Makings of the Best Spermatozoa: Molecular Determinants of High Fertility

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1. Introduction

What is the significance of sperm in reproduction? Recent studies have demonstrated surprising clues that have changed the answers to this question. In addition to providing half of the genome of a mammalian organism, sperm also contributes other molecules such as mRNA, microRNAs (miRNA), proteins, and metabolites that are vitally important for fertility (Feugang et al., 2009, 2010; Peddinti et al., 2008). Furthermore, molecular health of the sperm chromatin and DNA quality also play major roles in sperm viability. Put together, these recent advances have produced a wealth of new knowledge that can provide a systems biology point of view for fertility. These findings highlight the fact that changes in sperm DNA and expressed gene products provide information on the effects of environment on development and disease.

There is a comprehensive database of bull fertility records from thousands of artificial inseminations, which allow cattle producers to draw conclusions about fertility. Additionally, *in vitro* fertilization (IVF) in cattle is well established allowing us to exploit a method to study the molecular basis of sperm quality assessing and quantifying male infertility experimentally. Moreover, cow and human genomes and reproductive physiologies are similar; which makes it possible to apply to humans some of the knowledge produced in cattle. In consequence this review will mainly use the bull as the animal model, although it will also include studies on other mammals including humans and mice.

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Fertility variation is an essential factor limiting efficient production of cattle. There are several factors that influence sperm quality and fertility which can be divided into subcategories of compensatory (sperm viability, motility, etc.), and uncompensatory (molecular defects in the sperm) traits (Dejarnette, 2005). High fertility can be achieved for bulls suffering from compensable sperm defects by increasing the number of spermatozoa deposited in the cow's reproductive tract. Despite providing high numbers of sperm cells with normal morphology (motility and viability), bulls with non-compensable defects may never achieve adequate fertility, and the molecular mechanisms involved in these defects remain unclear. This gap in the knowledge base engenders millions of dollars of economic impact; in spite of this, there is no conventional method to adequately predict sire fertility. A thorough understanding of the mechanisms regulating bull fertility is essential for obtaining consistently high reproductive efficiency, ensuring lower costs and preventing serious time-loss for breeders.

Male infertility can be classified as pre-testicular, testicular and post-testicular, depending on its anatomic and physiologic origin. Patrizio and Broomfield have proposed a classification that includes male infertility with a single gene defect, which includes Usher's, Kallmann's and Immotile cilia syndromes; and male infertility with a chromosomal defect including Kleinfelter's, Noonan's and Prader-Will's syndromes as well as deletions on the AZF a, b or c regions of the Y chromosome. (Cram et al., 2001; De Kretser and Baker, 1999; Ferlin et al., 2007; Krausz and Giachini, 2007). Although sperm dysfunction is known to be a major cause of infertility, there is no pharmacological treatment to improve fertility. The only option for subfertile or infertile men is assisted reproductive technology (ART), which usually consists of treatments that might include intrauterine insemination (IUI), *in vitro* fertilization (IVF), or intracytoplasmic sperm injection (ICSI) depending on the severity of the dysfunction. It has been established that in men undergoing ICSI that 5% of those suffering from oligozoospermia, teratozoospermia and asthenoospermia show an abnormal karyotype compared to 20% of the ones with azoospermia. Our current limited understanding of the cellular and molecular mechanisms involved in sperm function is the main reason for the lack of clinical progress in this area.

2. Main theme summary

Male fertility, the ability of the sperm to fertilize and activate the egg and support early embryonic development is an essential factor for animal reproduction and development. Despite producing adequate numbers of sperm with normal morphology and motility, fertility of some bulls remains poor. This review summarizes molecular phenotypes of the sperm that are associated with sperm viability, methods to study these phenotypes, and implications of new biomolecular markers for improving fertility. There are several assays and tests which are utilized to predict sperm viability and potential fertility. The pitfall of these particular methods is their reliance on phenotypic data of sires which can be misleading and does not predict actual fertility. Further, no diagnostic assessment based on molecular characteristics of the sperm has been identified to accompany said analyses. Some males with normal sperm motility, morphology and cell number have been suffering from subpar fertility causing a decline in male fertility in mammals and other species (Saacke et al., 2000). Molecular mechanisms responsible for this developmental problem are currently unknown. Addressing this issue is imperative to prevent deficiency in reproduction, as well as aggressively pursuing solutions to remedy reproductive health disparities in multiple species.

2.1 Spermatogenesis

In mammals, spermatozoa are produced in the testis, subjected to maturation in the epididymis and stored in the cauda region of this organ. Upon ejaculation, gametes are mixed with accessory sex gland (ASG) secretions, starting their journey to fertilization. In this context, constituents of the sperm themselves and of fluids surrounding them potentially modulate the fertilizing capacity of these cells. In male offspring, germ cells, called prospermatogonia (De Felici, 2009) remain in mitotic arrest in the seminiferous tubules until the male reaches puberty. Morphologically three spermatogonial subtypes have been identified in human testis: type A pale spermatogonia, type A dark spermatogonia and type B spermatogonia. Type A pale spermatogonia are derived from the spermatogonial stem cells and they are maintained in the testis by mitosis as precursor cells. On the other hand, type A dark spermatogonia generate type B spermatogonia, which will undergo meiosis producing spermatocytes (Sadler, 2000). Serial cross-sections of a seminiferous tubule show that sperm cells differentiate in cycles, known as spermatogenic cycles, which are initiated by a surge in gonadotropin releasing hormone (GnRH) from the hypothalamus. Each cycle represents the time it takes for the recurrence of the same cellular stage within the same segment of the tubule. Each stage of the cycle follows in an orderly sequence along the length of the tubule. The number of stages in the spermatogenic cycle is species-specific with six stages in man (~64 days) and twelve stages in both the mouse and the bull (Phillips et al., 2010). During each spermatogenic cycle, spermatogonia proliferate by mitosis and after several stages primary spermatocytes are formed. Each primary spermatocyte will enter meiosis and through the first meiotic division will produce two secondary spermatocytes, each of which will finish meiosis becoming round haploid spermatids. The last part of the process is spermiogenesis (spermatid to spermatozoon), characterized by (*1*) formation of the acrosome; (*2*) condensation of the nucleus; (*3*) shaping of flagellum including mid-piece, and (*4*) loss of the cytoplasmic residues (Sadler, 2000). Spermatogenesis is ended by the delivery of these tailed cells (spermatozoa) into the seminiferous tubule lumen (Lie et al., 2009). Spermatozoa will then be transported into the epididymis, where they will be stored and will acquire forward motility. However, final maturation of sperm cells (sperm capacitation) is only completed in the female reproductive tract.

In contrast, mammalian oogenesis is accomplished through three developmental stages: the initiation of meiosis, the formation of a follicle around each oocyte during the perinatal period, and finally the cyclic growth of follicles and maturation of the oocytes within. Primordial germ cells in a female divide mitotically and differentiate into oogonia. The peak number of female primordial germ cells is reached at the transition from mitosis to meiosis (Gondos, 1981), but this number is drastically reduced, as a result of apoptosis, before the female is born (Hartshorne et al., 2009; Morita and Tilly, 1999). Most of them undergo mitosis whereas some are arrested in prophase of meiosis I and become primary oocytes. Near the time of birth, all primary oocytes undergo meiosis, passing through leptotene, zygotene, and pachytene stages before arresting in the diplotene stage (McLaren, 2003), which is a resting stage that is maintained by effect of oocyte maturation inhibitor (Sadler, 2000; Elsik et al. 2009). Oocytes do not complete the first meiotic division, so they remain in a meiotic arrest until the female reaches puberty. The events that coordinate the initiation of meiosis are not completely understood, however, several studies have proposed that retinoic acid (RA) is the molecular switch that determines meiotic entry in the developing

ovary (Bowles et al., 2006; Koubova et al., 2006; Wang and Tilly, 2010). During each estrous or menstrual cycle, a cohort of follicles is recruited; these follicles will grow and develop an antrum or cavity therefore being known as antral follicles. From this cohort, only a subset of follicles (in polytocous species) or only one follicle (in monotocous species) is selected for dominance and ovulation becoming preovulatory follicles (McGee and Hsueh, 2000). Prior to ovulation oocytes resume meiosis, this can be recognized by dissolution of the nuclear envelope known as germinal vesicle breakdown (GVBD). However, meiosis is stopped again and oocytes are ovulated at the metaphase of the second meiotic division (MII oocytes). The final stage of meiosis will only be completed if the oocyte is fertilized; otherwise, the oocyte degenarates in 24 hr post ovulation (Sadler 2000).

Fertilization mostly occurs in the ampullary region of the Oviduct by a sperm that completes capacitation and acrosome reaction. The sperm's entrance into the oocyte triggers a release of calcium from storage sites into the ooplasm in a wave like pattern (Kline and Kline, 1992; Swann and Yu, 2008), giving rise to a set of events known as oocyte activation. This activation includes the release of cortical granules leading to the block of polyspermy known as zona reaction (Abbott and Ducibella, 2001) and the cell cycle resumption, leading to the culmination of meiosis and the extrusion of the second polar body. Within around 24 hours of fertilization, the paternal and maternal genomes are assembled into pronuclei (PNs), which replicate their DNA. Chromosomes then come together at syngamy, the last step of fertilization that culminates with the formation of the one cell embryo, the zygote followed by embryogenesis.

