for 30 minutes (min). Five hundred (500) µl of 0.1% Poly-L-Lysine solution was spread on one side of each cover slip, which were then incubated at 37°C for 10 min. Excess Poly-L-Lysine solution was removed and the cover slips were allowed to dry at 37°C for 60 min.

Bull semen straws were removed from liquid nitrogen and thawed at 37°C for 30 seconds (sec). The samples were transferred into 1 mL of PBS (pH 7.4) supplemented with protease inhibitors and 10 mM EDTA (pH 8.0) in a sterile centrifuge tube, then centrifuged at 2,000 *g* at RT for five min. Sperm pellets were resuspended in 1 mL of 20

mM ChAPS in PBS and incubated at RT for 20 min. Samples were then centrifuged at 2000 *g* at RT for five minutes and pellets were resuspended in 1 mL of PBS and mixed by pipetting. Next, 50 µL of sperm suspension was incubated with 450 µL of decondensation solution (10 mM DTT and 1 mg/ml of heparin) at RT for 30 min. Five hundred µl of sperm suspension was uniformly distributed over at least two coverslips and sperm was allowed to settle at RT for 10 minutes. The solution was then decanted from the coverslips and cells were fixed with 200 µL of 4% formaldehyde in PBS at 4°C for 10 min. Excess of fixation solution was then removed. In order to permeabilize the cells, the coverslips were overlaid with 1 mL of 0.2% Triton X-100, 0.1% bovine serum albumin in PBS at RT for 15 min on a shaker machine.

The cover slips were dehydrated with 200 µL of 50% ethanol for one min and this procedure was repeated with 70% ethanol, 95% ethanol and 100% ethanol. The cover slips were then allocated in small petri dishes with the lysine coat face up. Next, each coverslip was immersed in 1 mL of 100% methanol and incubated at -20°C for 20 min, and the excess solution was removed. The coverslips were then washed three times with 1 mL washing buffer (PBS + 0.1% Triton X) for 1 min each, overlaid with 1 mL of blocking buffer (1% BSA + 0.1 % Triton X-100 in PBS) for 60 min and placed on a shaker machine. Once the blocking buffer was removed, slips were incubated with primary antibody overnight at 4°C. Primary antibodies were mouse monoclonal anti trimethylated H3K27 (H3K27me3) (Abcam, Cambridge, MA, USA; catalog # 6002; 1/200 dilution) and rabbit polyclonal anti acetylated H3K27 (H3K27ac) (Abcam, Cambridge, MA, USA; catalog # 4729; 1/200 dilution). The next day, slides were washed twice in 1 mL of washing buffer for 5 min each and coverslips were probed with

secondary antibody of goat anti-mouse IgG H&L (DyLight® 650) against H3K27me3 (Abcam, Cambridge, MA, USA; catalog # 96874; 1/5,000 dilution) and donkey anti-rabbit IgG-FITC against H3K27ac (Santa Cruz, Dallas, Texas, USA; catalog # 2090; 1/5,000 dilution) and at room temperature (RT) for 1 hour (h). Coverslips were then overlaid with 500 µl of DAPI solution (2.5 µg/mL) for 10 min at RT. Excess DAPI solution was removed and slides were washed with 500 µL PBS. One drop of mounting medium (VECTAshield, H-1000) was placed on each coverslip with the coated side face down, and all four sides of the coverslip were sealed with clear nail polish. Once the nail polish was dry (10-15 min, RT), samples were stored and protected from light until they were analyzed using a confocal fluorescence microscope (Zeiss LSM 510) under 40X and 63X magnifications using immersion oil.

Evaluation of H3K27ac *versus* H3K27me3 in bull sperm by flow cytometry

Flow cytometry was performed in the present study to quantify the enrichment of acetylated and trimethylated H3K27 in bovine sperm. Semen straws from 10 Holstein bulls, five high fertility (HF) and five low fertility (LF), were removed from liquid nitrogen and thawed at 37°C for 30 sec. The extenders were then separated from the samples by centrifuging at 1000 *g* at 4°C for 5 min and pellets were washed twice in PBS with 0.1% Bovine Serum Albumin (BSA) and again centrifuged at 1000 *g* at 4°C for 5 min. The pellets were then fixed in 1 mL of 4% formaldehyde at RT for 1 hour in separate centrifuge tubes. The samples were then centrifuged at 5000 *g* at 4°C for 5 minutes and pellets were resuspended in 250 µL of PBS and immediately permeabilized in 250 µL of 0.1% Triton X-100 in 0.1% sodium citrate in PBS on ice for 2 min. The pellets were resuspended in 500 µL of PBS, filtered through a flow cytometric tube using

a cell strainer cap (Becton Dickinson Labware; catalogue no. 352235), and then incubated with the primary antibodies at 4°C overnight. The primary antibodies used were mouse monoclonal anti trimethylated H3K27 (H3K27me3) (Abcam, Cambridge, MA, USA; catalog # 6002; 1/200 dilution) and rabbit polyclonal anti acetylated H3K27 (H3K27ac) (Abcam, Cambridge, MA, USA; catalog # 4729; 1/200 dilution). Next day, samples were centrifuged at 3000 *g* at 4°C for 5 min, washed once in 500 µL washing buffer, centrifuged at 3000 *g* at 4°C for 5 minutes and incubated with secondary antibodies at RT for two hours. The secondary antibodies were goat anti-mouse IgG H&L (DyLight® 650) (Abcam, Cambridge, MA, USA; catalog # 96874; 1/200 dilution) and donkey anti-rabbit IgG-FITC (Santa Cruz, Dallas, Texas, USA; catalog # 2090; 1/200 dilution). Following the incubation, the samples were washed twice in PBS with 0.1% BSA (3, 000 *g* at 4°C for 5 min). Sperm samples were then analyzed using the BD-FACSCalibur flow cytometer (BD Bioscience, USA).

Statistical methods employed for flow cytometric data

Mean values of H3K27me3, H3K27ac, and H3K27me3&H3K27ac were determined for each replicate within each repeat for each bull and were used for further analysis. One straw was distributed into two replicates and experiment was repeated three times. The effects of fertility on H3K27me3 and H3K27ac were assessed using mixed model analysis with PROC MIXED with SAS for Windows 9.4 (SAS Institute, Inc., Cary, NC). Group, fertility score and their interactions were included as fixed effects. In addition, separate linear mixed models with fertility score as the fixed effect were fit for both the high and low fertility groups to better understand the relationship between fertility score and both H3K27me and H3K27ac. Separate linear mixed models were also

fit with H3K27me3 or H3K27ac as response variables with group as a fixed effect as well as H3K27ac or H3K27me3, respectively. Another linear mixed model was fit to assess the effect of fertility group on H3K27me3&H3K27ac. In each of the linear mixed models, repeat within bull and bull were included as random effects and the KENWARDROBER denominator degrees of freedom option was used. Furthermore, separate analyses of variance using PROC MIXED were performed to determine to what extent there were differences between fertility groups for fertility score and breeding. Regression line plots were made using PROC SGPLOT. The regression line was determined using the model-predicted intensity values for each value of fertility score using the mixed effects model. Diagnostic plots of residuals for each outcome were assessed to ensure the assumptions of the statistical method had been met. An alpha level of 0.05 was used to determine statistical significance.

Analysis of H3 conservation

H3 amino acid sequences were obtained from the UniProt online database (Apweiler et al., 2004) for eight mammalian species: *Bos taurus, Bos mutus, Canis lupis familiaris, Equus caballus, Homo sapiens, Macaca mulatta, Mus musculus, and Rattus norvegicus*. The sequences were first aligned using Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/) (Goujon et al., 2010; Sievers et al., 2011), and then the percent identity matrixes for the sequences were obtained. In this case, sequences were highly similar for a meaningful phylogenetic tree to be generated. The Conserved Domains Database was used to obtain protein sequence and structure data were chosen to identify protein domains, *i.e*., distinct units of protein structures and

functions (Marchler-Bauer and Bryant, 2004). A CD-Search was performed using bovine H3.1 amino acid sequence. H3's conserved domains were identified.

Analysis of H3 Interactome

Gene ontology data, gene related sequences, and relevant resources were obtained from the NCBI Gene database (Brown et al., 2015). Following a comprehensive search using the bovine H3.1 amino acid sequence, gene ontology data were retrieved to provide more insights into the functions of H3. We employed Cytoscape version 3.4.0 (Shannon et al., 2003) to analyze the interactome of H3. A merged-network was generated in Cytoscape by entering the query keywords "hist1h3a", "hist1h3b", and "hist1h3c" into Ctyoscape's database search bar and importing the results found from the BioGrid and Uniprot databases. Cytoscape's ClusterViz app was then utilized for cluster searching by using its EAGLE algorithm (Wang et al., 2015).

Results

Cellular localization of H3K27me3 and H3K27ac in bull sperm

We determined the cellular localizations of H3K27ac and H3K27me3 in the sperm of both low and high fertile bulls using immunocytochemistry and confocal microscopy. Both H3K27ac and H3K27me3 signal intensities were detectable in the sperm head. While a weak signal of H3K27ac was present all-around the sperm head (Fig. 10), a strong signal was detected for H3K27me3 appearing as a crown around the sperm head (Fig.11).

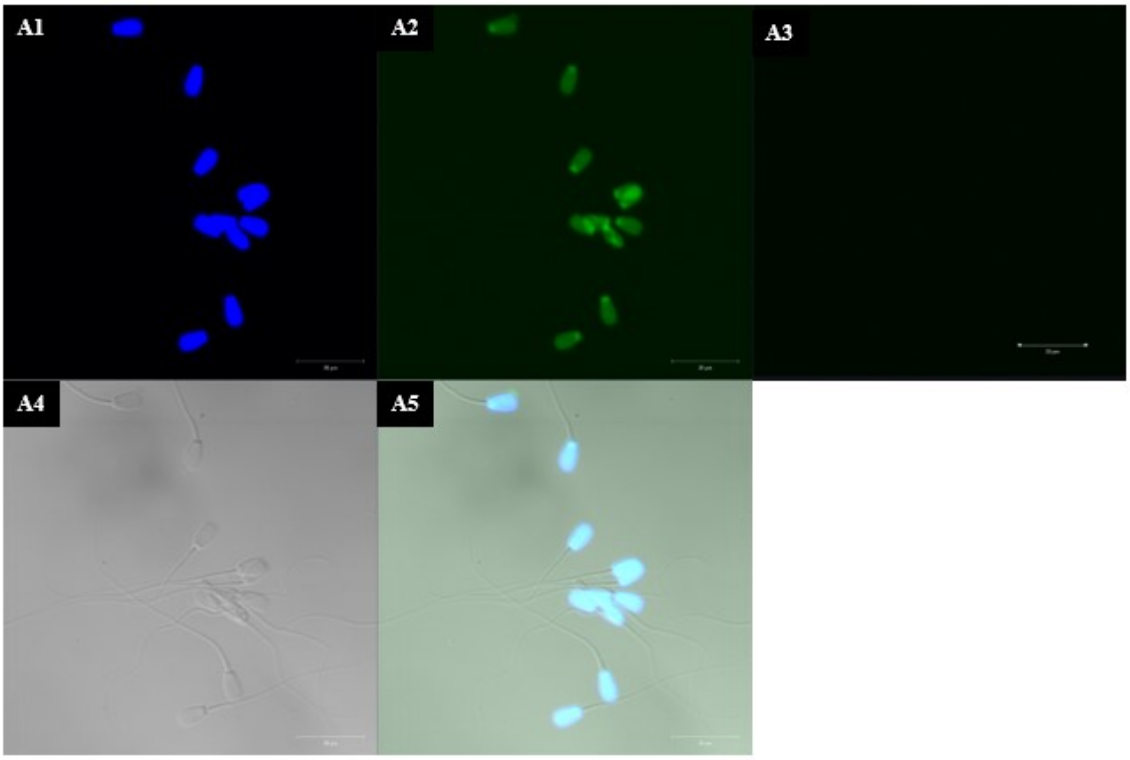


Figure 10 Cellular localization and intensity of H3K27ac in bull sperm.

