August 2014

Rapid Method of Processing Sperm for Nucleic Acid Extraction in Clinical Research

Matthew K. de Gannes
University of Massachusetts Amherst

Rapid Method of Processing Sperm for Nucleic Acid Extraction in Clinical Research

A Thesis Presented

By

MATTHEW K. DE GANNES

Submitted to the Graduate School of the
University of Massachusetts Amherst in partial fulfillment
of the requirements for the degree of

MASTER OF SCIENCE

May 2014

Public Health

Environmental Health Sciences

Rapid Method of Processing Sperm for Nucleic Acid Extraction in Clinical Research

A Thesis Presented

By

MATTHEW K. DE GANNES

Approved as to style and content by:

J. Richard Pilsner, Chair

Alexander Suvorov, Member

Brian W. Whitcomb, Member

Paula L. Stamps, Graduate Program Director
Department of Public Health

ACKNOWLEDGMENTS

It gives me pleasure to mention my advisor, J. Richard Pilsner, for his care and support over the last two years. His constant dedication and eagerness to challenge his students to achieve excellence have been key contributors to both my personal and professional development. I will forever appreciate his invaluable guidance throughout my future endeavors.

I would also like to thank my colleagues from the laboratory- Haotian Wu, Maggie Kwong, Gianna Luchetti, Justin English, Samuel Hildebrand, and Kara Page- who have worked many days and nights to help me perfect the methods described in this manuscript.

I would also like to express gratitude to the members of my committee, Alexander Suvorov and Brian W. Whitcomb, for their helpful comments and suggestions throughout the project.

Thanks are also due to Kathleen Arcaro and her laboratory, for allowing us access to her hemocytometer for counting sperm cells.

Finally, I would like to say a special thank you to all those whose support and friendship helped me to maintain my focus, and provided me with the encouragement to remain strong and work hard when the going got tough.

ABSTRACT

RAPID METHOD OF PROCESSING SPERM FOR NUCLEIC ACID EXTRACTION IN CLINICAL RESEARCH

MAY 2014

MATTHEW K. DE GANNES, B.S., GETTYSBURG COLLEGE

M.S., UNIVERSITY OF MASSACHUSETTS AMHERST

Directed by: Professor J. Richard Pilsner

Background: Sperm contain compact nuclei, inhibiting DNA extraction using traditional somatic cell techniques. Previous methods extracted quality sperm DNA using reducing agents, but with lengthy lysis procedures and no means of stabilizing DNA. These limitations hamper efficient clinical research.

Objective: We sought to optimize an efficient method of extracting high quality, molecular weight DNA from human sperm suitable for clinical research.

Methods: Sperm from semen samples provided by three volunteers were isolated using modified PureCeption Gradient protocol. We tested 1) proteinase K in the presence of DNA/RNA Shield, 2) dithiothreitol (DTT) and tris(2-carboxyethyl)phosphine (TCEP) as reducing agents, 3) QIAshredder for sperm cell homogenization, and 4) the stability of sperm DNA by performing DNA extractions using modified Quick-gDNA MiniPrep protocol on sperm samples immediately (baseline) or after four weeks of storage at $4^{O}C$ in DNA/RNA Shield. DNA was amplified by PCR using *ALU* primers and digested with

Hinf1 restriction enzymes. Imprinted DNA methylation was assessed using MassARRAY.

Results: Treatment with proteinase K produced similar DNA concentrations (30.1±0.28ng/μL and 33.4±0.21ng/μL) compared to without proteinase K in DNA/RNA Shield (28.9±0.00ng/μL and 30.9±0.85ng/μL). No sperm cells were observed after 1 minute with 25mM TCEP treatment compared to 20 minutes with 100mM DTT. Lysis with 50mM TCEP produced greater DNA concentrations (17.2±0.50ng/μL and 21.3±0.71ng/μL) compared to 50mM DTT (12.6±0.28ng/μL and 12.3±0.35ng/μL). QIAshredder with 50mM TCEP increased DNA concentrations (25.9±0.35ng/μL and 21.7±0.49ng/μL) compared to 50mM TCEP alone (18.6±0.99ng/μL and 12.3±0.35ng/μL). DNA concentrations at baseline (36.2±2.75 ng/μL, 32.2±1.38ng/μL, and 44.3±3.93ng/μL) were similar to those after 4 weeks (40.0±2.98ng/μL, 37.6±1.38ng/μL, and 38.7±3.93ng/μL). DNA from both storage times was successfully amplified by PCR using *ALU* primers and efficiently digested with *Hinf1* restriction enzymes. MassARRAY revealed similar percentages of methylation at baseline and 4weeks of storage for *SNURF* (1.43±1.02%, 1.55±0.95%), *PEG10* (3.69±0.66%, 4.28±1.52%), and *H19* (88.93±3.24%, 91.78±2.00%) imprinted loci.