2.2 Chromatin structure and DNA integrity of spermatozoa

2.2.1 Sperm chromatin and condensation

Since genetic material in the sperm is essential for fertilization in mammals, DNA is tightly packaged in the sperm head for its protection. In the course of this packaging DNA would be more condensed than that of somatic cells by replacing histones, with arginine and cysteine rich protamines. Histones are first replaced by transition proteins (TPs) and then by protamines. Nucleoprotamines package DNA over ten times more efficiently than nucleohistones, bringing DNA replication or RNA transcription to a halt during sperm maturation (Shaman et al., 2007; Miller et al., 2010). In somatic cells, DNA is packaged in a structure known as a selonoid; however, sperm DNA is packed as a loop named doughnut or torus (Ward, 1993). Once mammalian sperm chromatin is packaged into protamines, DNA is tightly coiled into a compact doughnut shape, known as a protamine toroid. Protamine-DNA toroids attach to a proteinaceous nuclear matrix via matrix attachment regions (MAR) similar to those of somatic cells. Several key factors play roles in chromatin remodeling such as protamine 1 (PR1), protamine 2 (PR2), TPs and MARs (Sharma et al., 2004). Towards the end of sperm maturation, 15% of spermatozoa still have histones associated with their DNA, whereas almost 85% have replaced them with protamines (Oliva, 2006) . There are two types of protamines found in sperm; P1, which are always present in mammals and are mature proteins, and P2, which are absent in some species and are generated by precursors (Mengual et al., 2003). A reduction in protamine content in the sperm nucleus is considered to be protamine deficiency; this seems to occur when there is a decrease in P2 levels, which alters the normal P1/P2 ratio by increasing the P1 to P2 levels (Oliva, 2006). Although the results are still being debated, one theory on the altered P1/P2

ratio is that there is an abnormal processing of the P2 precursor (de Yebra et al., 1998) that would increase the P1 to P2 ratio. A second theory for protamine deficiency is that there is a malfunction in the replacement of histones for protamines during spermiogenesis (Blanchard et al., 1990).

There are three basic reasons that could explain why DNA is more compact in the sperm cells compared to that of somatic cells. The first reason for this compaction is the optimization of the sperm cells' shape that enables their movement through the female reproductive tract. Secondly, sperm nuclei are protected by super-compaction from the effects of genotoxic factors. Lastly, sperm compaction assures the "frozen" state of the paternal genome, preserving its post-fertilization epigenetic function in the developing embryo (Miller et al., 2010). Histone-packaged chromatin is more susceptible to DNA damage than the bulk, protamine-packaged DNA that has an important function in embryonic development (Aoki et al., 2006). Protamines are essential for normal fertilization as their deficiency leads to much higher levels of sperm DNA strand breakage. It was observed that abnormal chromatin packaging can affect the accuracy of paternal gene expression (Tesarik et al., 2004). The relationship between sperm chromatin packaging anomalies and sub-fertility in human was shown in terms of sperm dysfunction caused by higher levels of DNA damage (Aoki et al., 2006; Oliva, 2006). Nicks in DNA occur in toroid linkers, between protamine-toroid loops and in sperm nuclear matrix. Endogenous DNAse digestion of DNA occurs at MAR regions. Finally, from a very condensed state, sperm DNA becomes decondensed right after fertilization. It has been shown that the sperm nuclear matrix is vital for activation of the fertilized egg (Shaman et al., 2007).

2.2.2 Sperm DNA integrity

Fundamentally, DNA integrity can influence sperm quality because sperm DNA damage is clearly associated with male infertility, but only a small percentage of spermatozoa from fertile males possess detectable levels of DNA damage (Spano et al., 2000). Among the main examined factors affecting DNA are defective sperm chromatin packaging, apoptosis, and oxidative stress (Agarwal and Said, 2003).

The first cause of DNA damage in sperm is defective and/or insufficient DNA packaging during spermiogenesis. As mentioned previously, sperm chromatin is a highly organized and compact structure consisting of DNA and heterogeneous nucleoproteins. It is condensed and insoluble in nature, which are features that protect genetic integrity and facilitate transport of the paternal genome through the male and female reproductive tracts (Manicardi et al., 1998). Sperm chromatin rearrangement takes place in the later stages of spermatogenesis that involves the replacement of histones with protamines. Protamines, the major nuclear sperm proteins, are essential for sperm head condensation, DNA stabilization and paternal genome protection (Aoki and Carrell, 2003). During condensation and de-condensation, sperm's DNA is more vulnerable to environmental changes; therefore, it is believed that DNA breaks mostly occur during this transition process (Aitken et al., 2004; Sharma et al., 2004).

Secondly, apoptosis-the process of programmed cell death that occurs in multicellular organisms- is another well-known reason of DNA damage in sperm. This is a natural and beneficial process for any organism to prevent their cells from uncontrolled proliferation (Vaux and Korsmeyer, 1999). In male reproduction, apoptosis controls the overproduction of male gametes. There are two pathways that control apoptosis; the intrinsic and extrinsic

pathways. In the extrinsic pathway, the Fas ligand (FasL) binds to the Fas positive cell which signals for the apoptotic death of a normal sperm cell, thus limiting the size of the germ cell population (Rodriguez et al., 1997). Infertile males have been shown to have an increase in the number of Fas positive cells. This would indicate that the correct amount of spermatozoa undergoing apoptosis is not occurring; therefore, the presence of spermatozoa that possess apoptotic markers, such as Fas positivity can indicate DNA damage and infertility (Agarwal and Said, 2003).

The last reason for DNA damage in sperm is oxidative stress caused by an imbalance between reactive oxygen species (ROS) and antioxidants. One feature of the semen of infertile males is the production of excessive levels of ROS; which can cause damages to DNA (Dizdaroglu, 1992). ROS have free radicals, which are unpaired electrons that tend to bind to other molecules and alter them. The sperm cell is highly susceptible to damage by these reactive molecules because of the lipid membrane structure covering its head and mitochondria. The sources of ROS in semen are sperm itself that generates reactive radicals as metabolites and the white blood cells found among sperm (Agarwal et al., 2003). One consequence of excessive ROS is peroxidative damage to the plasma membrane of sperm that causes spermatozoa to become dysfunctional and incapable of initiating fertilization (Irvine et al., 2000).

2.2.3 Methods to detect sperm DNA damage

The quality of sperm DNA is important in maintaining the reproductive potential of males. Damage to sperm nuclear DNA negatively affects assisted and natural fertility as well. Sperm DNA damage is significant in assisted reproductive techniques (Hartshorne et al. 2009) because these techniques by-pass the natural barriers of the reproductive tract that remove damaged sperm cells. ARTs can potentially allow genetically damaged sperm to fertilize the egg, which may cause decreased fertility in the offspring or pregnancy losses. DNA damage influences male fertility by affecting sperm functions through multiple avenues such as defective sperm chromatin packaging, apoptosis, and oxidative stress. Several assays have been developed to evaluate sperm chromatin and DNA integrity; TUNEL assay, sperm chromatin structure assay (SCSA), single-cell gel electrophoresis (SCGE: COMET) assay, the sperm chromatin dispersion (SCD) test and DNA breakage detection- fluorescent in situ hybridization (DBD-FISH) assay. There are also certain stains that can be used to detect DNA damage and chromatin abnormalities. For example, acridine orange (AOT) and Chromomycin A_3 using fluorescence microscopy whereas Toluidine blue (Gur and Breitbart) stain and Acidic aniline blue using bright field microscopy. Some of these tests can only detect DNA fragmentation induced by apoptosis such as TUNEL, whereas single- and double-stranded breaks associated with DNA damage (ss/dsDNA) can be determined by COMET assay (Collins et al., 1997). In this review only five major and commonly used assays are discussed.

2.2.3.1. Terminal deoxynucleotidyl transferase mediated dUTP nick-end-labeling (TUNEL) assay

This is the most common method of determination of DNA damage in the sperm. This method can be performed either by bright and (or) fluorescence microscopy or by flow cytometry. TUNEL assay detects either single or double fragmented DNA caused by endogenous endonucleases' activity during apoptosis and labels the 3'-hydroxyl DNA ends generated by virtue of terminal deoxyribonucleotidyl transferase (TdT) (Nagata et al., 2003).

Since nuclear DNA becomes fragmented by endogenous endonucleases during apoptosis, this assay is designed to detect DNA damages induced by apoptosis in the cell population. TUNEL assay involves fixing and permeabilizing of the cell, incubation steps with DNA labeling, and then staining and detection steps. Flow cytometry is a technique used for counting and examining microscopic particles; it uses a beam of light that is directed into a stream of fluid (containing the potentially damaged DNA), and quantifies the amount of damaged DNA from the non-damaged DNA in TUNEL assay (Anzar et al., 2002; Martin et al., 2007; Martin et al., 2004). In addition to this, in the fluorescence detection, the location of DNA damage induced by apoptosis can be determined. However, the fluorescence microscopic method is based on the microscopic observation of individual cells, and thus, flow cytometry provides more accurate data.

2.2.3.2 Sperm chromatin structure assay (SCSA)

Since abnormal sperm with damaged DNA is more vulnerable to DNA denaturation *in situ,* the SCSA measure the susceptibility of sperm nuclear DNA to induced denaturation. It is based on the ability of acridine orange (AO) to stain differently double-stranded or single-stranded DNA. For SCSA sperm is acid or heat treated to induce denaturation followed by AO staining (Agarwal and Said, 2003). Acridine orange binds to double-stranded DNA (native DNA) as a monomer, producing a green fluorescent color at 515–530 nm. On the other hand, AO intercalates single-stranded DNA (denatured DNA) as an aggregate, emitting a red fluorescence 630nm. The SCSA relies on visual interpretation of fluorescing spermatozoa under a microscope (Eggert-Kruse et al., 1996), which could cause a biased interpretation due to color confusion from individual to individual. An alternative parameter of the SCSA is the DNA fragmentation index (%DFI), which represents the population of cells with DNA damage (Evenson et al., 2002).