Immunocytochemistry and confocal microscopy were used to evaluate localizations and intensities of H3K27ac in bull sperm. (A1) DNA stained with DAPI (blue) in a bull sperm. (A2) H3K27ac histone stained with FITC conjugated secondary antibody (green) in bull sperm. (A3) Negative control (which is secondary antibody and DAPI only). (A4) Bright field images. (A5) Merged images of DAPI, bright field and FITC.

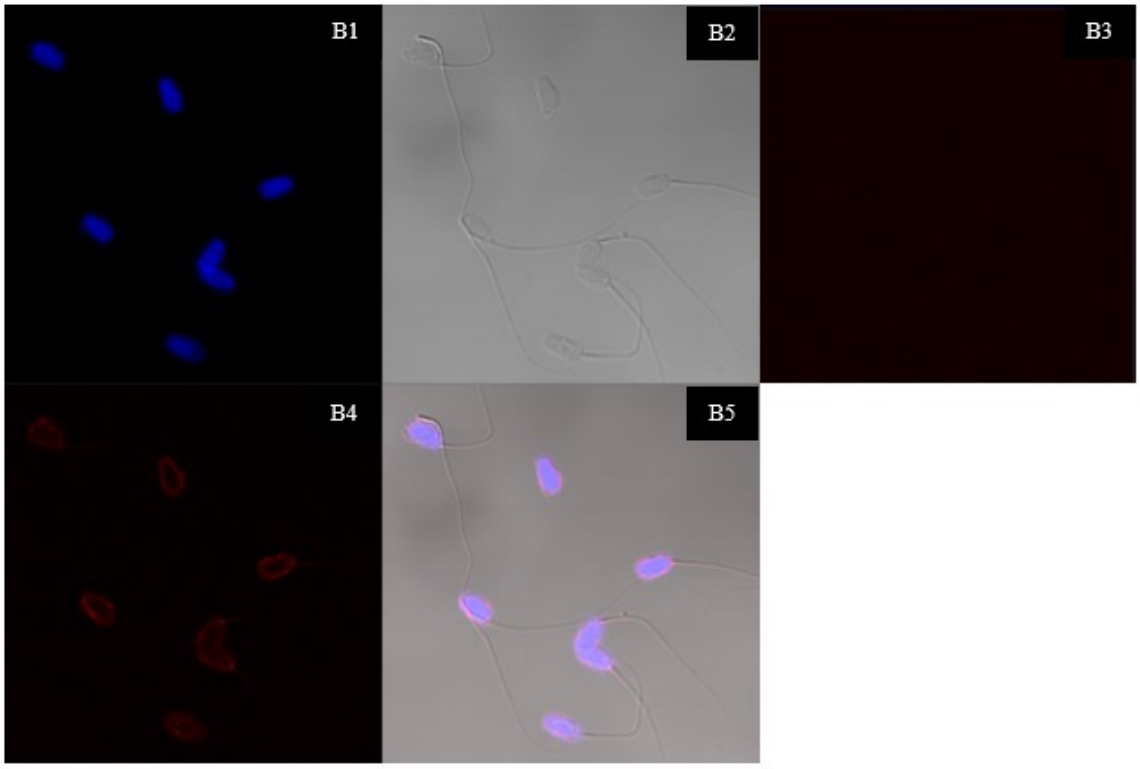


Figure 11 Cellular localization and intensity of H3K27me3 in bull sperm.

Immunocytochemistry and confocal microscopy were employed to illuminate spatiotemporal localizations and intensities of H3K27me3 in bull sperm, as determined by immunocytochemistry and confocal microscopy. (B1) DNA stained with DAPI (blue) in a bull sperm. (B2) Bright field images. (B3) Negative control (which is secondary antibody and DAPI only). (B4) H3K27me3 histone stained with DyLight 550 conjugated secondary antibody (red) in bull sperm. (A5) Merged images of DAPI, bright field and DyLight 550.

Flow cytometric analysis of H3K27ac and H3K27me3 expression in sperm from HF and LF bulls

Total of 150,000 sperm were analyzed by flow cytometry to investigate the expression of methylation of H3 and acetylation of H3 in sperm from five high fertility and five low fertility bulls. Flow cytometric measurements of sperm H3K27ac and H3K27me3 showed different histogram profiles between HF and LF bulls (Fig. 12A-E).

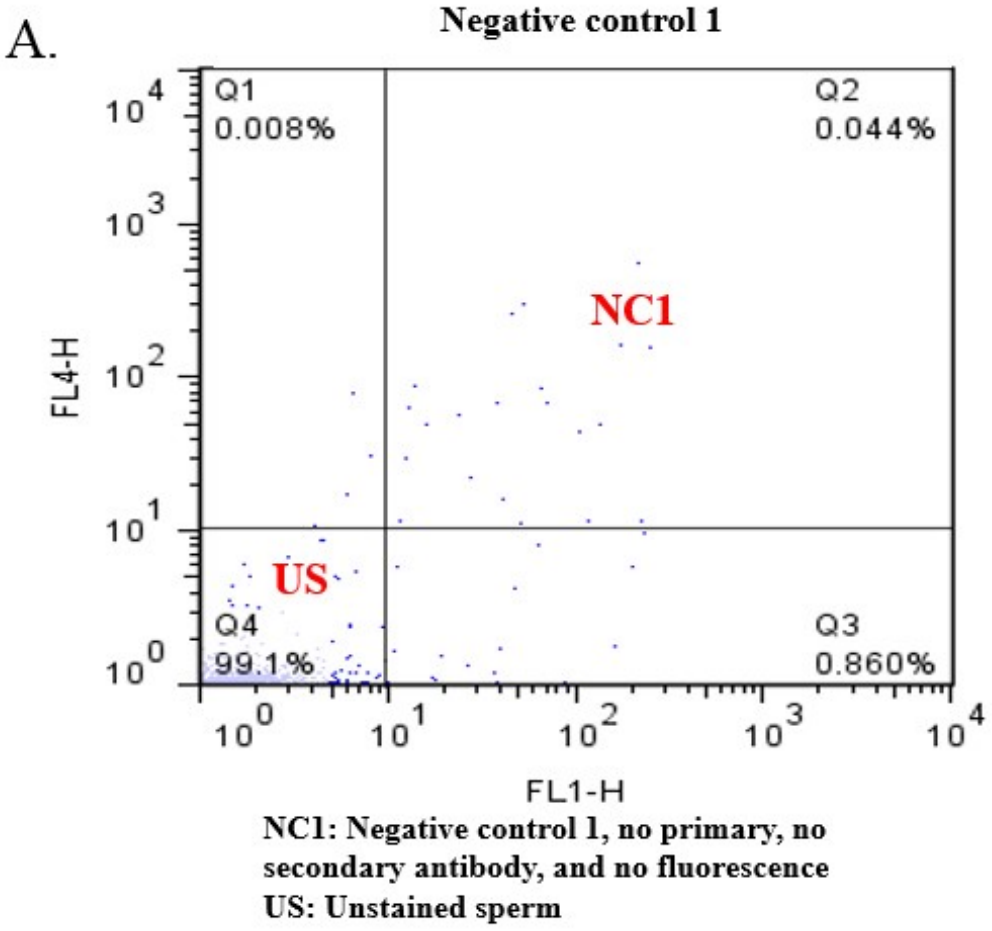
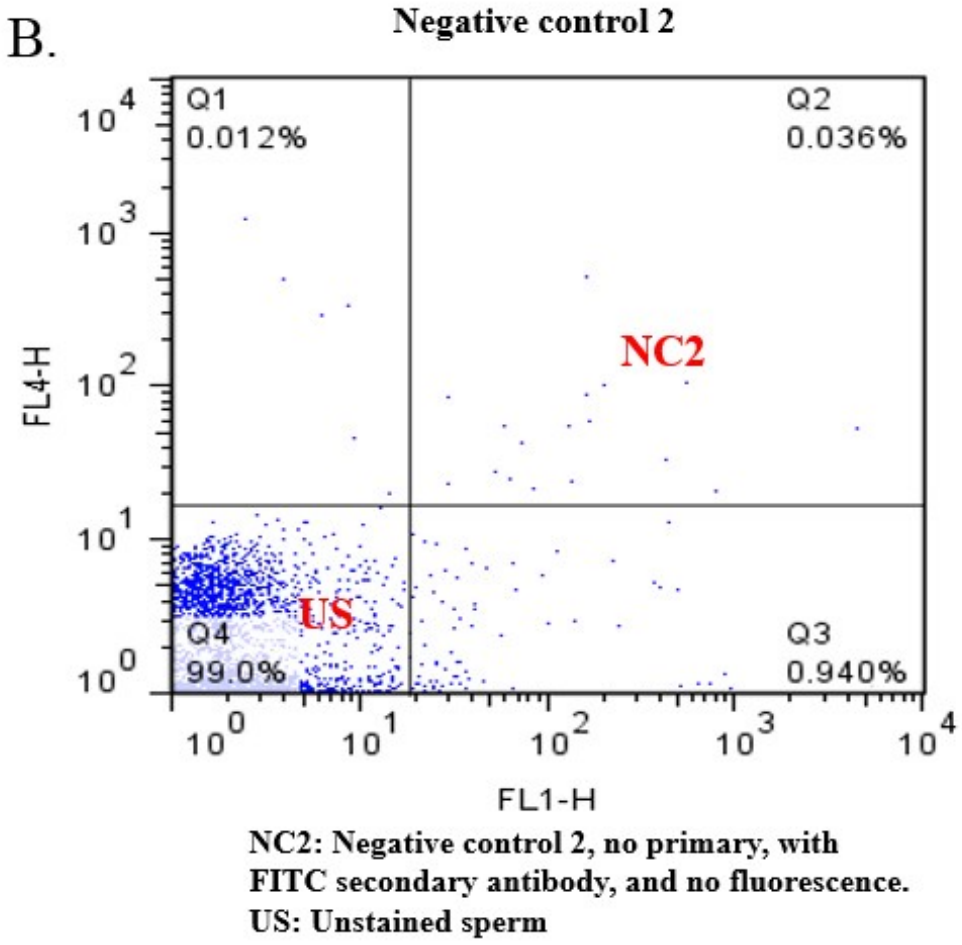


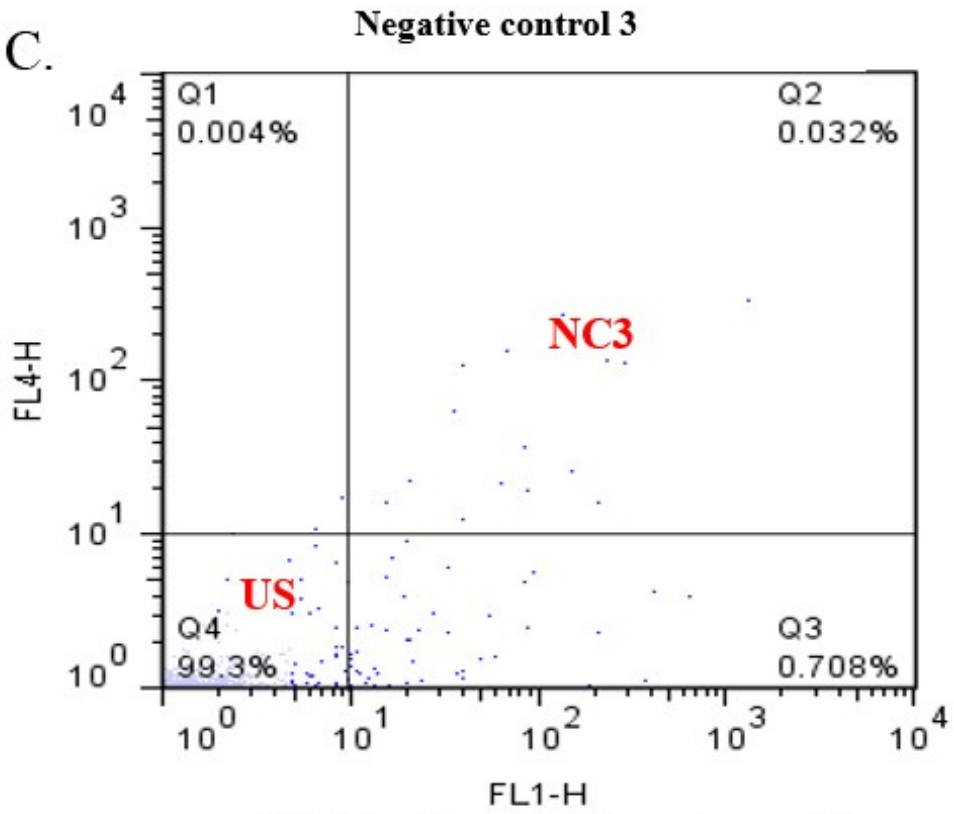
Figure 12 Differential expression of H3K27ac and H3K27me3 in bovine sperm.