Conclusions: We isolated high quality, molecular weight DNA from human sperm using 5 minute versus ≥ 2 hour lysis in other methods. DNA/RNA Shield stabilized sperm DNA over 4 weeks. Our methods may facilitate efficient clinical research essential to investigate the role of sperm genetics and epigenetics in male reproductive health.

TABLE OF CONTENTS

Page

APPENDICES

LIST OF TABLES

Table Page

LIST OF FIGURES

Figure Page

CHAPTER 1

INTRODUCTION

1.1 The Importance of Sperm to Male Reproductive Health

Epidemiological data estimate that 6-8% of the United States population was infertile between 1982-2010 [1] and male factors contributed to nearly half of these cases [2]. Furthermore, the decline in fertility observed between 1982-2000 was accompanied by an overall decline in human semen quality [3]. Several epidemiological and animal studies have revealed that there are key genetic [4-8] and epigenetic [9-13] factors essential to proper spermatogenesis and male reproductive health. An overview of the spermatogenesis process and the importance of key genetic and epigenetic factors to this process are discussed below.

1.2 Spermatogenesis and Sperm Biology

1.2.1 Stages of Spermatogenesis and Cell Division

In the seminiferous epithelium, germ cells form several concentric layers penetrated by somatic cells called Sertoli cells. The Sertoli cells extend around all the germ cells to nurture and maintain their cellular associations throughout spermatogenesis [14]. Germ cell differentiation is precisely regulated so that the same stages of spermatogonia, spermatocytes, round spermatids, and late spermatids are always found in association [15]. Because the differentiation process is spatially synchronized, a given cross section of seminiferous tubule most often has germ cells at the same stage of spermatogenesis. The presence of regularly repeating cell associations in a given tubule

cross section allowed Leblond and Clermont to identify 14 stages of spermatogenesis in the rat [16] and Oakberg to identify 12 stages in the mouse [17]. The process of spermatogenesis is summarized in **Figure 1** taken from *Anatomy and Physiology* [18].

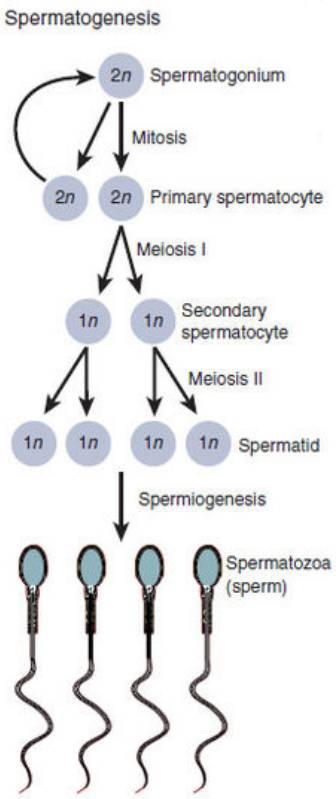

Figure 1: Summary of the process of spermatogenesis from primary spermatocytes to spermatozoa. This summary was taken from *Anatomy and Physiology* [18].