2.2.3.3 Single-cell gel electrophoresis COMET assay

Single cell gel electrophoresis (SCGE) also known as COMET assay is another technique to detect DNA damage in spermatozoa. In this assay, sperm cells are embedded in a slide containing an agarose matrix, and then lysed by either detergents or high salt leading to deproteinization. Afterwards, DNA is electrophoresed so that broken DNA strands migrate towards the anode, resulting in the formation of a Comet tail. At the end, two components are detected; a comet head that contains intact DNA and a comet tail that consists of damaged DNA; the higher the DNA density in the tail, the greater the extent of DNA damage. There are two types of SCGE techniques: in the neutral Comet, DNA migrates under neutral conditions, which allows for identification of double-stranded DNA breaks (DSB); in the alkaline Comet, DNA is denatured under alkaline conditions (pH>13). This technique detects both single-stranded DNA breaks (SSB) and DSB, but would not allow for differentiation between the two. Recently a two–tailed Comet assay has been developed (Enciso et al., 2009), which by revealing the total level of both SSB and DSB in individual cells, allows for a more precise and extensive analysis of the damage. However, since the technique is based on fluorescence microscopy the slides should be analyzed by an experienced observer for interpretation of the results.

2.2.3.4 DNA Breakage detection-fluorescence *in situ* hybridization (DBD-FISH)

This assay is based upon the detection of DNA breakage in cells embedded on a slide within an agarose matrix following a treatment with alkaline unwinding solution leading to the

single-stranded DNA (ssDNA) motifs. These ssDNA motifs serve as the ends for hybridization of probes. The *in situ* detection of DNA breaks and structural features in the sperm chromatin can be detected by DBD–FISH. Following neutralization, protein removal and hybridization with whole genome or specific DNA probes are performed, respectively. During incubation of these probes with the ssDNA motifs, they would intercalate to the specific area in the ssDNA leading to the nuclear halo detected. The intensity of these halos derived from ssDNA breaks hybridized by specific DNA probes are then detected by fluorescence microscopy. Spermatozoa with abnormal packaged chromatin are more sensitive to denaturation by alkali treatment, which results in more intense labeling (red fluorescence) by DBD–FISH (Agarwal and Said, 2003).

2.2.3.5 Sperm chromatin dispersion (SCD) assay

This test is based on the principle that sperm with fragmented DNA fail to produce the characteristic halo when mixed with an aqueous agarose following acid denaturation and removal of nuclear proteins. In detail, undamaged DNA of spermatozoa would be dispersed following acid treatment and lysis leading to deproteinized nuclei, which would be seen as a halo around the sperm head. In contrast to spermatozoa with undamaged or nonfragmented DNA, the halo is either limited or absent in spermatozoa with damaged or fragmented DNA. Although SCD test can be performed either by light microscopy or by fluorescence microscopy, it does not require any staining for detection because dispersed and nondispersed cells can be easily determined under the light microscope. Although SCD test is simple, fast, and cost-effective, its clinical significance is limited (Agarwal and Said, 2003).

2.2.4 Apoptosis in spermatozoa

Necrosis and apoptosis are two forms of cell death. Necrosis affects groups of neighboring cells as a result of acute cellular injury. Conversely, apoptosis influences single cells due to naturally occurring processes within the cell. In contrast to apoptosis, necrosis causes cell swelling and loss of plasma membrane integrity, producing a significant inflammatory response. Under normal circumstances, apoptosis is a mechanism used to remove unnecessary or damaged cells, and contributes to the maintenance of tissue homeostasis (Marchetti et al., 2002). Moreover, there must be a balance between apoptosis, cell death, mitosis, and cell gain. Apoptosis can be classified by the presence of three distinct stages: induction, execution, and degradation; each of these three stages involves activation of the mitochondrial pathway (Martin et al., 2007). Signals for the activation of apoptotic pathways can be either extrinsic or intrinsic. Extrinsic pathways involve expression of pro-apoptotic factors, such as CD95 and TNF receptor 1, on the cell surface. Intrinsic pathways are used to initiate apoptosis from within the cell in response to cytotoxic stimuli and pro-apoptotic factors, such as cytochrome C and endonuclease G, which signal the activation of caspases (Fulda and Debatin, 2006). Induction of apoptosis causes an opening in the mitochondrial pores, resulting in decreased mitochondrial membrane potential and the release of pro-apoptotic factors (Anzar et al., 2002; Martin et al., 2007). Apoptotic cells can be distinguished by translocation of phosphatidylserine (PS) from the inner leaflet to the outer leaflet of the cell (Anzar et al., 2002; Martin et al., 2004).

Apoptosis is a vital part of normal embryonic development; it has been found that abnormal apoptotic processes will result in abnormal development (van den Eijnde et al., 1997).

Additionally, apoptosis has been identified as an important mechanism for the continual replacement of the lining of the gastrointestinal tract in mammals; balance between cell gain and cell loss is crucial in this process (Hall et al., 1994). Apoptosis also plays a role in sperm cell maturation and germ cell apoptosis during spermatogenesis is essential in the production of sperm (Anzar et al., 2002; Marchetti et al., 2002). In human testis, 5% of spermatozoa undergo apoptosis to maintain germ cell population and to reduce abnormal spermatozoa that are being produced. However, increased level of sperm apoptosis may affect male fertility resulting in production of limited numbers of healthy spermatozoa. Increased levels of apoptotic sperm have been shown to have a direct impact on poor bull fertility by decreasing sperm viability (Anzar et al., 2002; Martin et al., 2007; Martin et al., 2004). Loss of sperm viability includes a decrease in mitochondrial membrane potential, increased membrane permeability, and DNA fragmentation. It is thought that cryopreservation of sperm cells induces apoptosis by causing PS translocation, targeting the cell for programmed cell death (Anzar et al., 2002). There are many hypothesized reasons as to why cryopreservation causes PS translocation. Some of these include physical and chemical stress due to low temperature and high salt concentration as a result of crystal formation, which are thought to destabilize the sperm plasma membrane (Anzar et al., 2002).

2.2.4.1 Annexin V assay

In the course of apoptosis, phosphatidylserine (PS) is relocated from the inner plasma membrane to the outer plasma membrane in the cell. Annexin V is a calcium-dependent phospholipid-binding protein that is especially sensitive to PS located on the apoptotic cell surface. Annexin V that detects the translocation of PS across the plasma membrane is generally used with Propidium Iodide (PI) that stains DNA to detect damage. Annexin V assay can be performed using flow cytometry to obtain more accurate and quantitative results. This assay can be performed using fluorescence microscopy to detect the location of apoptotic PS-translocation including its quantification (Anzar et al., 2002; Martin et al., 2007; Martin et al., 2004). In flow cytometry, four distinct populations can be identified through Annexin-V/PI assay: viable cells, necrotic cells, apoptotic cells, and late necrotic cells. Annexin V assay is generally used with TUNEL assay that is an established method to detect nicked DNA to confirm apoptotic-induced DNA fragmentation (Anzar et al., 2002).

2.2.4.2 Apoptotic genes and caspase activation

Another indicator of apoptosis is the activity of apoptotic genes and caspases, which can be linked back to the Fas model and nDNA apoptosis, as the Fas receptor activates caspases which can cause nDNA breaks. Expression of the pro-apoptotic gene (*bax*) (Elsik et al. 2009) and anti-apoptotic gene (*bcl-2*) can be detected using real-time RT PCR. Besides transcripts, protein expression from these two genes can be determined to assess apoptosis in the cell. Both of these proteins come from the same family, however, they each have distinctly different purposes. Bcl-2 inhibits the activation of caspase 9, which in turn would inhibit apoptosis as caspase 9 helps begin the apoptotic pathway. BAX is a pro-apoptotic protein that promotes the activation of caspases and creates pores in organelles that would help promote apoptosis. Both, Bcl-2 and BAX provide a signaling pathway that helps maintain the apoptotic balance in a cell and if this balance is disrupted it can disturb normal apoptotic levels (Fulda and Debatin, 2006).

2.3 Transcriptome of spermatozoa

2.3.1 mRNAs of spermatozoa

Spermatozoa deliver more than just the paternal genome into the egg. The newly transcribed and remnant sperm RNA from spermatogenesis are totally or partially transmitted to the oocyte (Eddy, 2002; Gur and Breitbart, 2006; Miller and Ostermeier, 2006a; Miller and Ostermeier, 2006b; Ostermeier et al., 2004). Diverse mRNAs have been found in human, rodent and bovine mature spermatozoa (Dadoune et al., 2005; Gilbert et al., 2007; Miller and Ostermeier, 2006a). Although the roles of these transcripts during embryonic and fetal development are still unclear, analysis of their profiles could serve as a diagnostic tool to assess male infertility (Bissonnette et al., 2009; Garrido et al., 2009; Lalancette et al., 2009), or it could have prognostic value for fertilization and embryo development (Boerke et al., 2007; Ostermeier et al., 2004; Sassone-Corsi, 2002).

Microarray technology has been successfully used for global gene expression profiling in human, mouse and bovine spermatozoa (Gilbert et al., 2007; He et al., 2006). However, in the case of high merit bulls, the great demand and cost of their semen 36 limit their availability for research needs. Determination of transcript abundance in mammalian spermatozoa is challenging. Several techniques including TRIZOL (Invitrogen), Guanidine Isothiocyanate and commercial kits (i.e., RNeasy from Qiagen, MirVana from Ambion) have been used to improve both the purity and the recovery rate of spermatozoal total RNA extraction (Gilbert et al., 2007; Lalancette et al., 2008b; Ostermeier et al., 2002; Ostermeier et al., 2004). Sperm RNA profiles revealed the absence or low amounts of ribosomal RNAs (28S and 18S) (Gilbert et al., 2007). These levels of ribosomal RNAs (i) correlate with the idea of low translational activity in mature spermatozoa (Boerke et al., 2007, Elsik et al. 2009) remain the principal signature of spermatozoal RNA profile compared to somatic cells (which contain both types of rRNAs).

Although the presence of RNAs within the spermatozoa is well-documented, both the identity and the role of these transcripts remain largely uncharacterized. It has been proposed that sperm RNA could play an important role in the developmental success of the early embryo. Sperm RNA population includes transcripts involved in a wide variety of cellular functions. The absence of genes associated with cell cycle regulation in the spermatozoon could indicate that the mature gamete does not require the cell cycle machinery. Considering the limited capacity of the spermatozoon to store RNA relatively to the oocyte, a specific role of sperm RNAs for early embryonic development could imply the targeting of particular pathways by the accumulation of specific transcripts during the later stages of spermatogenesis (Gilbert et al., 2007).