NC2: Negative control 2, no primary, with FITC secondary antibody, and no fluorescence.
US: Unstained sperm

Figure 12 (Continued)

C.



NC2: Negative control 2, no primary, with DyLight 550 secondary antibody, and no fluorescence.
US: Unstained sperm

Figure 12 (Continued)

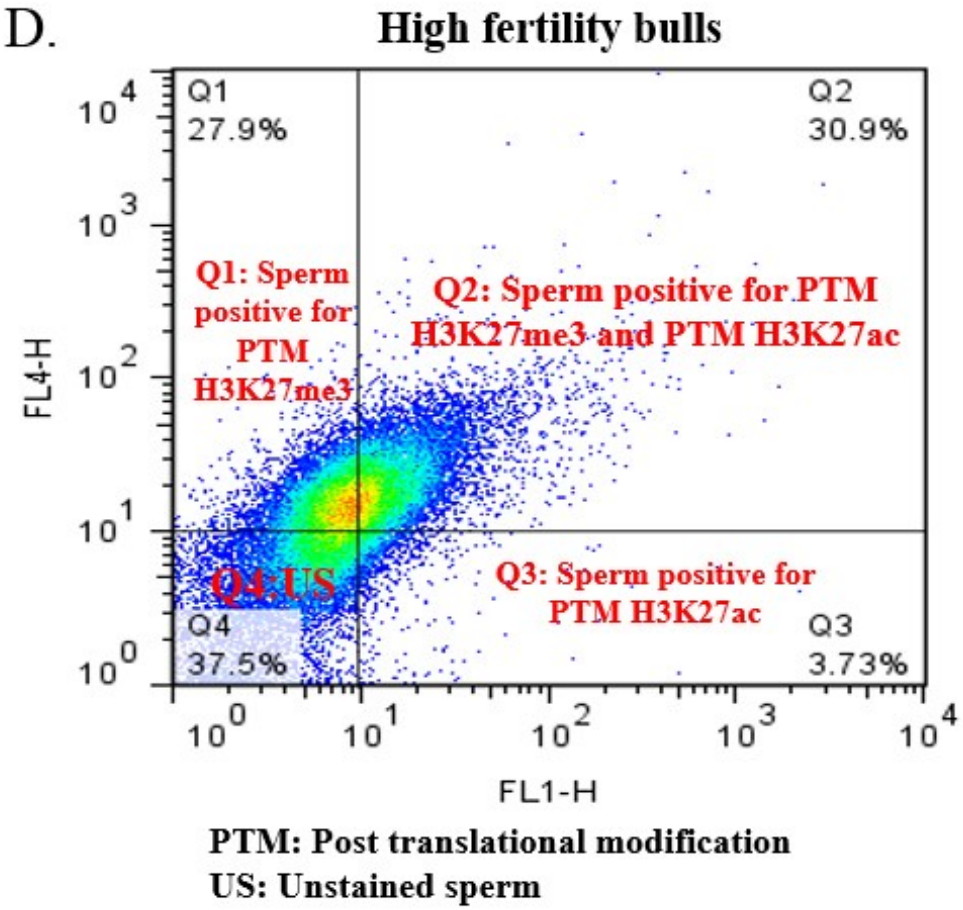


Figure 12 (Continued)

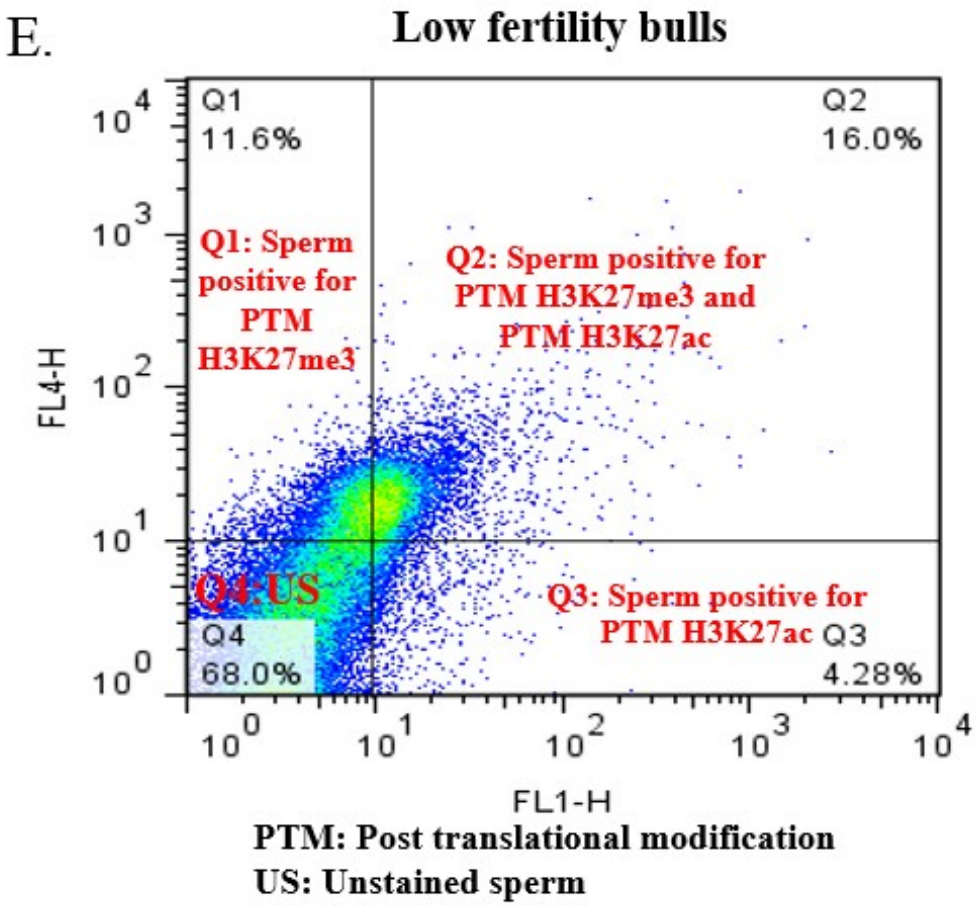


Figure 12 (Continued)

The negative control showing the absence of fluorescence and are unstained sperm (UN) (Fig. A-C). Percentage of sperm expressing H3K27ac, H3K27me3, double positive and unstained cells in high fertility bulls (Fig. D). The percentage of sperm in low fertility bulls expressing H3K27ac, H3K27me3, double positive and unstained sperm (Fig. E). The percentage of sperm expressing H3K27me3 are present in Q1 (Quadrant). Sperm positive for the both H3K27ac and H3K27me3 are present in Q2. The percentage of sperm in Q3 indicates expressing H3K27ac. The percentage of sperm which does not express either H3K27ac or H3K27me3 are unstained (UN sperm) in Q4.

Sperm from high and low fertility sires presented higher levels of methylation of histone 3 (H3K27me3) as compared to the levels of acetylation of histone 3 (H3K27ac; Table 7; Fig. 13A-C).

Table 7. Average value of unstained sperm, sperm positive for H3K27ac, H3K27me3 positive, double positive (H3K27ac + H3K27me3) positive from HF and LF bulls

		Unstained	H3K27me3	H3K27ac	H3k27 me3ac	Total H3K27me3	Total H3K27ac
Group	High fertility (HF) bulls						
1	11HO5929 Ally	47.58	20.19	2.41	29.81	50.00	32.21
	11HO9219 Alverez	55.10	11.62	9.30	24.00	35.61	33.30
	11HO4631 Oixie	49.53	19.18	2.49	28.80	47.98	31.29
	11HO9269 Stateside	42.08	8.17	8.74	40.99	49.17	49.73
	11HO6440 Sylvester	54.03	23.61	2.70	19.63	43.23	22.32
Group	Low fertility (LF) bulls						
2	11HO1222 Champ	66.93	14.13	2.94	16.02	30.15	18.95
	11HO8105 Decker	47.80	16.14	7.65	28.43	44.57	36.08
	11HO8128 Jetwave	47.30	16.59	2.66	33.46	50.05	36.19
	11HO8524 Midnite	43.25	9.74	5.57	41.43	51.17	47.00
	11HO8740 Navigator	47.67	23.14	2.99	26.18	49.32	29.17

Differences between average values of unstained sperm, H3K27ac positive sperm, H3K27me3 positive sperm, average of double positive (H3K27ac + H3K27me3) positive sperm, average of total positive H3K27ac and average of total positive H3K27me3 between high fertility (HF) and low fertility (LF) bulls.

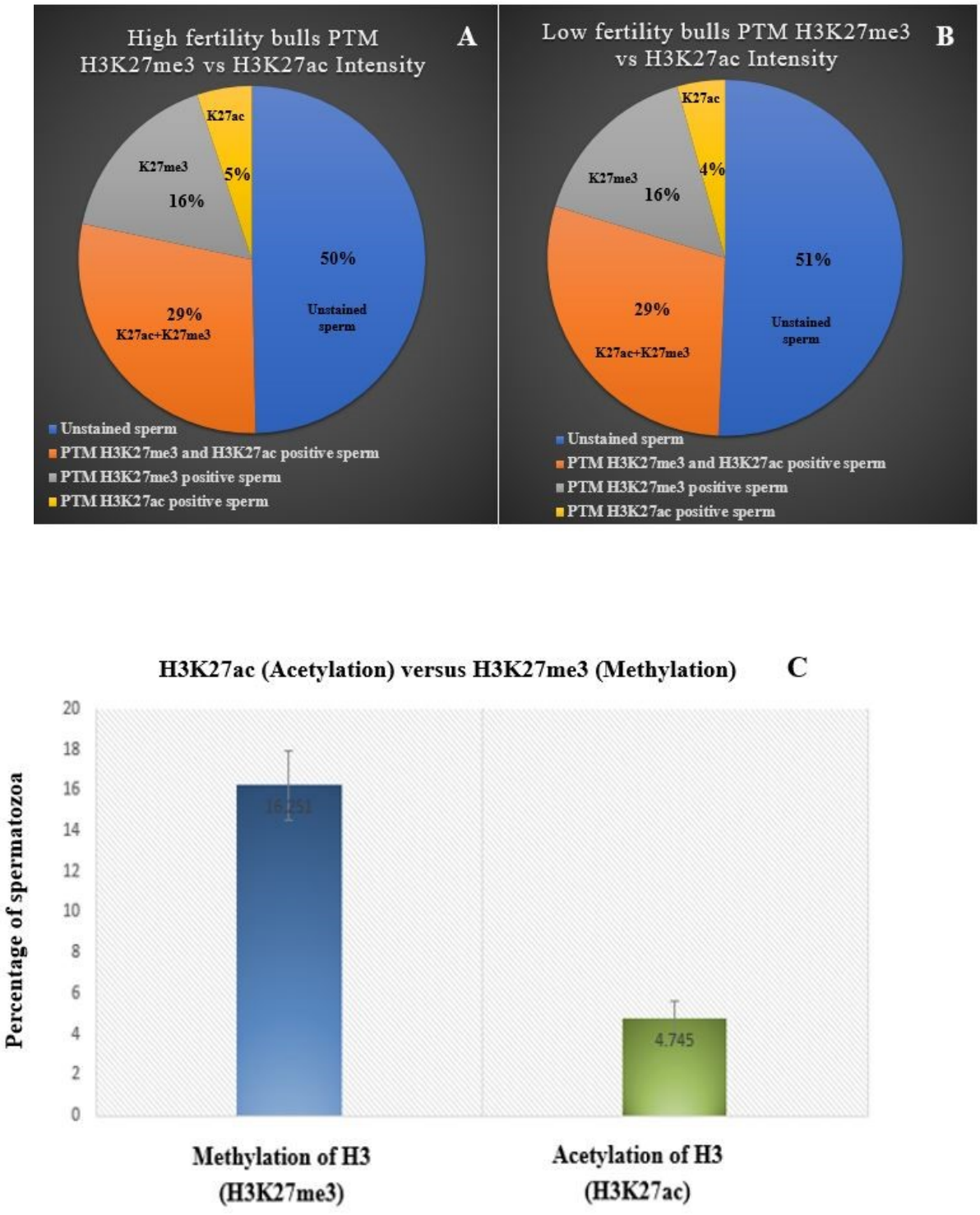


Figure 13 Differences between average of acetylation and methylation of H3K27.