Spermatogenesis is a precisely timed and highly organized process by which haploid spermatozoa are produced from diploid spermatogonial stem cells. The process begins at puberty, after which sperm are produced constantly (200 to 300 million daily) until age 35 in humans, when sperm production begins to slowly decline [18,19]. One cycle of spermatogenesis, from spermatogonia through mature sperm, takes approximately 64 days with a new cycle occurring every 16 days [18]. The cycle begins with mitosis of diploid spermatogonia on the basement membrane of seminiferous

tubules [18]. Type A1 spermatogonia, containing dark nuclei, divide mitotically and reproduce themselves (homonymous division), maintaining the spermatogonia population [20]. Type A2 spermatogonia, containing pale nuclei, may divide mitotically to produce type A3 and then A4 spermatogonia. The type A4 spermatogonium can 1) form another A4 spermatogonium (self-renewal), 2) undergo cell death (apoptosis), or 3) differentiate into an intermediate spermatogonium (heteronymous division) which is inducted into spermatogenesis. Intermediate spermatogonia divide once mitotically to form type B spermatogonia that also divide mitotically into primary spermatocytes. It is not known what causes the spermatogonia to take the path toward spermatogenesis rather than self-renewal [21].

Mitosis is followed by the first meiotic division where DNA is replicated and the chromosome number is halved in the primary spermatocyte [18]. Chromosome pairs undergo homologous recombination involving the formation of synaptonemal complexes in which double strand breaks occur [22]. Genetic material is then exchanged between maternal and paternal chromosomes during repair of the breaks. At the end of the first meiotic division, two secondary spermatocytes with identical chromosomes are produced [18]. The second meiotic division involves the separation of individual chromatid strands to produce four haploid, spherical cells called spermatids [18].

Following meiosis, the differentiation of spermatids occurs through a process called spermiogenesis. This occurs in 4 phases: Golgi, capping, acrosomal, and maturation [14]. The Golgi apparatus of these early spermatids produce vesicles and granules containing enzymes that will cover the developing sperm nucleus. A single large acrosomal granule within a larger vesicle indents the nucleus and the vesicle begins to

flatten into a small cap over the nuclear surface. The acrosomic vesicle becomes very thin, the granule flattens, and the new acrosome flattens over the nuclear surface of the elongating spermatid. The nuclei also begin to change shape as condensation of chromatin and replacement of histones with protamines occurs, and the nucleus becomes more compact [23].

The maturation phase has fewer changes in nuclear shape and acrosomal migration, but the nucleus continues to condense. The acrosome matures into a thin structure that protrudes at the apex, covering nearly all of the nucleus except for the portion connected to the tail [14]. The cytoplasm is reduced, resulting in the formation of cytoplasmic lobes and residual bodes which contain unused mitochondria, ribosomes, lipids, vesicles, and other components [14]. The end result of this process occurs in the final stage of spermatogenesis, in the portion of the tubule nearest the lumen, where spermatozoa are formed [18]. The spermatozoa are released into the lumen and then migrate through a series of ducts toward the epididymis where they mature further and slowly acquire the ability to move on their own. The motility of sperm is dependent on ATP produced by tightly packed mitochondria that fill the mid-piece of the sperm. The ATP powers the flagellum, which extends from the neck through the tail of the sperm, enabling the sperm to move [18]. The structure of a fully mature sperm is illustrated in **Figure 2** taken from *Anatomy and Physiology* [18].

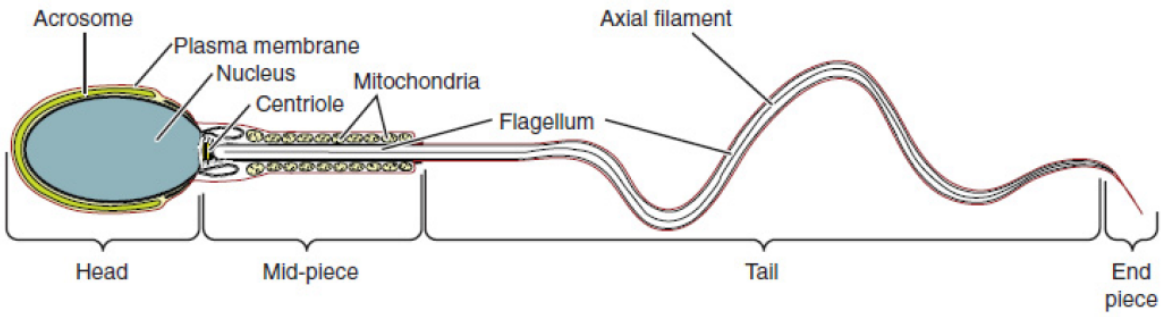

Figure 2: The structure of a fully mature sperm. Each sperm cell is divided into a head containing DNA, mid-piece containing mitochondria, and tail providing motility**.** This diagram was taken from *Anatomy and Physiology* [18].