2.3.2 Small non coding RNAs (snc-RNAs) of spermatozoa

Highly specialized gene regulation is necessary for the expression of spermatogenic genes during the complex developmental processes leading to mature sperm formation. Different small non-coding RNAs, microRNAs (miRNA), small interfering RNAs (siRNAs), and Piwi-interacting RNAs (piRNAs) are noted for their germline expression. The following section briefly describes how these small non-coding RNAs (sncRNAs) function at the molecular level.

MicroRNAs are negative regulators of mRNA translation and stability. They regulate gene expression by binding to the 3′ untranslated region of specific mRNA containing complementary sequences to those within the seed region of miRNAs. MicroRNAs are found in various tissues and, especially in the testis, they are important for proliferation and early differentiation of spermatogenic stem cells (Hayashi et al., 2008). PiwiRNAs protect the germline from invading transposons. PiRNAs are germ line specific and found abundantly during spermiogenesis where they are involved in the repression of retrotransposons during spermatogenesis (Frost et al., 2010).

Piwi proteins and their variants, MIWI and MILI are regulatory proteins playing essential roles in spermatogenesis. These proteins rely upon their interaction with miRNA and piRNA to influence gene regulation. Spermatogenic arrest in MIWI deficient testis implies the importance of small non-coding RNA mediated gene regulation with the help of other RNA processing molecules. MILI is shown to be an essential factor for meiotic differentiation during spermatogenesis. In MILI null mice spermatogenesis was blocked at the early prophase stage of first meiosis (Kuramochi-Miyagawa et al., 2004)

So far, 22 testis-preferential and 6 testis-specific miRNAs have been identified in male germ and Sertoli cells in mouse (Ro et al., 2007). Specifically, Mir122a is predominately expressed in post-meiotic germ cells, and it suppresses the transcription of transition protein 2, a testis-specific protein involved in chromatin remodeling during mouse spermatogenesis (Yu et al., 2005). Mirn34b is highly expressed in adult testis compared to the prepubertal testis, indicating its potential role in the differentiation of male germ cells (Barad et al., 2004). Compared to adult testis, 14 miRNAs are upregulated and 5 miRNAs are downregulated in immature testis. Characteristic miRNA signatures for testis and ovary are elucidated in mouse providing an insight into the sex-based functional roles of miRNA (Mishima et al., 2008).

MiRNAs are initially transcribed as pri-miRNA where the miRNA portion pair up to form dsRNA. A dsRNA specific ribonuclease, Drosha converts the pri-mRNA into precursor miRNA (pre-miRNA) in the nucleus. Exportin-5 transports pre-miRNA from the nucleus to cytoplasm. Dicer, a member of the RNase III superfamily cleaves pre-miRNA to yield 19-22 bp dsRNA with 1-4 bp 3′ overhang at either end. The single stranded mature miRNA which gets incorporated into RNA-induced silencing complex (RISC) controls the target mRNA expression. Identification of miRNA expression is the first step towards understanding their biological role and the pathway involved. Array based methods and real-time PCR are used for miRNA profiling (Barad et al., 2004). Besides, bioinformatics tools have been designed to scan mRNA sequences for binding sites of known and registered miRNAs (Rusinov et al., 2005). Several novel miRNAs have been identified in germ cells. MiRNAs belonging to miR-17-92 cluster and are found to be highly expressed in primordial germ cells (PGCs). The Mir-290-295 cluster is preferentially expressed in PGCs, and the expression is notably reduced in differentiated cells indicating its role in the pluripotency of embryonic stem (ES) cells (Hayashi et al., 2008). Comparison of miRNA expression patterns between immature and mature mouse testes revealed significant differences. From the miRNA microarray data, a range of miRNAs in immature testis showed higher expression levels compared to the mature testes. Through computational search for putative targets, mammalian developmental and spermatogonial genes are found to be potential targets for differentially expressed miRNAs. Among those are Brd2, a gene highly expressed in diplotene

spermatocytes and round spermatids and expressed at low levels in spermatogonia being targeted by mmu-miR-127, Usp42, a gene expressed during spermatogenesis and embryogenesis in mouse being targeted by mmu-miR-411 and mmu-miR-29b. Also, several other genes exhibiting germ cell-specific expression are predicted to be the targets for identified miRNAs (Yan et al., 2007). This explains the need for recruiting specific miRNAs in adult testis to regulate spermatogenesis. Further, miRNAs such as Mirn-24-1, Mirn-25 were identified from sperm derived samples which excluded cytosolic materials but retained nuclear and perinuclear components, mimicking the actual sperm components entering the oocyte during fertilization. However, those miRNAs are found to play a limited role only in fertilization, postulating that sperm-borne miRNAs are majorly directed towards spermatogenic regulation (Amanai et al., 2006).

Recently, a testis specific miRNA, miR-34c was identified. TGIF2 (TGFβ-induced factor homeobox 2) and NOTCH2 (Neurogenic locus notch homolog protein 2) are identified to be its direct targets indicating the possibility that these pathways are involved in regulation. TGIF2 is an inhibitor of the TGFβ pathway which plays a major role during spermatogenesis. NOTCH signaling pathway controls inhibition of cell differentiation, proliferation control, and stem cell count (Bouhallier et al., 2010). These two key pathways requiring miRNA mediated regulation reinforces the essential role of miRNA in spermatogenesis. Dgcr8 is a gene encoding an RNA-binding protein specifically required for miRNA processing. Dgcr8-deficient oocytes matured normally and produced healthy-appearing offspring, even though miRNA levels were reduced. The mRNA profiles of wild-type and Dgcr8 null oocytes are identical, whereas Dicer null oocytes showed hundreds of misregulated transcripts. These findings show that miRNA function is globally suppressed during oocyte maturation and preimplantation development where siRNA instead of miRNA, obviating the Dgcr8 requirement take up the regulatory role. This possibly explains the abnormalities of Dicer null oocytes (Suh et al., 2010). In addition, a recent study showed a reprogramming of major small RNA class from siRNA/piRNA to zygotically derived miRNA during pre-implantation development (Ohnishi et al., 2010), thus, manifesting the indispensable role of miRNA regulation in the shaping up of early embryo. Although maternally derived small RNAs decrease as fertilization proceeds, a class of small RNA called L1 functions till the 8–16-cell stage of embryo. Zygotically derived siRNAs and piRNAs also participate in regulating early development. The potential involvement of these small RNAs in pre-implantation development is confirmed by Dicer knock down studies resulting in reduced expression of N-myc, a component associated with pluripotency in embryo. In general, both maternal and zygotic small RNAs are involved in preventing retrotransposon invasion of the genome. MiRNAs are the most predominant small RNAs found in the inner cell mass of blastocyst (Ohnishi et al., 2010).

Transcripts for the RNA-induced silencing complex (RISC) catalytic components- EIF2C2, EIF2C3, and EIF2C4 are found to be up-regulated during preimplantation development in the mouse. RISC incorporates miRNA to function; hence, miRNA plays a role in early preimplantation as well. Similarly, gene knockout of Dicer renders the embryo non-viable due to unsuccessful preimplantation differentiation (Amanai et al., 2006). Several distinct miRNAs have been identified and characterized from the bovine. The miRNA candidates are found by homology searching and partially verified by cloning from small cattle RNA library where distinct and homologous miRNAs are identified (Long and Chen, 2009). The
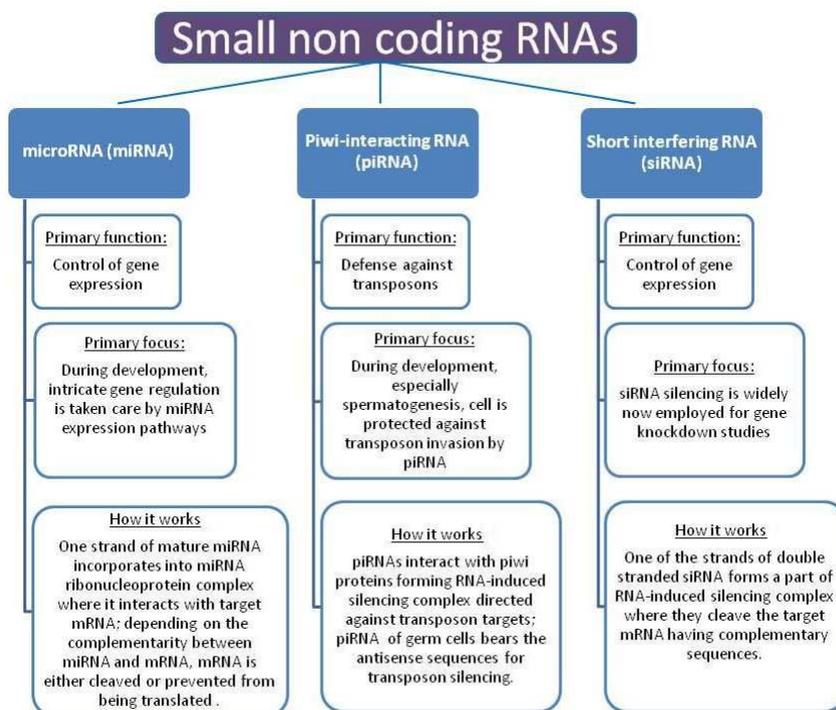
Small non coding RNAs

microRNA (miRNA)

Primary function:
Control of gene expression

Primary focus:
During development, intricate gene regulation is taken care by miRNA expression pathways

How it works
One strand of mature miRNA incorporates into miRNA ribonucleoprotein complex where it interacts with target mRNA; depending on the complementarity between miRNA and mRNA, mRNA is either cleaved or prevented from being translated.