Bulls, irrespective of fertility group (A&B), showed significant methylation of histone 3 (H3K27me3) compared to the acetylation of histone 3 (H3K27ac; $p = 0.0032$; C).

Further, the H3K27me3 signal intensity was inversely proportional to signals of H3K27ac between high and low fertility bull spermatozoa ($p = 0.0032$; Fig. 14; $y = -0.5668x + 18.4277$).

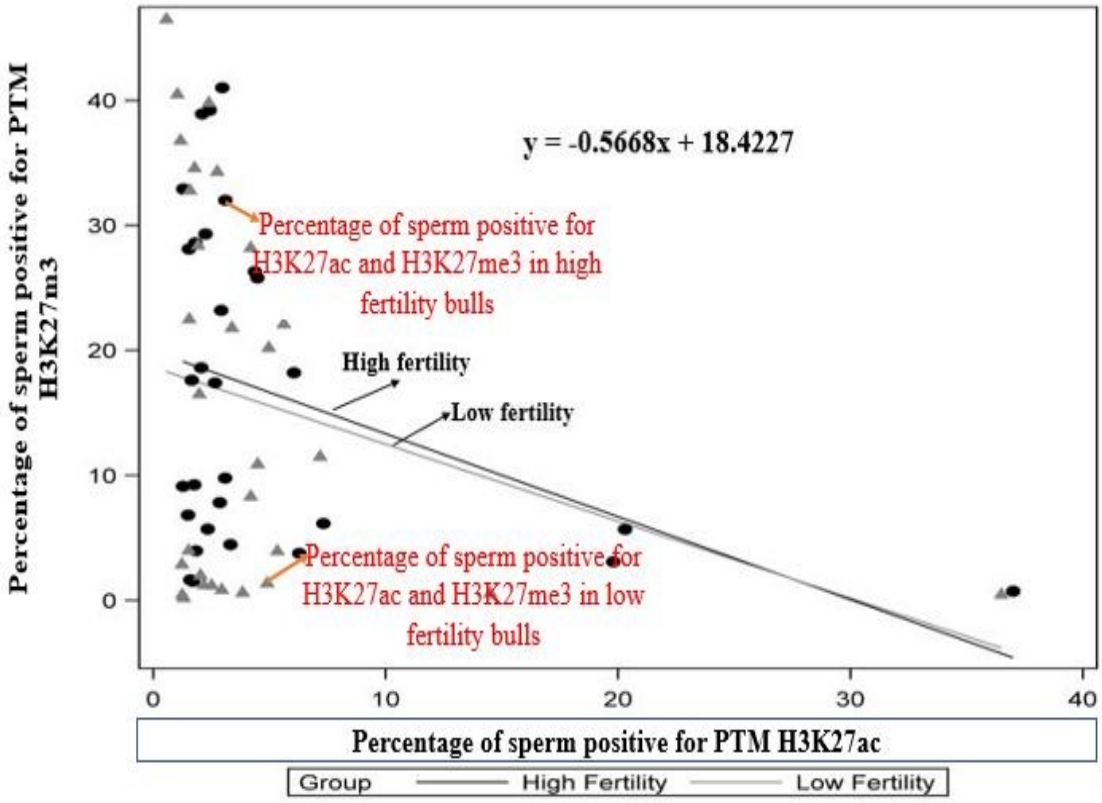


Figure 14 Differences between sperm acetylation and methylation of H3K27.

Regression plot depicts the inverse relationship of H3K27me3 and H3K27ac within two groups ($p = 0.0032$; $y = -0.5668x + 18.4277$).

Conservation of H3 across mammals

Based on the Clustal Omega sequence alignment and percent identity matrix of both H3 protein as well as *H3* transcript among mammals, H3 is highly conserved among the mammals selected (96.32-100%; Table. 8).

Table 8 Percent identity matrix nucleotide and amino acid calculated between different mammals for *Histone 3*.

Nucleotide Percent Identity Score								
Histone 3	*Bos. taurus*	*Homo sapiens*	*Mus musculus*	*Rattus norvegicus*	*Equus caballus*	*Macaca mulatta*	*Canis lupus*	*Bos mutus*
Bos taurus		82.76	84.69	83.88	85.52	80.41	89.50	90.06
Homo sapiens	100		84.10	85.87	85.68	81.51	83.02	86.26
Mus musculus	100	100		93.61	87.01	81.87	88.13	88.89
Rattus norvegicus	100	100	100		86.20	80.81	87.94	87.43
Equus caballus	100	100	100	100		81.62	90.93	91.52
Macaca mulatta	96.32	96.32	96.32	96.32	96.32		81.96	83.33
Canis lupus	96.32	96.32	96.32	96.32	96.32	96.32		93.57
Bos mutus	96.32	96.32	96.32	96.32	96.32	96.32	96.32	
Amino Acid Percent Identity Score								

H3 amino acid and nucleotide sequences were aligned and the percent identity matrix was determined using Clustal Omega. The amino acid sequences are 96.32-100% identical with one another and nucleotide sequences were significantly similar.

Because H3 amino acid sequences are highly conserved, all of its domains are also conserved. A superfamily contains conserved domain models which generate overlapping annotations for the same protein sequences, while multi-domains are computationally detected domains that likely contain multiple domains within them (Marchler-Bauer et al., 2014). Amino acids 1-57 of H3 were identified as belonging to the PAF (PCNA-Associated Factor) superfamily and amino acids 1-132, as part of the Histone multi-domain. The entire sequence was identified as belonging to the H4 superfamily. The PAF superfamily contains PCNA-associated factor protein, which is involved in chromatin binding (Brown et al., 2015). Histone multi-domain and H4 superfamily contain the core histones H2A, H2B, H3 and H4 (Marchler-Bauer et al.,

2014). The conserved domain data revealed involvement of H3 in PAF superfamily, H4 superfamily and histone multi domain.

Interactomes of H3 with other sperm specific proteins and significant gene ontology (GO) terms

The interactome of H3 contains 209 nodes and 224 edges and the cluster identified by the ClusterViz app harbors 28 nodes and 30 edges. This cluster includes chromatin assembly factor 1 (CHAF1B), centromere protein A (CENPA), codanin 1 (CDAN1), PHD finger protein 1 (PHF2), hepatoma-derived growth factor (HDGF) and integrin (ITGA4) (Fig. 15).

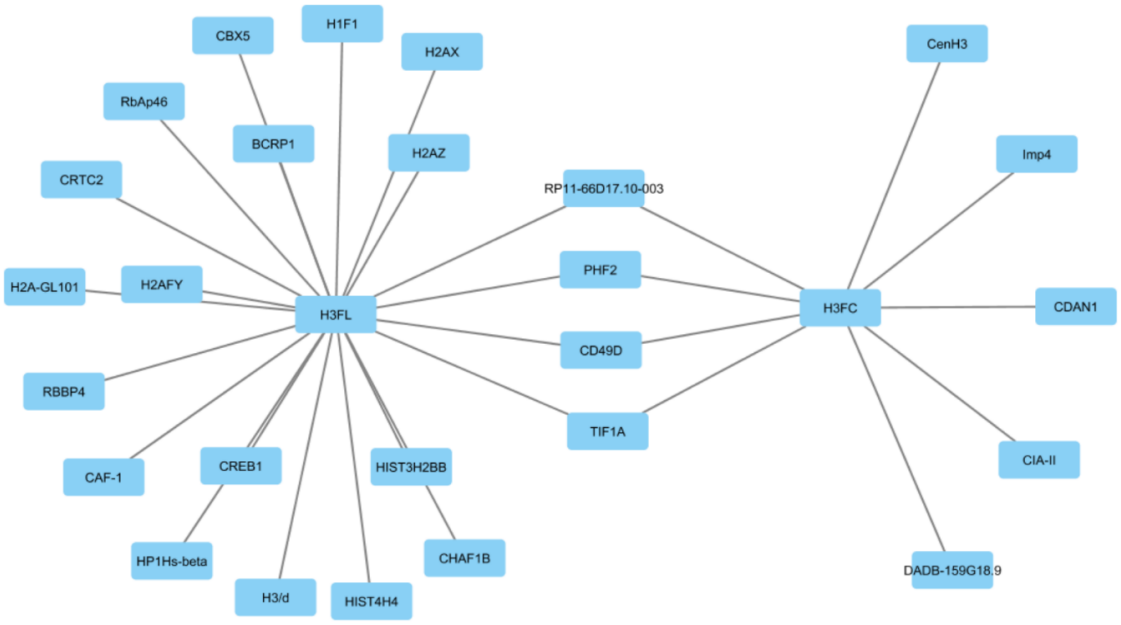


Figure 15 Cluster from H3's interactome generated by Cytoscape's. ClusterViz application (with 28 nodes and 30 edges).

Such proteins are involved in regulation of H3K27 methylation, nucleosome assembly, regulation of DNA replication and chromatin assembly (Fig. 16) (Stark et al., 2006). Figure 6 shows the full H3 interactome while Figure 16 presents the largest cluster from within H3 interactome. Gene ontology data of H3 retrieved from the NCBI Gene database indicate that H3 is significantly involved in key processes including DNA binding, protein binding, histone binding, and protein heterodimerization.

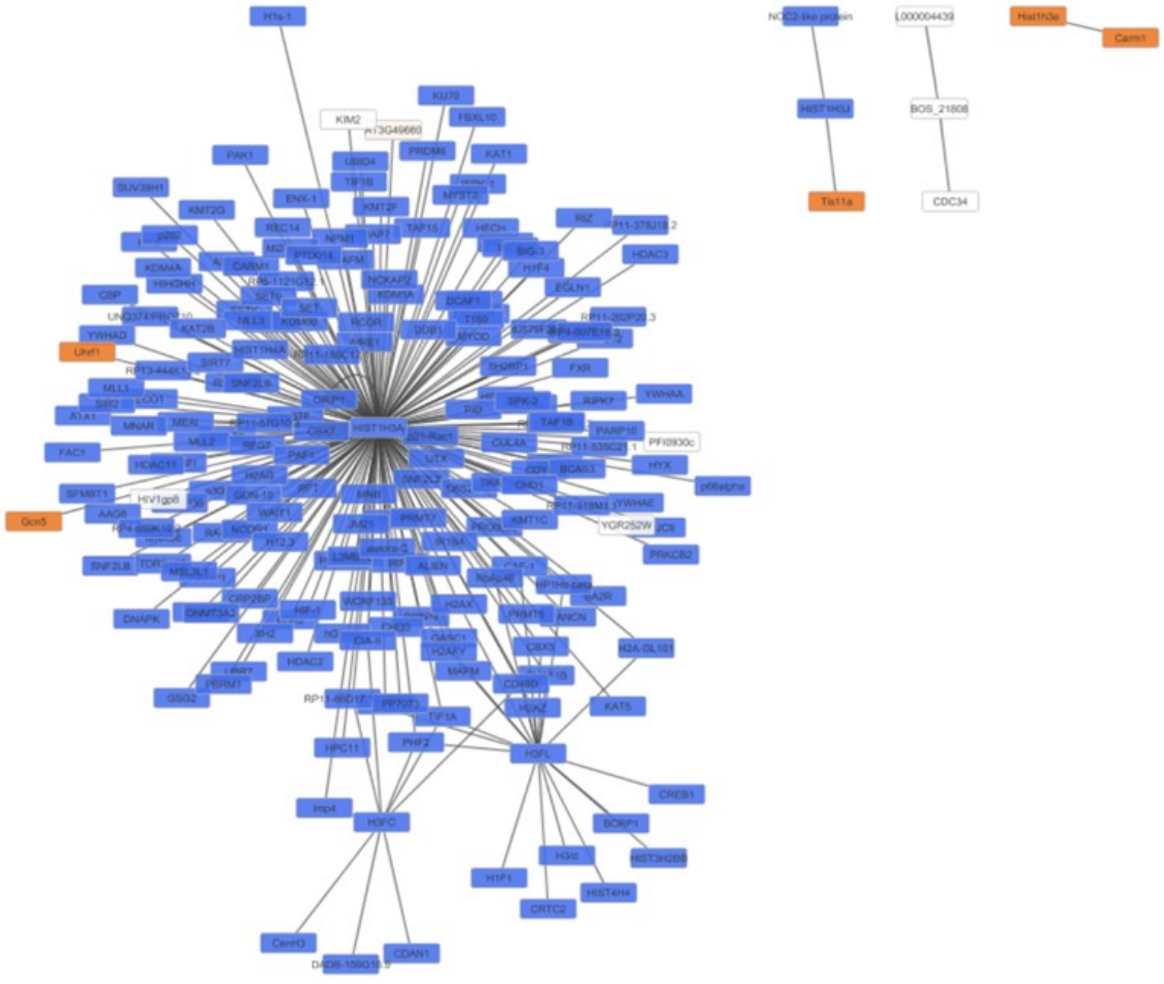


Figure 16 The interactome of H3 generated by Cytoscape, containing 209 nodes and 224 edges.