1.2.2 Histone Replacement with Protamines

The sperm cell has a specialized architecture which allows it to traverse the potentially hostile environment of the female reproductive tract and fertilize the human egg [24]. Dramatic changes to sperm chromatin structure occur during spermiogenesis, whereby 90-95% of histones are replaced with protamines [25]. Protamination of sperm chromatin allows the nuclear compaction necessary for sperm motility and helps to protect the genome from oxidation and harmful molecules within the female reproductive tract [25]. Furthermore, because the higher order packaging of DNA after protamination precludes transcriptional activity, protamination is a nontraditional form of epigenetic regulation unique to sperm cells [23].

The replacement of histones with protamines is a multistep process and is summarized in **Figure 3** taken from Carrell et al. [26]. First, there is an increase in site-specific acetylation, methylation, phosphorylation, and ubiquitnation of histones which facilitate their replacement by testis-specific histones (H-t) that are expressed during spermatogenesis [26,27]. The hyperacetylation of H4-t is a key factor that produces a relaxed chromatin structure important for facilitating topoisomerase-induced double-

stand breaks and replacement of histones with transition proteins [28,29]. Transition proteins 1 and 2 (TP1 and TP2) are proteins of intermediate basicity that bind to DNA, allowing removal of histones and subsequent protamine compaction [30].