Piwi-interacting RNA (piRNA)

Primary function:
Defense against transposons

Primary focus:
During development, especially spermatogenesis, cell is protected against transposon invasion by piRNA

How it works
piRNAs interact with piwi proteins forming RNA-induced silencing complex directed against transposon targets; piRNA of germ cells bears the antisense sequences for transposon silencing.

Short interfering RNA (siRNA)

Primary function:
Control of gene expression

Primary focus:
siRNA silencing is widely now employed for gene knockdown studies

How it works
One of the strands of double stranded siRNA forms a part of RNA-induced silencing complex where they cleave the target mRNA having complementary sequences.

Fig. 1. Diversity of snc-RNAs

cattle genome sequence provides the resource for *in silico* miRNA and target prediction (Elsik et al., 2009). The bovine miRNA genes have been annotated based on the sequence similarity with other species miRNA (Strozzi et al., 2009). The miRNA expression pattern among 11 tissues revealed that most miRNAs are ubiquitously expressed while few are tissue specific (Jin et al., 2009). The role of miRNAs has been elucidated to understand the developmental processes and gene regulation, for example, miR-133 plays a role in the development and physiology of the rumen (Gu et al., 2007). Embryonic tissues also have a distinct profile of miRNA expression including miR-122a and miR-199a as embryo-specific miRNAs (Coutinho et al., 2007). Further, miRNAs were mapped to find how they are clustered in the chromosome (Jin et al., 2009). Depending on miRNA clustering and functionality they may serve as potential molecular markers. Finally, the study on miRNA expression in cattle is important because it serves as a mammalian model organism and their functional genomics are comparative. Conserved miRNAs among mammalian species are expected to have similar evolutionary roles.

All these seminal studies indicate the need to unravel the indispensable role of small non coding RNAs, especially miRNAs and piRNAs in regulating the germ cell development and beyond. With the increasing association of male infertility with genetic and epigenetic factors, exploring the regulatory events towards spermatogenesis and following fertilization will be worthwhile.

2.3.3 Long non coding RNAs (lnc-RNAs) of spermatozoa

In the diverse RNA species, a small number of long RNA transcripts are assigned with functions other than protein coding. This family of RNA is called long non-coding RNAs (lncRNAs). From the genome, these RNAs are transcribed as interlaced and overlapping transcripts that are greater than 200 nt in length (Carninci et al., 2005). Although it is doubtful whether the long non-coding RNAs are biologically meaningful or merely transcriptional ''noise'', few long ncRNAs have been functionally characterized. Experimental observations suggest that these ncRNAs contribute to the complex networks of gene regulation involved in cell functions (Mattick, 2001). Mercer et al have identified 849 long transcripts with little or no protein-coding potential that were transcribed in the adult mouse brain. They are observed to be posttranscriptionally modified and exhibit highly specific expression, suggesting that these transcripts are functional and are likely to play a role in cell function (Mercer et al., 2008). Dosage compensation in mammals involves X-chromosome inactivation (XCI) mediated by Xist, a lncRNA (Penny et al., 1996). Xist mediated transcriptional inactivation of one X chromosome is the result of stable high level Xist expression in inactive X chromosome (Panning et al., 1997).

The non-coding RNAs which take up either housekeeping or regulatory role are evolutionarily conserved. Analyses of 3,122 long ncRNAs from the mouse showed a proportion of conservation in their sequence which comparable to the density of exons within protein-coding transcripts (Ponjavic et al., 2007). Two long ncRNAs associated with trimethylated H3K4 histones and histone methyltransferase MLL1 in mouse embryonic stem cell are shown to be developmentally regulated, contributing to the pluripotency of the cell (Dinger et al., 2008). Quantitative real-time RT–PCR analysis of 15 long, conserved non-coding transcripts was performed in different normal human tissue and in breast and ovarian cancers. The results showed altered expression of many of these non-coding transcripts in both cancer types. This study indicates that lncRNAs may play an important

lncRNA	Organism	Function	Protein partner	References
steroid receptor activator RNA (SRA)	Mammals	NR co-activator	SRC-1, SLIRP, SHARP, SKIP, and others	(Lanz et al., 1999)
Evf-2	Mouse	Activator of DLX-5/6 enhancer	Dlx-2	(Feng et al., 2006)
7SK small nuclear RNA	Human cell lines	Suppressor of P-TEFb (general transcription inhibition)	HEXIM-1	(Yik et al., 2003)
B2 RNA	Mouse	General transcription inhibitor	RNA polymerase II	(Espinoza et al., 2004)
6S RNA	Bacteria	General transcription inhibitor	Bacterial RNA polymerase	(Trotochaud and Wassarman, 2005)
heat shock RNA-1 (HSR1)	Mammals	Activator of HSF1 (master regulator of heat shock genes)	eEF1A	(Shamovsky et al., 2006)

Table 1. Few long non-coding RNAs and their roles in transcription regulation. Adapted from Shamovsky and Nudler, 2006

function in both normal cells and in cancer development (Perez et al., 2008). Owing to the functions of lncRNA in cellular regulation and development, Lee et al has suggested that lncRNA may also play a role in male germ cell development (Lee et al., 2009). Recently, a novel non-coding RNA has been identified in the rat epididymis, named HongrES2. It is a 1.6 kb mRNA-like precursor that gives rise to a new microRNA-like small RNA. Upon over-expression of this small RNA in the cauda epididymis, a reduced expression of CES7 protein is observed. CES7 is a carboxylesterase with cholesterol esterase activity, which is necessary during sperm capacitation process. Also, tyrosine phosphorylation of the sperm protein during capacitation was affected by high expression of this microRNA-like small RNA indicating the requisite of low level expression for normal sperm maturation process in epididymis (Ni et al., 2011). In summary, although there exist significant numbers of lncRNAs in the body, their expression dynamics and functions in sperm are not well studied.

2.4 Methylome of sperm DNA

Epigenetics is the study of changes in gene expression without causing a change in the structure of DNA. Genes may be over or under-expressed which could be beneficial or detrimental to the organism depending on what genes are being activated or silenced. Epigenetics can shed some light on how non-genetic factors such as environment and nutrition can influence gene expression. There are several mechanisms involved in epigenetics; such as chromatin re-modeling, and the most studied epigenetic phenomenon, DNA methylation. In the following section, DNA methylation in sperm and its impacts on fertility will be described.

Cytosine methylation is the most common covalent modification of DNA in eukaryotes. DNA methylation is essential for normal development and is associated with a number of key processes including genomic imprinting and X-chromosome inactivation. In the course of gametogenesis, imprinting of parental (paternal and maternal) allele or alleles occurs, which is closely associated with the methylation of cytidines. DNA methylation involves the addition of a methyl group to the cytosine, which occurs in the CpG islands, regions composed of cytosine and guanine. DNA methylation often leads to transcriptional silencing of a gene (Zilberman and Henikoff, 2007). Either the loss or gain of methylation at specific genes can lead to developmental abnormalities (Gehring and Henikoff, 2007). Despite the clear importance of DNA methylation, the extent to which changes in DNA methylation are involved in mammalian gene regulation is unclear (Goll and Bestor, 2005). There have been a number of studies done on DNA methylation, varying from the mechanism of methylation itself to its different methods of study and the analysis of the methylation results. The role of DNA methylation in the regulation of mouse and human insulin gene expression was examined (Kuroda et al., 2009). These findings suggested that insulin promoter demethylation may play a crucial role in beta cell maturation and tissue specific insulin gene expression. A high correlation between DNA methylation and chronological age has been described; and interestingly, these highly correlated associations take place close to genes involved in the DNA binding and transcription process (Hernandez et al., 2011).

Although differential DNA methylation has been demonstrated in human and chimp sperm, associations of specific DNA methylation patterns with male fertility have not been

adequately investigated. De novo methylation is known to occur during germ cell maturation and spermatogenesis and this creates hypomethylated regions in the male germ line that are associated with promoters; especially sperm-specific promoter regions that are associated with germ cell functions at distinct stages of spermiogenesis (Houshdaran et al., 2007). With the use of immunostaining, the connection between DNA methylation and fertility was attempted, however, no association was found between sperm DNA methylation and fertilization rate or embryo quality (Benchaib et al., 2005). Houshdaran et al. (2007) suggest that if erasing the initial methylation pattern in epigenetic reprogramming does not occur correctly, that there is a possibility of epigenetic defects in the sperm that would ultimately affect male fertility. Additionally, Henckel et al. (2009) suggest that there is a mechanistic link between DNA methylation and histone methylation in which H3.3 is required for DNA methylation. Brykcznska et al. (2010) suggest that molecular genetic experiments will be needed to explain the extent and functional significance of methylation at specific histone loci.

Generation of genome-scale DNA methylation profiles at nucleotide resolution in mammalian cells has become as a powerful technology for epigenetic profiling of cell populations (Meissner et al., 2008). There are three different methods for studying DNA methylation; bisulfite conversion, methylation–sensitive restriction enzymes, and affinity purification. It is known that methylated cytosines and unmethylated cytosines have similar characteristics and it is hard to discern which is which with standard sequencing (Zilberman and Henikoff, 2007). In order to overcome this obstacle, the bisulfite conversion test can be performed on the sperm by treating the DNA with bisulfite which converts non-methylated cytosine residues to uracil, but leaves methylated cytosine residues unaffected. PCR amplification of converted DNA replaces the uracil with thymine and analysis of the PCR product can be used to quantify the extent of methylation at each cytosine (Eckhardt et al., 2006). A second method for studying DNA methylation is by by the use of methylation–sensitive restriction enzymes. This method cleaves DNA at specific methylated-cytosine residues in CpG islands which have lost their methyl group, leaving only hypermethylated DNA intact (Yegnasubramanian et al., 2006). Digestion is followed by amplification of the products, and they can be detected when digestion of the products are inhibited by presence of methylation. The third method of study is the affinity purification and is the most recently discovered. This method takes advantage of the methyl-binding domain (MBD) that binds to the methylated CpG sites. A MBD that has been attached to a CG site can be expressed in *E. coli* and affinity purified and then methylated DNA can be purified (Zhang et al., 2006).