Each node represents a gene that encodes for a protein interacting with H3. The nodes are color-coded by species of origin: blue indicates *Homo sapiens*, orange indicates *Mus musculus*, and white indicates species other than *Mus musculus* and *Homo sapiens*.

Discussion

Mature sperm of human and bovine are bound to protamines at approximately, 97.1% in human and 86.6% in bovine (Samans et al., 2014), hence bovine sperm retains around 13.4% of histones. Abnormal retentions of these histone affect the histone: protamine ratio. Post translational modifications of the histones in bovine sperm are completely unknown. Lack of such knowledge is important because it is hindering advances in the fundamental science of mammalian gametes and reproductive biotechnology. Our study is the first to evaluate levels of two prominent histone modifications in high and low fertility bulls. Most of the acetylation modifications occur prior to histone to protamine exchange in sperm. This massive acetylation relaxes the sperm chromatin and facilitates exchange of nuclear proteins (Buffone et al., 2004). Trimethylation of histone 3 at lysine 27 is associated with repression of transcription as well as with control of gene expression in male reproductive tract, differentiation of spermatocytes and X- chromosome in activation (Glaser et al., 2009; Turner et al., 2005). The H3K27ac and H3K27me3 as two histone signatures in mature sperm are the marks of understanding the differences of low and high fertility in bulls as well as infertility in human population.

Our findings suggest that abnormalities in H3K27 acetylation and methylation in bovine sperm might be reflective of abnormal spermiogenesis as well as indicator of

ability of the sperm to fertilize and activate the egg and support embryo development. The present study reports newly generated knowledge concerning the epigenetic regulation of bull fertility via acetylated and trimethylated H3 lysine27 in sperm. Using immunocytochemistry, flow cytometry, and tools in bioinformatics, we present evidence that posttranslational modifications of H3 (H3K27ac and H3K27me3) were localized in sperm head; while H3K27ac was detected all around the sperm head and H3K27me3 was consistently around the sperm head, just like a crown. The methylation of H3K27me3, which is a mark for transcriptional repression, were significantly higher than those of acetylation of H3K27ac, a marker for transcriptional activation. These findings agree with previous experimental evidence showing that methylation of histones cause transcriptional inactivity in mammals including humans (La Spina et al., 2014; Santos and Dean, 2004). The findings demonstrate that acetylated and trimethylated H3K27 are differentially retained in sperm from bulls with different fertility indexes measured *in vivo*.

Histone 3 (H3) is conserved across mammals, modulates chromatin structure, and it could be associated with bull fertility. The percent identity matrix showed that H3 is highly conserved (96.32-100%) across mammalian species such as *Bos taurus, Bos mutus, Canis lupis familiaris, Equus caballus, Homo sapiens, Macaca mulatta, Mus musculus and Rattus norvegicus*. Analysis of H3 protein sequence showed that H3 interacts with proteins such as chromatin assembly factor 1, centromere protein A, codanin 1, PHD finger protein 1, hepatoma-derived growth factor, and integrin. In addition, proteins interacting with H3 are involved in subcellular processes such as

regulation of H3K27 methylation, nucleosome assembly, regulation of DNA replication and chromatin assembly.

Epigenetic modifications have a penetrating effect on functioning and fate determination of all body cells including sperm (Bernstein et al., 2007). Gene expression is influenced by changes in chromatin states through epigenetic mechanisms involving posttranslational modifications of histones (Lindeman et al., 2011; Ruthenburg et al., 2007). While posttranslational modifications of H3K27ac are involved in transcriptional activation (Cao et al., 2002; Czermin et al., 2002; Kuzmichev et al., 2002; Müller et al., 2002; Santos and Dean, 2004; Taguchi, 2015), those of H3K27me3 result in transcriptional silencing (Lee, 2009; Santos and Dean, 2004; Tsai et al., 2010; Zhao et al., 2008). Our study is the first to demonstrate the degree of posttranslational changes of H3K27ac *versus* H3K27me3 in bull sperm. We also demonstrated that both H3 modifications (H3K27ac and H3K27me3) are detectable in bull sperm using immunocytochemistry. These results are consistent with those reported by other authors (de Oliveira et al., 2013; Flores et al., 2011; Zhang et al., 2006) .

Chromatin compaction, retaining of posttranslational histone modifications and the incorporation of protamines determine the state of molecular and cellular health of sperm (Brunner et al., 2014; Dogan et al., 2015) and abnormal retention of histones are indicator of immature sperm (de Oliveira et al., 2013). These facts are consistent with our study that confirms large numbers of sperm exhibiting no methylation or acetylation marks. Further, the difference in degree of methylation and acetylation could be a reason for excessive population of sperm with abnormal epigenetic status that make them unable to fertilize, activate the egg and to contribute to embryo development as well.

In conclusion, conservation of the amino acid sequences present in H3 confirms the validity of using bull as a model for the study of mammalian sperm. For this reason, our findings have potential implications for improving the diagnosis of male subfertility of both bulls and other mammals, including men. Through the interactome data, we further demonstrate the importance of H3 for understanding of epigenetics and mammalian fertility. Most important, because H3K27 is associated with male fertility, this may be a suitable candidate for epigenome editing research.

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CHAPTER IV

SPERM CELLULAR AND NUCLEAR DYNAMICS ASSOCIATED WITH BULL FERTILITY

Abstract

Special structure and shape characteristics of sperm and the molecular chemistry of their nuclear proteins have been found to indicate and influence fertility of a bull and the subsequent development of the embryo. Some bulls, despite production of abundant sperm with normal to better motility and morphology, still have low fertility because of molecular defects to the cell and its proteins which interfere with sperm physiology. The intent of this study was to both ascertain those cellular characteristics and the dynamics of the nuclear proteins protamine1 (PRM1) and protamine2 (PRM2) in the sperm of Holstein bulls having different levels of fertility. To accomplish this objective, key sperm parameters were analyzed using computer-assisted sperm analysis (CASA). Sperm plasma membranes, acrosome status, DNA fragmentation and levels of nuclear proteins PRM1 and PRM2 were analyzed using PI, FITC-PNA, and in acridine orange (AO) and flow cytometry in sperm from Holstein bulls showing different *in vivo* fertility phenotypes. Average pathway velocity, amplitude of lateral head displacement, and straightness as determined by CASA were different ($p< 0.05$) for sperm from high and low fertility bulls. However, no differences were observed between the high and low fertility bulls with regard to the characteristics of sperm plasma membrane and acrosome,

and DNA fragmentation. Expression levels of PRM1 and PRM2 in sperm from the high and low fertility bulls were found to be inversely related to each other ($p < 0.0001$). These results are significant because in addition to advancing the fundamental science of mammalian male gamete, these markers can be used to evaluate semen quality and predict bull fertility, and enhance mammalian reproductive biotechnologies such as *in vitro* fertilization, and embryo culture.

Introduction

During spermiogenesis, spermatids become spermatozoa, and most of the histones are imultaneously replaced by protamines to protect the paternal genome. The chromosomal DNA of spermatozoa is compressed into a less than nanometer space in the cell nucleus by protamines and histones. Histones are highly alkaline proteins, whereas protamines are small arginine rich proteins and abundantly present in spermatozoa (Takeda et al., 2016). Nuclear remodeling and condensation during spermiogensis result in sequential displacement of histones first by transition nuclear proteins and then primarily by protamines, namely protamine 1(PRM1) and protamine 2 (PRM2) (Balhorn et al., 1984; Kistler et al., 1996). The histone and protamine transition is essential for efficient chromatin compaction in order to silence sperm gene expression, protection and maintenance of sperm DNA integrity in the female reproductive tract, and for appropriate size of sperm head for better hydrodynamicity (Braun, 2001).

Mammalian sperm chromatin is not entirely packed with protamines, as 1-15% portion of mature sperm DNA is bound to histones (Erkek et al., 2013; Gatewood et al., 1987; Hammoud et al., 2009; Samans et al., 2014). Few of these histones are specific to spermatozoa such as TH2B, TH2A, H2AL1, H2AL2, H3.3A and H3.3B (Govin et al.,

2007) and linker histones H1T2 and HILS1 (Martianov et al., 2005) with various functions. Protamines are important for their efficient compression of sperm DNA into a 10-fold smaller sperm chromatin compaction configured as protamine toroids wherein DNA is attached to the nuclear matrix (Ward, 2010). Protamines are positively charged which triggers increased condensation with the negatively charged paternal genomic DNA (Lewis et al., 2003; Oliva, 1995; Queralt and Oliva, 1995). Because of cysteine amino acids in protamines, tight disulphide bridges are created between the protamine molecules resulting in a stabilized nucleoprotamine complex (Lewis et al., 2003; Vilfan et al., 2004).

Most primates carry single copy of PRM1 and PRM2 (Nelson and Krawetz, 1993; Nelson and Krawetz, 1994; Queralt and Oliva, 1993; Schlueter et al., 1996). The presence and functions of PRM2 in bull sperm are not fully understood. The genomic sequences of the PRM1 and PRM2 genes are organized to create a loop domain in conjunction with the transition protein 2 gene (TNP2) plus a sequence called gene4 (Martins et al., 2004; Wykes and Krawetz, 2003). This three-dimensional spatial organization may facilitate a coordinated expression of these genes during spermatogenesis. While the protamines (PRM1 and PRM2) and transition protein (TNP2) genes are expressed at high levels and their functions have been extensively studied in human and mice, only limited information is available for bovines.

While we know that protamines play a crucial role in bovine sperm development and reproduction, the mechanisms regulating sperm physiology are unclear. Protamines are thought to shield the sperm genetic material and its delivery to the egg (Mengual et al., 2003; Oliva and Dixon, 1991). Damage of sperm by endogenous or exogenous

agents, such as nucleases, free radicals or mutagens occurs because of the compromised protamination (Alvarez et al., 2002; Irvine et al., 2000). As determined by sperm chromatin structure assay (SCSA), abnormal protamination leads to DNA-fragmentation causing and lower success in intracytoplasmic sperm injection (ICSI) and in *in vitro* fertilization (IVF) rates (Evenson et al., 1980; Evenson and Wixon, 2005). Levels of protamines in human sperm are directly related to sperm DNA integrity (Aoki et al., 2005). Developmental defects after fertilization have occurred with decreased DNA integrity in mice (Lewis and Aitken, 2005; Suganuma et al., 2005; Tesarik et al., 2004). Cited studies about protamines could help increase understanding of epigenetic programming, and to develop novel approaches for early diagnosis and treatment of infertility (Bao and Bedford, 2016). The objectives of the study were to determine sperm phenotypes associated with bull fertility using a computer-assisted sperm analysis system and flow cytometry and to ascertain the differential expression of PRM1 and PRM2 in bull spermatozoa by using flow cytometry.