2.5 Proteome of spermatozoa

Proteins and their cofactors, located both in the cytoplasm and on the membrane play important roles at different stages of gametogenesis, fertilization and embryonic development. Cell-cell interaction is the main function of membrane proteins during fertilization. The participation of such proteins in sperm, especially cytoplasm proteins injected into the occytes, are required for egg activation and subsequently early embryonic development. An area of focus for some key players is that of sperm-egg fusion. The integrin, a disintegrin and metalloprotease (ADAM) and Protein Kinase C (PKC) families are principal groups of proteins significant for fertilization and its succeeding steps. Current knowledge and understanding of molecular mechanisms of the proteomics during sperm

egg fusion as well as the complicated networks of these proteins throughout embryonic development is still at the dawn of investigative process. Pioneering experiments have however been able to classify some specific proteins as potential molecular markers of fertility. Among them are Fertilin α and β. Both Fertilin α (ADAM1) and Fertilin β (ADAM2) are members of the ADAM family and together form a heterodimer. Fertilin β was initially discovered through initial implications of being the antigen to the antibody PH30 that prevented the fertilization of Guinea pig oocytes (Primakoff et al., 1987). There are a total of five members of the ADAM family that have been discovered. All of which are localized in the testis in the mouse (Heinlein et al., 1994; Wolfsberg et al., 1995). These proteins have been implicated as essential contributors during sperm egg fusion. Results of experiments conducted by Cohen et al. (2008) showed appearance of CRISP1 involvement in the first step of sperm binding to the zona pellucida. They also observed that sperm testicular CRISP2 is also able to bind to the egg surface which is indicative of its necessity in gamete fusion.

Massive changes in transcription and translation occur during spermatogenesis (Alcivar et al., 1990; Hake et al., 1990; Hecht, 1988). Considering the limited contents in the sperm, proteins that exist in the spermatozoa are speculated to have importance in sperm maturation and fertilization. Jmjd1a, for example, is a regulator of histone modification through demethylation in the testes, and its deficiency induced many infertile symptoms, indicating the essential role in spermatogenesis (Liu et al., 2010). In spermatids, Kinesin family member C 1 (KIFC1) motor which is a member of the Kinesin-14 subfamily associates with nucleoporin-containing complex and is postulated to be involved in acrosome elongation and motility (Yang et al., 2006; Yang and Sperry, 2003). TLRR is a leucine-rich repeat protein which interacts with KIFC1 (Wang and Sperry, 2008). Associating with the manchette, TLRR binds with testis specific isoform of protein phosphatase-1 (PP1) and was suggested to take part in cytoskeleton modulation (Wang and Sperry, 2008) Izumo was identified through the production of monoclonal antibody that inhibited sperm–egg fusion (Okabe et al., 1987; Rubinstein et al., 2006). The expression of Izumo was found to be testis-specific and was not detectable on the surface of fresh sperm but only became apparent after initiation of the acrosome reaction (Rubinstein et al., 2006)

In addition, a number of enzymes mediating the process of spermatogenesis have also been discovered. The Testis Specific Serine Kinases (TSSKs) are a collection of kinases in germ cells or in testis, which include TSSK1, TSSK2, TSSK3 and others (Bielke et al., 1994; Blaschke et al., 1994; Kueng et al., 1997; Zuercher et al., 2000). Since Specific Serine Kinases are only synthesized postmeiotically in spermatogenesis, they were proposed to act during sperm maturation, capacitation and fertilization. Sosnik et al. demonstrated Tssk6's essential role in maintaining sperm structural integrity and suggested its function to regulate actin dynamics (Sosnik et al., 2009). Trypsin is another enzyme that is active during spermatogenesis. In addition to being secreted into the intestine to digest proteins, trypsin also functions in the initiation of meiosis, spermiogenesis, and fertilization (Miura et al., 2009). Miura et al. also implicated the involvement of trypsin in the entry site of the meiosis cycle. The proteins mentioned here are sperm specific for involvement in the fertilization process. Their continuous investigation further proves the complexity of the multifaceted fertilization process on a molecular level. Moreover, it rationalizes the necessity for incessant investigation into the mounting issues of subfertility in males across species.

2.5.2 Approaches for the study of sperm proteomes

To date there are several popular and accepted techniques utilized in proteomic studies to investigate sperm proteomes related to sperm functions such as motility, oocyte-sperm fusion, and fertilization. Since sperm is transcriptionally silent, proteomic approaches provide precious information on sperm proteins coded during spermatogenesis. The most common method SDS-PAGE, the precursor to 2D-DIGE, produces the direct and global two dimensional views of several hundred proteins from a sample. Its combination with dyes and software achieves the precise and fast process of an image. Western Blotting and Immunocytochemistry are primarily used to detect the quantity and localization of the target proteins, respectively. Elemental composition of proteins can be determined by mass spectrometry techniques. It is well documented that many sperm proteins that remain as remnants in spermatozoa are critical for fertilization and embryo development. Collectively these techniques have aided in the advancement of sperm-specific protein identification as well as provided an enhanced understanding of protein function in motility, capacitation, acrosome reaction and fertilization (du Plessis et al., 2011).

2.5.2.1 SDS-PAGE electrophoresis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) is a proteomic approach in biochemical experiments to separate proteins. SDS-PAGE was first published as a method in 1970 (Laemmli, 1970). The principle of SDS-PAGE relies on the same attachment of SDS in each unit of peptic length, which induces the same charge per mass unit. Proteins are denatured by SDS, which is an anion detergent and rounded with negative charge. Therefore, the charge of protein is relative with its size which enables researchers to separate proteins according to their molecular weight. The negatively-charged proteins migrate across the gel towards the anode in the electric field. The running system is determined by the concentration and size of the gel and molecular weight of protein samples. Following electrophoresis, Coomassie Brilliant Blue R-250 is commonly used to stain and visualize the proteins on the gel. The molecular weight of target proteins can be approximated in the gel by markers whose molecular weights have already been determined. The amount and purity of protein samples can be viewed according to the densities and numbers of the bands using software. The blotting application in addition to SDS-PAGE expands its usage. However, unlike native electrophoresis, SDS-PAGE cannot be applied to keep protein's native state due to the presence of the detergent. A limitation of this technique is the inability of proteins with similar or same molecular weight to be differentially separated; in addition SDS-PAGE is not a suitable method for the detection of low-molecular weight proteins. Despite minimal shortcomings, SDS-PAGE is a useful and critical tool for proteomics as a result of its broad application in protein size modification, protein identification, purity examination and protein quantification.

2.5.2.2 Two-dimensional differential in gel electrophoresis (2D- DIGE)

Using the same fundamental principles of SDS-PAGE, two-dimensional differential in-gel electrophoresis (2D-DIGE) was developed to separate proteins by molecular weight as well as isoelectric point (PI). The 2D-DIGE technique was not described in literature until 1975 (O'Farrell, 1975). In 1997, 2D-DIGE was developed to run more than one sample by pre-labeling samples with fluorescent cyanine dyes (Unlu et al., 1997). Trichloroacetic Acid in Acetone Protocol (TCA precipitation) or Bio-Rad protein assay is carried out to extract proteins. Accuracy during quantification in experiments possessing more samples is better

determined by use of an internal control. A linear or nonlinear (Bjellqvist et al., 1993) program is run following the loading of samples onto the IPG strips. The first dimension will separate the proteins according to their PI. In the second dimensional step, IPG strips are loaded on the SDS-PAGE where proteins will be separated again by their charge or molecular weight. To visualize the protein spots, both Coomassie Brilliant Blue and silver staining (Merril et al., 1981; Oakley et al., 1980) are utilized. However, neither stain possesses the quality or sensitivity of detection methods that rely on fluorescent compounds and radiolabeling of proteins (Gorg et al., 2004). The diluted protein samples and internal controls are pre-labeled with the fluorescent cyanine dyes (CyDye, Cy2, Cy3, and Cy5 separately) before being loaded onto the IPG strips so that groups of samples can be visualized individually at different wavelengths.

The length of IPG strip varies from 7-24 cm and its pH range is between 2.5 and 12. More than 5,000 proteins can be resolved in the gel and this technique can detect as little as 1 ng of protein per spot (Gorg et al., 2004). This is the only technique to parallel large sets of protein mixtures. The protein expression level, isoforms or modification can be reflected from the protein mapping. Since the dyes cannot bind to the proteins without lysine, 2D-DIGE has varying sensitivity towards proteins too few or too many lysine.

2.5.2.3 Western blotting (also known as immunoblotting)

Protein signals can be detected through antibody specific binding in Western Blotting. The antibodies bind to target proteins which work as antigens on a membrane and emit signals by the conjugated enzymes. SDS-PAGE electrophoresis in addition to a positive control is needed so that the proteins can be separated on the gel and later transferred to a membrane. The positive control works as a marker to confirm the existence of target protein. The transfer process can be monitored by reversible staining or Ponceau S staining of the membrane. Transfer of proteins from the gel onto the membrane can be developed on several apparatuses. Wet transfer is less likely to possess background due to the drying of the membrane. It has been suggested that large proteins (>100 kDa) are transferred more effectively during wet transfer. By utilizing the semi-dry transfer, transfer time can be reduced to an hour or less and is currently the most widely used method. Dry transfer takes approximately eight minutes or less, however, the application is restricted by its transfer efficiency. Nitrocellulose and Polyvinylidene fluoride (PDVF) are the most popular membranes in Western blotting. Fixing or staining of gels will lower the efficiency of transfer, especially for the proteins more than 50 kDa (Perides et al., 1986). Post transfer staining might be applied by using compatible reversible stains such as Ponceau-S Red, CPTS (Copper Phthalocyanine Tetrasulfonic Acid) amido Black and Spyro Ruby (need laser to visualize) or non-compatible stains like Coomassie Brilliant Blue and Colloidal Gold (known to block some epitopes) (Millipore). In order to prevent non-specific binding and to decrease the occurrence of background noise during development, the membrane is incubated in blocking buffer at room temperature. Traditional blocking buffer is usually produced by adding blocking agent (0.5% casein, 5% nonfat milk and 5% Bovine serum albumin) in TBS (50 mM Tris.HCl, pH 7.4 and 150 mM NaCl). Followed by three rounds of washing, the membrane is then incubated with primary antibody, which binds to the target protein. Following washing, secondary antibodies conjugated with horseradish peroxidase or alkaline phosphatases are added to the membrane. The horseradish peroxidase or alkaline phosphatase enzymes react with substrates and emit light or other detectable signal. Final signals can be detected and visualized by X-ray film or digital images.