Materials and methods

Determination of sperm phenotypes associated with bull fertility using computer-assisted sperm analysis system (CASA).

Cryopreserved semen straws from 10 Holstein bulls (Alta Genetics, Inc., Watertown, WI, USA) were used for CASA (Table. 9).

Table 9 Fertility data of bull sperm used for this study.

Bulls	**Fertility group**	**Number of breeding's**	***In vivo* fertility scores**	**Std. dev.**
High fertility (HF) bulls				
1	HF-1	748	3.0	1.406
2	HF-2	965	3.0	1.371
3	HF-3	682	2.9	1.339
4	HF-4	3647	2.8	1.311
5	HF-5	6051	2.6	1.198
Low fertility (LF) bulls				
6	LF-1	1020	-3.6	-1.658
7	LF-2	927	-3.6	-1.664
8	LF-3	901	-3.6	-1.649
9	LF-4	1105	-3.7	-1.5979
10	LF-5	987	-3.7	-1.73

Methods for determination of bull fertility have been previously described (Peddinti et al., 2008). For this study the fertility phenotypes were separated into high fertility (HF) and low fertility (LF) groups (de Oliveira et al., 2013). For determination of cellular characteristics of the sperm, semen straws (250 µl) were thawed in a water bath at 37°C for 60 sec. The semen was then transferred to 2.0 ml centrifuge tubes, diluted to 5×10^6 sperm/ml in 0.0067 M phosphate buffered saline (HyClone, GE Healthcare Life

Sciences, Logan Utah) pre-equilibrated at 37°C in a water bath. Sperm parameters of each ejaculate were then assessed immediately after thawing the semen in the straws.

Sperm motility was performed with a computer-assisted sperm analysis system (CASA; IVOS) with the following software setting: frame rates: 45 frames/s, number of frames: 60 frames/object, velocity limit for slow sperm: 30 μm/s, velocity limit for medium sperm: 60 μm/s, minimal linearity (straightness) for progressive fast sperm: 90%, frames min (amplitude of lateral head displacement): 7, and filter for particles: 20%. To measure these, a 10 μL aliquot of semen sample was placed on a pre-warmed standard count 4 chamber slide (Leja® Products B.V. Luzernestraat, Nieuw Vennep, Netherland) and at least 200 sperm were counted using a phase contrast microscope at magnification of 100X. Three aliquots from each semen sample were placed in a standard chamber and evaluated with randomly selected fields of view for each sample. The semen variables evaluated were: percentage of motile and rapidly progressive motile spermatozoa (fraction of all cells moving with average path velocity >60 μm/s and straightness >90%); average path velocity (length of a derived ‘‘average’’ path of sperm-head movement per unit time); amplitude of lateral head displacement (width of the head movement envelope) and straightness (ratio [straight line, starting point to end point, velocity/average velocity along the whole length of path] ×100).

Evaluation of sperm plasma membrane, acrosome status and DNA chromatin damage using flow cytometry

Sperm plasma membrane and acrosome status were assessed using flow cytometry as described by Nagy et al. (Nagy et al., 2003), with some modifications. An aliquot of 120 μg of fluorescein isothiocyanate-labelled peanut agglutinin (FITC-PNA;

which binds specifically to outer acrosomal membrane) was added to 1 ml PBS for the preparation of the staining solution, and then divided into equal aliquots (100 µl) after being filtered and stored at -20°C. Thawed straws were diluted 1:1 with PBS, and then 60 µl of the diluted semen was mixed with 10 µl of FITC-PNA and 2.5 µl of propidium iodide (PI). The sample was gently mixed, incubated in the dark at 37°C for 20 min and added 10 µl of Hancock's solution (Schäfer and Holzmann, 2000) for semen fixation, followed by FACSCalibur flow cytometry analysis (BD Bioscience San Jose, CA 95131-1807, USA).

Flow cytometric detection of sperm DNA chromatin damage was carried out according to the method as described by Rybar et al. (Rybar et al., 2003). The semen sample was diluted with TNE buffer (0.15 M NaCl, 0.01 M Tris HCl, 0.001 M disodium EDTA; pH 7.4) to a sperm concentration of 2×10^6 cells/ml and immediately mixed with 400 µl of 0.08 M HCl, 0.15 M NaCl, and 0.1% Triton X-100 (pH 1.2). Following 30 sec incubation, 1.2 ml of acridine orange (AO) staining solution (0.037 M citric acid, 0.126 M Na_2HPO_4, 0.0011 M disodium EDTA, 0.15 M NaCl; pH 6.0) containing 6 µg/ml electrophoretically purified AO (Polysciences, Inc, Warrington, Pa) was added. Sperm samples were then analyzed using the BD-FACSCalibur flow cytometer (BD Bioscience San Jose, CA 95131-1807, USA). Ten thousand events were measured for each sample; this permitted the state of condensation of the sperm chromatin to be analyzed, as the DNA condensation is directly related to acridine orange uptake.

Flow cytometric evaluation of PRM1 and PRM2 expression in bull spermatozoa

To quantify the enrichment of PRM1 and PRM2 in bovine spermatozoa, the signal intensities of these proteins were recorded using flow cytometry. Semen straws

from five HF and five LF Holstein bulls were removed from liquid nitrogen and thawed at 37°C for 30 sec. Samples were washed twice in PBS with 0.1% Bovine Serum Albumin (BSA) and centrifuged at 2,000 *g* at 4°C for 5 min. Sperm were counted using a Neubauer-chamber and approximately 2-3 million cells were distributed in separate centrifuge tube and then fixed in 1 mL of 4% formaldehyde at RT for 1 hr. The samples were then centrifuged at 5,000 *g* and 4°C for 5 minutes and pellets were resuspended in 250 µL of PBS and immediately permeabilized in 250 µL of 0.1% Triton X-100 in 0.1% sodium citrate in PBS on ice for 2 min. The pellets were resuspended in 500 µL of PBS, filtered through a flow cytometric tube using a cell strainer cap (Becton Dickinson Labware; catalogue no. 352235), and then incubated with the primary antibodies at 4°C overnight. The primary antibodies used were rabbit polyclonal PRM1 (M-51; Santa Cruz Biotechnology, Dallas, TX, USA; catalog # sc30174; 1/250 dilution) against PRM1 and mouse monoclonal antibody PRM2 (Briar Patch Biosciences, Livermore, CA, USA; catalog # Hup 2B; 1/250 dilution) against PRM2. Next day, samples were centrifuged at 3,000 *g* at 4°C for 5 min, washed once in 500 µL washing buffer, centrifuged at 3,000 *g* and 4°C for 5 minutes and incubated with secondary antibodies at RT for one and half hours. The secondary antibodies were Donkey anti rabbit IgG (FITC; Santa Cruz, Dallas, Texas, USA; catalog # sc2090; 1/250 dilution) and goat anti-mouse IgG H&L (DyLight® 650; Abcam, Cambridge, MA, USA; catalog # 96874; 1/250 dilution) Following the incubation, the samples were washed twice in PBS with 0.1% BSA (3,000 *g* at 4°C for 5 min). The experiment was repeated five times and sperm samples were then analyzed using the BD-FACSCalibur flow cytometer (BD Bioscience, USA).

Statistical analyses

For CASA, statistical analyses were carried using GraphPad Prism software, version 5 (GraphPad, San Diego, CA, USA). Data were assessed for normality using the Shapiro-Wilk test and variables not normally distributed were arcsine transformed prior to the statistical analyses. Statistical differences between groups were calculated by t-test and considered significant at $P < 0.05$. The relationship between PRM1 and PRM2 statistical analysis was carried out using SAS for Windows 9.4 (SAS Institute, Inc., Cary, NC). To meet the assumptions of the statistical tests, values for PRM1 and PRM2 were natural log transformed (LPRM1 and LPRM2). Separate linear mixed models were fit with PROC MIXED for LPRM1 and LPRM2 as outcomes. Group was included as a fixed effect with bull included as a random effect in each model. In addition, separate linear mixed models were fit with LPRM1 and LPRM2 as response variables with LPRM2 and LPRM1 as fixed effects, respectively. Group as a fixed effect and bull as a random effect were also included in each of the models. Separate models with fertility score as the fixed effect were fit for both the high and low fertility groups to better understand the relationship between fertility score and both LPRM1 and LPRM2. Regression line plots were made using PROC SGPLOT. The regression line was determined using the model-predicted values of LPRM1 and LPRM2 for each value of fertility score, LPRM2, or LPRM1 as indicated using the mixed effects model. Diagnostic plots of residuals for each outcome were assessed to ensure the assumptions of the statistical method had been met. An alpha level of 0.05 was used to determine statistical significance.

Results

Determination of sperm phenotypes associated with bull fertility using CASA

Among the several indicators for bull fertility are quality and quantity of sperm delivered to the dam. During artificial insemination, increased sperm concentrations are required for successful fertilization of low fertility bulls than that for high fertility bulls. In fact, appropriate sperm concentration, evaluation of sperm parameters involving sperm motility, rapidly progressive motility, average path velocity, lateral head displacement and straightness using CASA showed surprising results. Analyses of semen samples by CASA showed statistically significant differences for high and low fertility bulls in average pathway velocity, amplitude of lateral head displacement, and straightness ($P < 0.05$; Table 10).

Table 10 Sperm parameters (mean values ± S.D) of frozen-thawed bull spermatozoa with different fertility phenotypes.

Evaluations	**Fertility phenotypes**	
Semen parameters	**High fertility**	**Low Fertility**
Motile spermatozoa (%)	55.6±2.4	52.4±3.6
Rapidly progressive motile spermatozoa (%)	40.5±2.4	38.5±3.0
Average path velocity (µm/s)	91.5±1.7[a]	82.61±3.4[b]
Amplitude of lateral head displacement (µm/s)	8.8±0.4[a]	7.69±0.4[b]
Path straightness (%)	70.4±1.9[a]	73.66±1.6[b]

Different superscript letters in the same row indicate significant differences ($p < 0.05$).

Evaluation of sperm plasma membrane, acrosome status and DNA chromatin damage using flow cytometry

Sperm plasma membranes are rich in polyunsaturated fatty acids and essential proteins, and they have significant influence on sperm capacitation, acrosome reaction and successful sperm-egg fusion. Sperm acrosome status and DNA fragmentation are directly dependent on integrity of sperm plasma membrane and hence are potential indicators of viable bull spermatozoa. Sperm plasma membrane integrity, acrosome status, and DNA fragmentation assessments by flow cytometry revealed no statistical differences ($P > 0.05$) between high and low fertility bulls (Fig. 17).

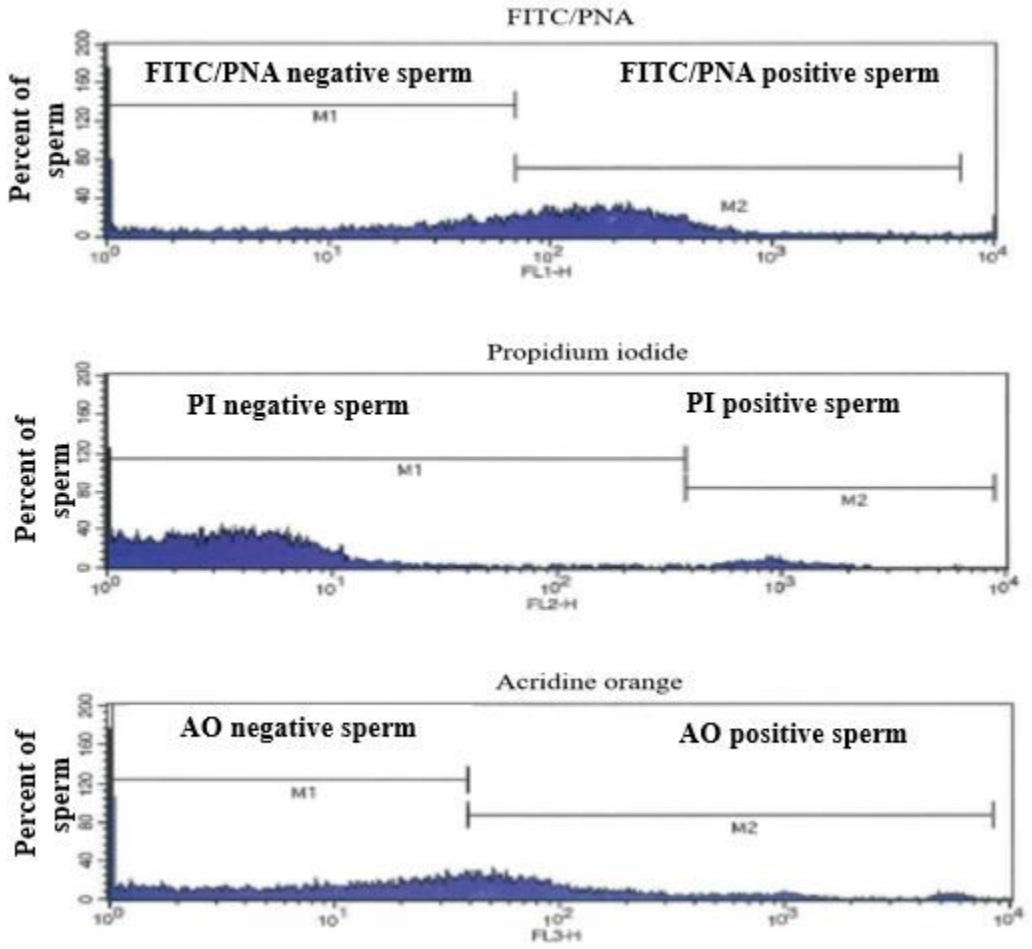

Figure 17 Assessment of plasma membrane, acrosome status, and DNA chromatin damage of bull spermatozoa.

Data were generated using FITC-PNA, Propidium iodide, and acridine orange stain followed by flow cytometry analysis on sperm of high and low fertility bulls.

Flow cytometric analysis of PRM1 and PRM2 expression in sperm from HF and LF bulls

Protamines are arginine and cysteine rich and are essential for sperm head formation, its good hydrodynamicity, and the necessary and appropriate speed to meet the egg in isthmic-ampullary junction. In addition, protamines are the main proteins in sperm

chromatin and are involved with the packaging of nuclear DNA of sperm and hence protecting the paternal genomic information. While most all mammals have both PRM1 and PRM2 genes in their genomes, in bulls PRM1 is expressed, and the presence of PRM2 protein requires much further study. For this, our analysis of the PRM1 and PRM2 expression for a total of 150,000 spermatozoa were carried out by flow cytometry to determine the expression of protamine1 and protamine2 in sperm from the high fertility and the low fertility groups of five bulls. Flow cytometric measurements of PRM1 and PRM2 sperm showed different histogram profiles in sperm from the HF and LF groups (Fig. 18A-C; Fig 19A&B).

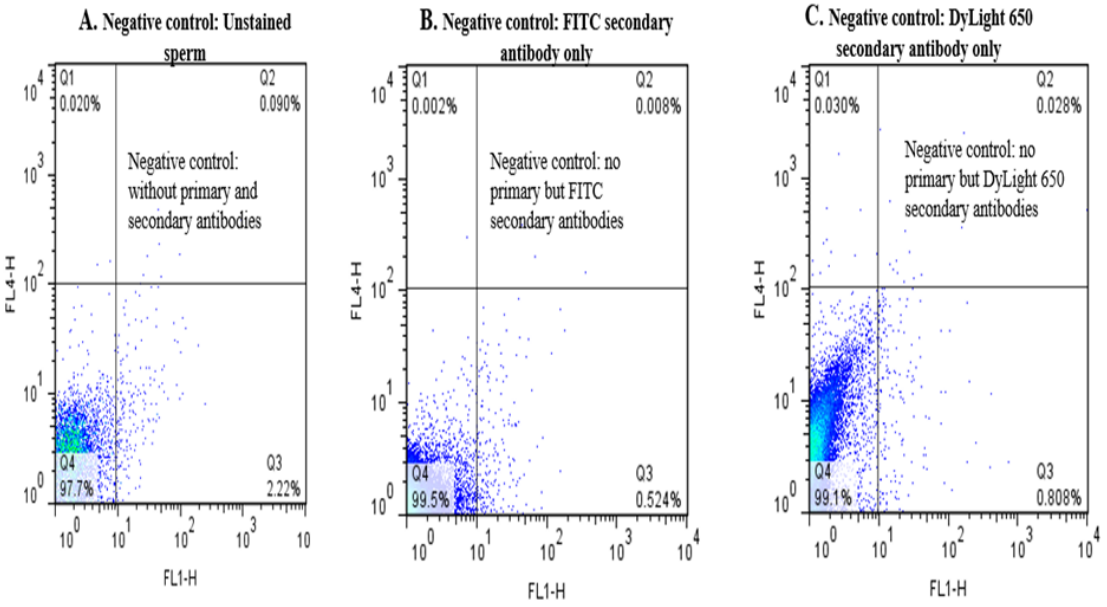

Figure 18 Flow cytometry negative controls.

The negative control showing the absence of fluorescence and unstained sperm (Q4; Fig. A). Negative control FITC as secondary antibody (Q4; Fig. B) to target PRM1. Negative control for DyLight 650 as secondary antibody (Q4; Fig. C) to target PRM2.

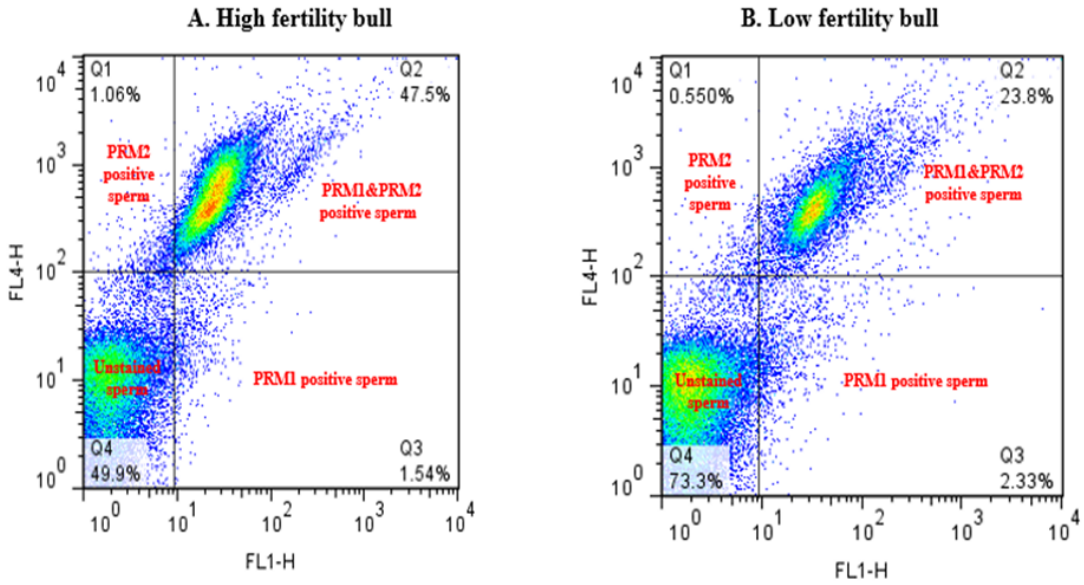

Figure 19 Differential expression of PRM1 and PRM2 in bovine spermatozoa.

Percentage of spermatozoa expressing PRM2 (Q1), double positive (PRM1 and PRM2 together) (Q2), PRM1 (Q3), and unstained cells (Q4) in high fertility (A) and low fertility bulls (B).

The degree of expression of LPRM1 and LPRM2 was not significantly different between high and low fertility bulls (Fig. 20A&B; Fig. 21A&B).

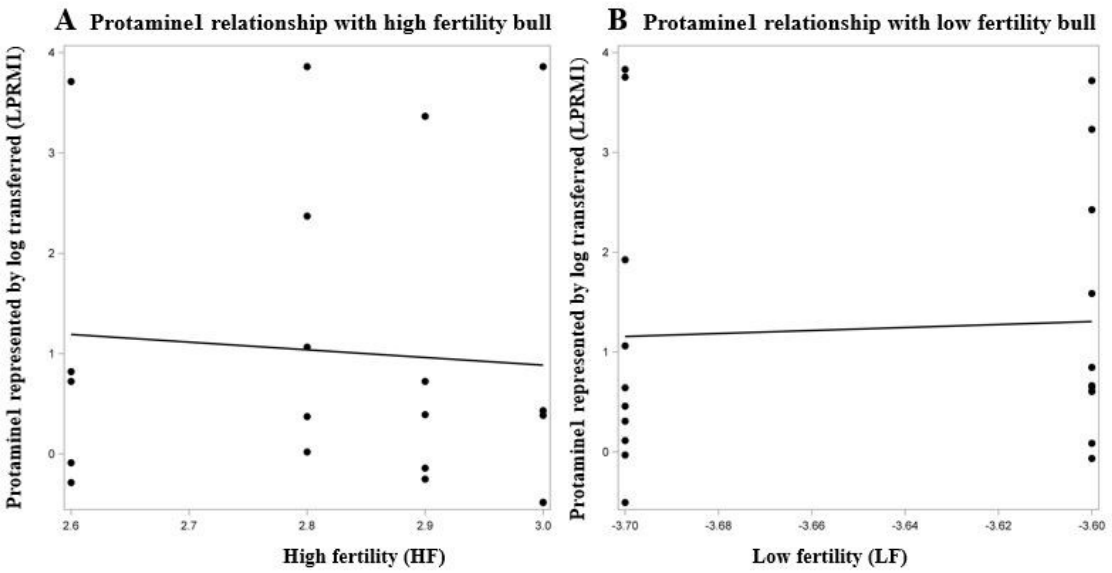

Figure 20 Differential expression of PRM1 in HF and LF bull sperm.

Regression plot depicts the relationship of PRM1 with high (A) and low (B) bull fertility score.

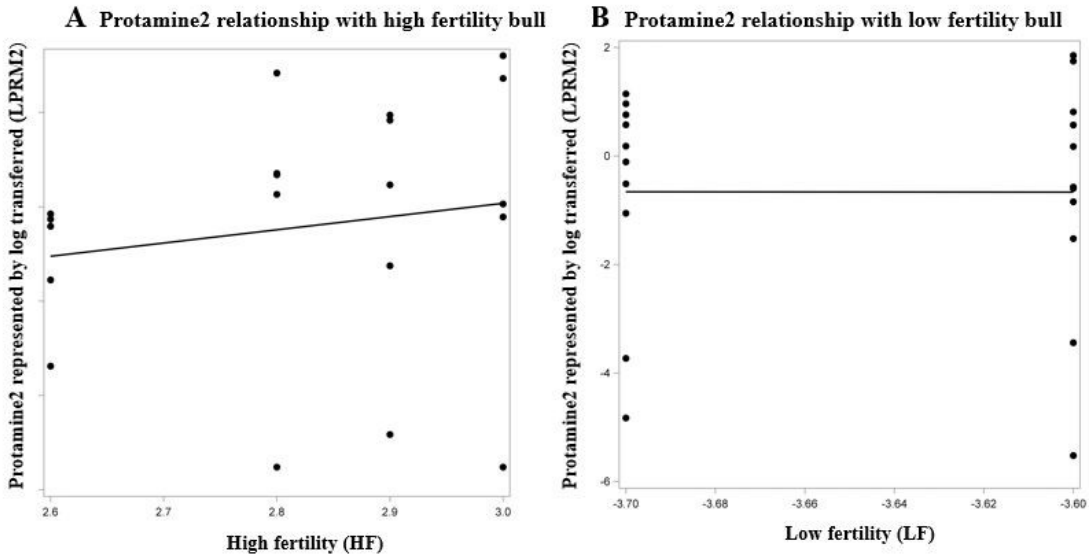

Figure 21 Differential expression of PRM2 in HF and LF bull sperm.

Regression plot depicts the relationship of PRM2 with high (A) and low (B) bull fertility score.

However, LPRM1 and LPRM2 expression levels were inversely related to each other ($p < 0.0001$; Fig. 22).