Therefore, electrophoretic separation of proteins cannot be examined by staining the gel until completion of the transfer step. The capacity of analyzing and identifying target proteins has led to the popularity of Western Blotting. Protein concentration can be quantified by implementing this technique according and calculation of subsequent signal intensity. However, it cannot show the localization of this protein in the cell, which can be performed by immunocytochemistry. Although Western Blotting has been widely utilized, its reliability is reduced by its nonspecificity and possible degradation of target protein causing visualization of multiple bands.

2.5.2.4 Immunocytochemistry

Immunocytochemistry is used to visualize the presence and localization specific proteins and other molecules within the cell. It is further also utilized for the investigation of protein processing and interaction. The application of immunocytochemistry is expanded by combination with other techniques, such as Formaldehyde-Induced Fluorescence, Autoradiography and Microinjection. However, since the immune system is adjustable and flexible, immunocytochemistry is questioned for its specificity. Secondly, immunocytochemistry cannot characterize the charge, size or hydrophobicity of the molecules (Larsson and Sjoquist, 1988). Compared to other immunoassays, it is expensive and difficult to quantify the signals to determine the amounts of proteins expressed.

2.5.2.5 Mass spectrometry

Mass spectrometry (MS) is a powerful technique used to ionize and determine elemental composition regarding mass-to-change ratio. An ion source, mass-selective analyzer, and an ion detector are the three chief modules in a MS instrument. The procedures in this analytical technique generally include five steps. First, a sample is vaporized prior to an ion source. Secondly, the ion source converts gas phase sample molecules into positively charged ions. Then the magnetic field in the instrument accelerates all the ions. The high speed ions are finally separated based on various masses. Lastly, the detector collects the data for analysis.

Tandem mass spectrometry is the main MS employed to identify proteins. Electrospray ionization (Elsik et al. 2009) and matrix assisted laser desorption ionization (MALDI) are two other major developed methods of MS, which are able to analyze molecules of very high molecular weight. The importance of mass spectrometry is not limited to protein identification but rather expands to its wide application in isotope dating, atom probe, protein characterization and so on. Mass spectrometry is a high throughput technique coupled with other techniques, such as SDS-PAGE and 2D-DIGE. The combination of MS and liquid chromatography (LC) enhances protein separation and identification of more than 1,000 proteins simultaneously (Liu et al., 2004; Peng et al., 2003; Sadygov et al., 2004; Washburn et al., 2001). However, MALDI has its limitation of protein quantification due to its nonlinear signal intensity and ESI-MS/MS has a high requirement of high purity protein samples and poor tolerance for electrolytes and detergents (Barnidge et al., 1999).

2.6 Proteome of seminal fluid

As mentioned previously, sperm proteins (D'Amours et al., 2010), DNA integrity (Ward, 2010) and mRNA profile (Lalancette et al., 2008a) are some of the sperm components that

influence male fertility, and the seminal plasma is the principal medium enclosing the sperm. Seminal fluid contains a diverse cohort of proteins, including chaperones, proteins with redox activity, ion and phospholipid-binding proteins, carriers of lipophilic substances, glycosidases, proteases and protease inhibitors, among others, which in turn modulate numerous functions, from sperm protection, capacitation and acrosome reaction to sperm-oocyte interaction and fertilization. Knowledge of seminal plasma composition is more advanced for the bovine than for other farm animals and, for this reason, the present review will focus on the bovine, unless otherwise specified.

Proteins acting to prevent oxidative stress and immune reactions are expressed in the seminal plasma. Sperm usually produce reactive oxygen species (ROS) as part of their normal physiology (Aitken et al., 2004) but, if in excess, ROS have detrimental effects on spermatozoa. Thus, antioxidant systems must be present in the reproductive fluids to control these effects. Proteins participating in such systems include albumin, acidic seminal fluid protein (aSFP), glutathione peroxidase and transferring, among others. Albumin is present in both cauda epididymal and accessory sex gland (ASG) secretions of the bovine (Moura et al., 2007b; Moura et al., 2010) and its protective effect on sperm comes from its ability to absorb lipid 22 peroxides (Alvarez and Storey, 1995). Bovine aSFP is a typical component of the ASG fluid (Moura et al., 2007b; Wempe et al., 1992), although evidence exists that it is expressed in the cauda epididymal fluid as well (Moura et al., 2010). aSFP has redox activity superior to that of glutathione peroxidase (Einspanier et al., 1993; Schoneck et al., 1996), binds to ejaculated bovine sperm (Dostalova et al., 1994) and inhibits sperm motility in a reversible manner (Schoneck et al., 1996). Thus, aSFP protects sperm against oxidative stress in both pre- and post-ejaculation media and its inhibition of motility may be of importance during sperm storage in the cauda epididymis. Besides reactive oxygen species, transitional metal ions, like iron, can generate lipid peroxide radicals, with potential damage to sperm integrity (Agarwal et al., 2005; Wakabayashi et al., 1999). As a consequence, the expression of transferrin in the seminal fluid is a strategy to deal with excessive iron. Moreover, through its ability to bind iron, an essential ion for bacterial growth, transferrin represents a line of defense against pathogenic microorganisms (Farnaud and Evans, 2003). Most studies classically define transferrin as an epididymal protein (Belleannee et al., 2011; Moura et al., 2010) but it has also been detected in the seminal vesicle fluid of rams (Souza et al., 2011). Clusterin is another seminal protein designated to protect sperm and appears as multiple isoforms in the bovine cauda epididymal (Moura et al., 2010) and accessory sex gland fluid (Moura et al., 2007b). It prevents oxidative damage to cells (Reyes-Moreno et al., 2002), agglutinates abnormal spermatozoa in bulls (Ibrahim et al., 1999) and acts like a chaperone, protecting sperm from the toxic effects of protein precipitation (Humphreys et al., 1999). Clusterin has the ability to inhibit complement-induced sperm lysis (Ibrahim et al., 1999; O'Bryan et al., 1990), another form to preserve sperm cell integrity.

Proteins of the seminal fluid play important roles in capacitation, one of the early events occurring after sperm comes in contact with ASG secretions. In the bovine, Binder of Sperm Proteins (BSP) represents the most abundant group of seminal fluid proteins (Manjunath et al., 2009; Moura et al., 2007b). BSPs are characterized by two tandemly-arranged, fibronectin type II domains, which bind to membrane phospholipds (Kim et al., 2010) and induces cholesterol efflux, a crucial step in capacitation (Manjunath and Therien, 2002). Moreover, bovine BSPs mediates the interaction between sperm and the

oviductal epithelium and contributes to sperm survival in that region of the female tract (Gwathmey et al., 2006). Interestingly, homologues of BSPs also represent the major constituents of seminal plasma from other ungulates, such as *Bos indicus* bulls (Moura et al., 2010), and rams (Rego et al., 2011). Albumin is another seminal fluid protein that affects sperm capacitation through its ability to modulate membrane efflux of cholesterol (Go and Wolf, 1985; Visconti and Kopf, 1998).

Phospholipase A_2 (PLA$_2$) and osteopontin (OPN) are two of seminal plasma proteins participating in acrosome reaction and fertilization-related events. PLA2 exists as a secreted form in the seminal plasma (Moura et al., 2007a; Soubeyrand et al., 1997) and in membrane extracts from bull sperm (Ronkko et al., 1991). Sperm-anchored PLA$_2$ synthesizes arachidonic acid, which is converted to prostaglandin E_2, a step leading to acrosome reaction. Sperm PLA$_2$ is also implicated in sperm-egg fusion (Yuan et al., 2003) and secreted PLA$_2$ stimulates cytokine release by immune cells (Granata et al., 2005), exerting antimicrobial action in the seminal plasma (Bourgeon et al., 2004; Weinrauch et al., 1996). Osteopontin is typically involved in cell adhesion, tissue remodeling and activation of intracellular signaling (Denhardt, 2004; Mazzali et al., 2002). OPN comes mainly from accessory sex glands (Cancel et al., 1999) and binds to sperm at ejaculation, as observed for the BSPs (Souza et al., 2008). Information about OPN roles in male reproduction has been demonstrated by experiments using in vitro fertilization (IVF). In this regard, incubation of bovine oocytes with OPN or fertilization of oocytes with sperm previously treated with OPN enhanced fertilization, cleavage rates and blastocyst development at days 8 and 11 (Goncalves et al., 2008; Monaco et al., 2009). Following this same conception, studies conducted with the swine species confirmed that OPN, when added to IVF media, also increased fertilization and embryo survival (Hao et al., 2006). These results indicate that OPN works during sperm-oocyte interaction and even influences post-fertilization events, although by mechanisms not yet completely understood. Given OPN´s biochemical features, it is plausible that OPN adheres to sperm and this complex binds to the oocyte, facilitating sperm penetration, fusion and fertilization, as suggested before (Souza et al., 2008). Also, interaction of sperm OPN with integrins and/or CD44 receptors in the oocyte could trigger intracellular signaling and affect early embryo development (Souza et al., 2008).

Several proteases and glycolytic enzymes are present in the seminal plasma and reportedly take part in remodeling of sperm membrane components during epididymal transit and capacitation. Proteases and constituents of the seminal plasma that interact with the extracellular matrix act during mammalian fertilization as well, when sperm contacts and crosses barriers formed by cumulus cells and the zona pellucida. Proteins belonging to these categories include enolase, fucosidases, glucosaminidases, galactosidases, leucine aminopeptidase, dipeptidyl peptidase, matrix metalloproteinase 2, angiotensin-converting enzyme, plasma glutamate carboxipeptidase, arylsulfatase A, cathepsins, among others. As expected, activity of these enzymes needs to be modulated to avoid undesirable cleavages and protect tissue integrity, and protease inhibitors are in fact secreted by the seminal plasma, such as tissue inhibitor of metalloproteinase 2, alpha 2 macroglobulin, serine protease inhibitors and cystatin (Kelly et al., 2006; Moura et al., 2007b; Moura et al., 2010).

As a typical fluid, the seminal plasma contains proteins with affinity to lipophilic substances, such as apolipoprotein A1 (Apo-A1), epididymal secretory protein E1 (also known as cholesterol transfer protein) and prostaglandin D-synthase (PGDS). Apo-A1 is

part of the high-density lipoprotein complex (Rader, 2006) and interacts with ejaculated sperm. HDL present in the female reproductive tract is involved in sperm capacitation (Therien et al., 1997) but it is still unknown if Apo-A1 directly contributes to that event. Epididymal secretory protein E1, the second most abundant protein in the bovine cauda epididymal fluid (Moura et al., 2010) , binds to sperm (Kirchhoff et al., 1996) and alters the ratio of cholesterol and phospholipids of membranes, which occurs during sperm capacitation (Manjunath and Therien, 2002). PGDS, in turn, is a typical component of the epididymis and distinctively binds to the upper region of acrosome of both epididymal and ejaculated bull sperm. PGDS may act as a transporter of retinol and other lipophilic substances in the epididymis and semen (Leone et al., 2002) but how this putative role exactly affects sperm function is still unclear.

2.6.1 Approaches for the study of seminal proteome

Usually, approaches to analyze the seminal fluid proteome include 2-D electrophoresis (2-D SDS-PAGE) and/or chromatography, allied with mass spectrometry. Availability of several options of "strips" with immobilized pH and with different sizes (from 7 to 24 cm, in most cases) practically allows endless combinations of methods for electrofocalization of the majority of seminal proteins. For the second dimension, choices of gel mashes separate proteins based on their mass, especially those between 10 and 150 kDa. Typically, 2-D gels of bovine seminal plasma obtained with 18- or 24-cm "strips", within the 3-10 pH range, show at least 400 spots. Higher rates of spot detection are possible using strips with narrow pH intervals for electrical focalization, among other alternatives. Sensitive stains, such as SyproRuby (Bio Rad, USA), help detection of more spots in 2-D gels of seminal proteins but silver-staining has limitations. Silver stains are not saturable and thus, as it stains low abundance proteins, it will also increase the size of the BSP spots, reaching a point where these spots overshadow large sections of the gels. Despite the valuable pieces of information generated by 2-D SDS-PAGE, this method still limits the detection of low abundance proteins expressed in complex samples, such as the seminal plasma, or those that are too acidic or too basic, or with kDa values below or above 10 and 200, respectively. The gel-based method provides a quantitative "snapshot" of protein expression that can be analyzed and compared among different samples by imaging software (Gorg et al., 2004). The capacity of measuring this relative abundance has been improved with the development of two-dimensional differential in-gel electrophoresis, which allows distinct samples to be separated within a single map (Unlu et al., 1997). This method reportedly reduces the between-gel variability and allows more precise comparisons between samples related to contrasting phenotypes or treatments. Chromatography is another method for protein separation, using matrices such as ion-exchange, gel-filtration, affinity and multidimensional (Stroink et al., 2005). Given the complexity of seminal plasma, pre-separation of samples by chromatography enhances the detection of components during proteome-based studies. For instance, separation of seminal proteins in a gel-filtration column enhances spot detection in 2-D gels, given that a "zoon" is formed at different kDa ranges. This strategy isolates the BSP proteins in specific sections of the gels, allowing detection of low abundance proteins in others. The seminal constituents can also be divided based on biochemical attributes, such as heparin- and gelatin-biding capacity. After

separation of proteins, either by 2-D SDS-PAGE alone or coupled with chromatography, identification of spots is mandatory and today mass spectrometry remains the cost effective technique for this purpose. N-terminal can be used to corroborate results from that method. In conclusion, several approaches are available for separation of proteins into comprehensive maps, relative quantification, and identification of such components, which are the foundations to establish the seminal plasma proteome.

3. Conclusion and future directions

Conventional evaluation of sperm biophysical characteristics has a large number of limitations which have not been successful to identify sub fertile or high fertile semen so far. While differences in lifetime fertility among males is the biggest concern, the day to day variation in semen quality causes major economic losses to animal breeding companies when expensive semen must be discarded. Identification of molecular determinants of fertile spermatozoa has great potential for enhancing the economic well-being of animal breeding companies and human IVF laboratories. In animal agriculture, these molecular fingerprints will allow us to assess semen quality which is more powerful than any previous sperm selection criteria, and should provide a foundation for early identification and selection of animals with superior genetics in the animal breeding and for male factor human infertility. Implementation of new approaches is crucial to reduce the cost of male fertility prediction, increase sire fertility and increase the overall financial margins in the dairy industry.

Chromatin structure and integrity of spermatozoa DNA is associated with sperm viability and thus fertility. Development of reliable molecular tests that provide quantitative measures of abnormalities in chromatin structure and DNA damage is expected to be a significant advance in predicting fertility. Diverse sets of transcripts in human and livestock spermatozoa have been well documented. Due to the low amount of transcription and translation in the spermatozoa, the "meaning" of these transcripts remains a mystery. These transcripts might play important roles during spermatogenesis, in egg activation and/or sustaining early embryonic development. Regardless of the significance of these transcripts in mature spermatozoa, there is a potential for spermatozoa transcripts to be used as predictors of sperm viability.

DNA methylation has been speculated for many years to have a close association with reproduction and fertility. Benchaib et al. (2005) attempted to determine the relationship between sperm DNA methylation level and fertilization and pregnancy rates. It was found that DNA methylation level in human sperm could represent a new approach to study the ability of sperm to lead to pregnancy. Jammes et al. (2011) proposed that in humans, abnormal epigenetic programming, via DNA methylation, is a possible mechanism compromising male fertility. Another study with Houshdaran et al. (2007) proposed that abnormal epigenetic programming of the germline is a possible mechanism compromising spermatogenesis of some men currently diagnosed with infertility (Houshdaran et al., 2007). Additional studies are necessary to clearly define the roles of DNA methylation in fertility.

A number of spermatozoa proteins have been identified using proteomics approaches followed by reductionist methods. Researchers have determined differentially expressed

proteins in sperm from males with high and low fertility; enabling a list of proteins to be used as potential markers to predict fertility. Further validation of these proteins as well as posttranslational modifications of these proteins is important for considering these as reliable biomolecular markers. Because seminal plasma proteins play vital roles in sperm physiology and fertilization, studies have evaluated empirical associations between semen criteria, such as sperm motility of fresh or frozen semen, and seminal constituents as well. A classic study published in 1993 (Killian et al., 1993) showed that fertility of Holstein bulls was related to the relative abundance of four protein spots depicted in seminal plasma 2-D gels, two of which were identified as osteopontin (OPN) and prostaglandin D-synthase (Cancel et al., 1997; Gerena et al., 1998). This study was conducted with 35 bulls and later on, the evaluation of accessory sex fluid proteome from other 37 Holstein bulls confirmed that OPN had greater expression in high fertility than in low fertility sires. In contrast to BSP 30 kDa (now named BSP 5) and PLA_2 spermadhesin Z13 is inversely related to fertility. Similar results were obtained in the swine species (Hao et al., 2006). Other studies have identified a seminal fluid, 30-kDA heparin-binding protein as a marker of bull fertility. This protein binds to sperm and was first named "Fertility Associate Antigen" and later identified as a DNAse I like protein (Bellin et al., 1996; Bellin et al., 1998).

In conclusion, sperm viability is surely determined by multifactor mechanisms, from those inherent to the cells to constituents of seminal proteins. Because fertility is a complex trait with low inheritance rate, studies on both genetic and epigenetic aspects of sperm viability are crucial in understanding the nature and mechanisms regulating male fertility. The molecular attributes of the spermatozoa and the methods including the *"omics"* approaches reviewed in this chapter are certainly testaments for great progresses. However, much needs to be accomplished to fully understand the complexity of male fertility and develop ways by which to diagnose and prevent infertility. For example, there is a need for systems physiology of sperm where all components of sperm viability, i.e., spermatozoal proteins, transcripts, small molecules, and chromatin dynamics should be all considered to determine "the best spermatozoa". It would then be possible to include all of these biomolecular markers in a comprehensive evaluation scheme to define potential male fertility.

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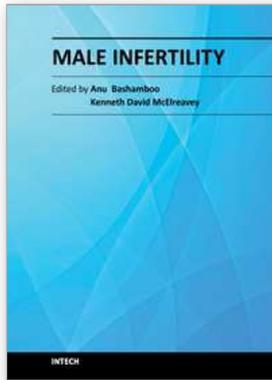
Male Infertility

Edited by Dr. Anu Bashamboo

Male infertility is a multifaceted disease where genetic, epigenetic and environmental factors all contribute to the development of the phenotype. In recent years, there has been an increasing concern about a decline in reproductive health, paralleled by an increase in demand for infertility treatments. This calls for a detailed and thorough understanding of normal and aberrant testicular function and the environmental influences on the establishment and integrity of the male germ cell. This is crucial for understanding the complex pathophysiology of male infertility and eventual success of Assisted Reproductive Technologies.

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