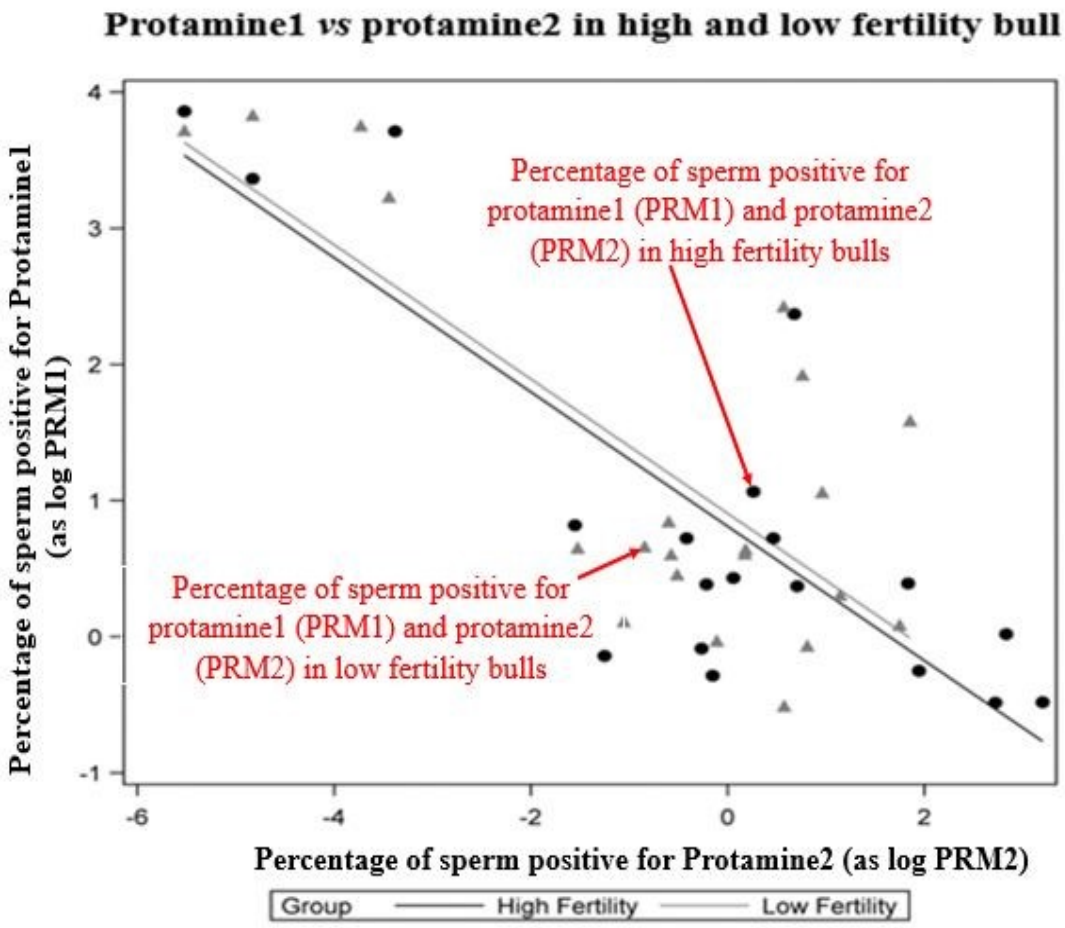

Figure 22 Relationship between the expressions of PRM1 and PRM2 in bull sperm. Regression plot depicts the inverse relationship of log transformed PRM1 and PRM2 ($p < 0.0001$).

Discussion

High fertility is essential for efficient, profitable and sustainable production of cattle. The influence of sperm nuclear proteins on sperm head shape, size, motility, and fertilizing ability, all aid in determining male fertility of mammals. Although producing ample sperm with good motility and morphology, some bulls suffer from noncompensable infertility due to some molecular defects in their sperm (Blaschek et al., 2011; Memili et al., 2012). We used computer-assisted sperm analysis system and flow

cytometry to test our hypothesis that sperm cellular phenotypes as well as PRM1 and PRM2 levels are associated with the degree of bull fertility. This pioneering study took on a critical issue to improve and simplify the knowledgebase and biotechnology for cattle reproduction.

We demonstrated that average pathway velocity, amplitude of lateral head displacement, and path straightness were different in sperm from high and low fertility bulls. Our observation that the proportion of rapidly progressive motility and lateral head displacement spermatozoa was significantly lower in low fertility bulls compared with high fertility bulls is in accordance with previous reports (Birck et al., 2010; Hoflack et al., 2007; Singh et al., 2016). Surprisingly, we also determined that sperm plasma membrane integrity and acrosome status as well as sperm DNA fragmentation status were not different between high and low fertility bull samples. A functional plasma membrane is dependent on membrane ion channels (Petrunkina et al., 2001; Petrunkina et al., 2004) and directly related to sperm fertilizing potential (Tartaglione and Ritta, 2004). However, the mechanisms underlying the alterations of membrane functionality and the influence on fertility are not fully understood. Previous studies also showed non-significant relationship between sperm characteristics and plasma membrane integrity related to the HF and LF bulls (Mishra et al., 2013).

The analysis of the expression PRM1 (represented by LPRM1) and PRM2 (represented by LPRM2) showed different histogram profiles and the log transformed PRM1 and 2 expression levels were inversely related, showing as LPRM2 increasing while the expression of LPRM1 decreased ($P < 0.0001$). To confirm the PRM2 expression dynamics separately in spermatozoa, monoclonal PRM2 antibody was used to

show decreased expression, of which results remain unexplained. Possible reasons could be that both PRM1 and PRM2 protamines, if present together in sperm, have a synergistic effect, where PRM2 aids in incorporation of PRM1 or that the absence of one protamine allows augmentation of the other protamine or even allows absence of a different protein in sperm nucleus. Alternatively, expression of PRM2 in minute amounts could be an indicator of a healthy sperm population in a bull. This assumption is well supported by research findings of Cho et al., (2001) (Cho et al., 2001), showing that PRM1 requires PRM2 to get incorporated into sperm chromatin. Also, decreased PRM2 in the majority of infertile individuals was found because of an abnormal PRM1/PRM2 ratio (Aoki et al., 2005; Aoki et al., 2006). This abnormal ratio is associated with decreased fertility, DNA fragmentation, and morphological changes in bull sperm (Aoki et al., 2005; Aoki et al., 2006; Carrell and Liu, 2001; Nasr-Esfahani et al., 2004; Razavi et al., 2003). Other researchers studying different mammals have shown that abnormal expression of protamines leads to conformational changes in sperm formation during spermatogenesis and hence a decrease in male fertility (Aoki et al., 2005; Carrell and Liu, 2001; Mengual et al., 2003).

Conclusions

In conclusion, average pathway velocity, amplitude of lateral head displacement and path straightness of frozen-thawed sperm evaluated by CASA, but not sperm plasma membrane, acrosome status or DNA integrity, were significantly distinct between bulls with contrasting *in vivo* fertility scores. Analysis of PRM1 and PRM2 expression by flow cytometry revealed different histogram profiles in sperm from HF and LF bulls and is a high throughput analysis method to test bull fertility and its suitability for breeding. Thus,

flow cytometry and CASA analysis are valid for evaluation of potential bull fertility and sperm nuclear proteins (PRM1 and PRM2) can be important biomolecular markers of fertility as well. Results of the present study will advance the fundamental science of mammalian male gamete and efforts for reliable prediction of bull fertility.

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CHAPTER V

OVERALL SUMMARY, CONCLUSIONS AND PROSPECTS

The bull is an ideal model for the comparative study of genetic and epigenetics of sperm nuclear proteins and their role in sperm chromatin dynamics because of the remarkable similarities in reproductive physiology throughout most mammals and humans. The focus of this dissertation was on testis specific histones 2B (TH2B; Chapter 2), posttranslational modifications (PTM) of histone 3 (H3K27me3/H3K27ac; Chapter 3), chromatin remodeling proteins, and protamines (PRM1and PRM2; Chapter 4). Sperm nuclear proteins are essential for male reproduction and critical to many causes of infertility. They determine the health of sperm by efficient packaging of chromatin, presence of specific PTM for early transcript release, which are essential for fertilization, egg activation and early embryo development. Increasingly many bulls produce ample sperm with normal motility and morphology but exhibit low fertility (Kumar and Sigh, 2015). Similarly, among the Western populations of the world, male infertility is a rising trend affecting one in 20 men (Zamudio et al., 2008). Improper packaging of sperm DNA, caused partly by reduced protamination, predisposes sperm DNA to damage, which then interferes with fertilization and early embryonic development. Although the paternal genome is believed to be reprogrammed between generations (Oswald et al., 2000), inheritance of epigenetic marks is also possible (Zamudio et al., 2008). Abnormal chromatin condensation in the sperm during spermatogenesis (pre-fertilization event) and

during pro-nucleus formation (post-fertilization) can induce reproductive problems causing infertility, improper egg activation, and imperfect embryo development (Ward, 2010). Despite advances in animal science and technologies, reproductive success influenced by paternal effects is still in urgent need of more sophisticated research in the lab to enable successful results to improve dairy production. Powerful molecular technologies, including precise measurements of genomic and epigenomic landscapes within single cells, are expected to enhance the production of new knowledge and to result in new and significant applications.

Testis specific histone 2B in bull sperm is a histone 2B variant which initiates replacement of histones, first by transition nuclear proteins and then by protamines. I have demonstrated the expression of these in bovine sperm (Chapter 2). The fluorescence signal intensity from TH2B was quantified and compared among sperm from bulls with different fertility phenotypes. Our results suggest that TH2B plays a key role in facilitating the histone to protamine transformation in bovine. However, this mechanism involves other key players, which are nuclear proteins and might also be associated with sperm chromatin called chromatin remodelers BRD4 and CHD5. These regulate histone to protamine transition in sperm chromatin in the mouse (Montellier et al., 2013; Shinagawa et al., 2015). Our study on the bovine functional genome also suggests that the chromatin compaction and eviction of histones is dependent on all three proteins together, BRD4, CHD5 and TH2B, where TH2B may be involved in catalyzing that transformation. We analyzed TH2B networks in the chromatin of bull sperm to depict the cellular locations of TH2B variant, and to detect the TH2B protein. All of these results are in accordance with findings on the functions of TH2B in the mouse as reported by

other researchers (Montellier et al., 2013; Shinagawa et al., 2014; Shinagawa et al., 2015). The results are significant because they help us better understand the mechanisms of chromatin compaction in bull sperm and that the results can be applicable in reproductive biotechnology of other mammals including humans.

Posttranslational modifications of histone 3 (H3) have two alternative functions, in the repression of the genome or its activation. In the repression state, genes are not transcribed, but in the opposite activation state they are likely to be transcribed. My studies on histone 3 (H3) lysine 27 trimethylation and acetylation are described in Chapter 3. I concluded that amino acid sequences of histone three are highly conserved among mammals. Thus, the findings have possibilities for improved diagnosis of male subfertility of bulls and other mammals, including man. The interactome data further demonstrated the importance of H3 for understanding reproductive epigenetics and mammalian fertility. Of special important, because H3K27 is so closely associated with male fertility, this may be a suitable candidate for epigenome editing research.

Genes for protamine 1 (*PRM1*) and protamine 2 (*PRM2*) are present in bovine spermatozoa. Although the presence of PRM1 protein in sperm has been confirmed, that of presence of PRM2 in bovine has been elusive in part because the relationship between protamines and sperm parameters are not well understood. In chapter 4, I present my investigations on the characteristics of sperm propulsion dynamics which are average pathway velocity, amplitude of lateral head displacement and path straightness as occurs for sperm that has been through the frozen-thawed cycle. The sperm plasma membrane, acrosome status or DNA integrity, were all analyzed for bulls with contrasting *in vivo* fertility scores. Analysis of the PRM1 and PRM2 expression using flow cytometry

revealed different histogram profiles for sperm from higher and lower fertility bulls. There is advantage to using flow cytometry, a high throughput analysis method, minimizing time required for testing bull fertility and their selection for breeding. Flow cytometry and CASA analysis are valuable methods for evaluation of semen and predicting bull fertility. Sperm nuclear proteins (PRM1 and PRM2) are shown to be potential biomolecular markers of fertility as well and should be investigated in large bull populations. These results will advance the fundamental science of mammalian male gametes and efforts for better prediction of bull fertility.

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