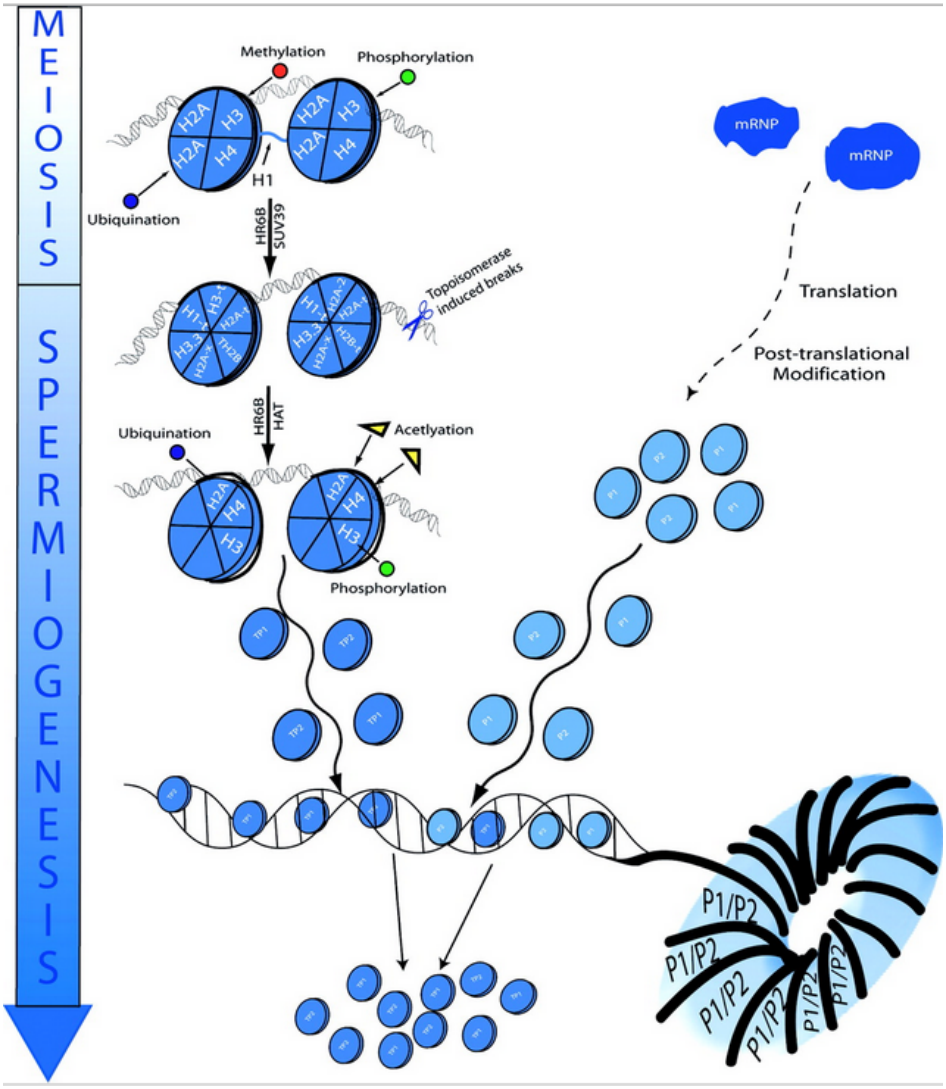

Figure 3: Diagram highlighting the key events in the transition of histones to replacement by protamines. Histones undergo site-specific acetylation, methylation, phosphorylation and ubiquitination which facilitate their replacement by testis-specific histones (H-t) during meiosis. Hyperacetylation of H4-t is a key factor that produces a relaxed chromatin structure important for facilitating topoisomerase-induced double-stand breaks and replacement of histones with transition proteins. Protamines 1 and 2, processed from a pool of RNP particles, undergo maturation before and during binding to the DNA and replacement of the transition proteins. Legend: HR6B, ubiquitin-conjugating enzyme E2B (UBE2B) (RAD6 homolog); HAT, histone acetyltransferase; Suv39, H3 Lys 9 histone methyltransferase. This diagram was taken from Carrell et al. [26].

In the next step, transition proteins are completely replaced by protamines. Protamines 1 and 2 (P1 and P2) are processed from a pool of ribonucleoprotein (RNP) particles and undergo maturation before and during binding to the DNA and replacement of transition proteins [26]. In healthy, fertile individuals, P1 and P2 are expressed in roughly equal quantities [31]. Efficient chromatin packaging and compaction of the sperm nucleus is dependent upon proper protamine replacement. The formation of disulfide bonds between the protamines and the formation of toroidal chromatin structures facilitates nuclear compaction [32]. Because this compaction renders the sperm nucleus resistant to damage from harmful molecules, high quality nucleic acids cannot be extracted from sperm using traditional somatic cell techniques [33].

Despite the replacement of histones with protamines, 5-10% of DNA in fertile men, and more in infertile men, remain bound to histones [34,35]. Ward has proposed a model illustrating the structural arrangement of histone- and protamine bound regions of DNA in mature sperm in which histones are interspersed between protamine toroids and may be bound to matrix attachment regions associated with linker regions [36,37]. The model shows the protection of protamine-bound DNA from damage by toroidal compaction, and the vulnerability of histone-bound and linker regions to DNA degradation by endonucleases [38]. This model has important implications in studies where the proper functioning of sperm and associated male fertility can be investigated through proper replacement of histones with protamines [39,40].

1.3 Spermatogenesis and Genetics

In mammals, many more germline cell divisions occur in the life of a sperm compared to an egg because sperm are produced continuously throughout adult life, unlike eggs where all cell divisions are completed before birth [4]. As the human male ages, the number of cell divisions and chromosome replications increases, rendering the sperm susceptible to increased *de novo* mutations [4,5]. Double strand breaks and DNA repair during spermatogenesis is important for the genomic integrity of future haploid sperm. One study showed that the loss of the nuclear protein PTIP, implicated in the DNA damage response, in male mice resulted in cessation of spermatogenesis, testicular atrophy, and a near complete lack of spermatozoa [22]. It has been proposed that the increased *de novo* mutations may be the result of reduced fidelity of DNA replication and efficiency of DNA repair mechanisms, which normally decline with age [41].

Epidemiological studies suggest associations of *de novo* mutation rates in the male germline with paternal age that increase the risk for disease in offspring, including achondroplasia, Apert syndrome, schizophrenia, and autism spectrum disorders [4-8]. One study estimated the odds ratio (OR) comparing non-synonymous to silent mutations in affected autistic individuals (probands) and their unaffected siblings across 200 families [6]. The total number of non-synonymous *de novo* single nucleotide variants (SNVs) was significantly greater in probands compared to unaffected siblings. Probands also had double the odds of having non-synonymous mutations versus silent mutations compared to unaffected siblings (OR = 1.93). The rate of these *de novo* SNVs was found to increase with paternal age [6].

These findings highlight the importance of sperm genetics to future health and development, and underscore the importance of stable and efficient methods for the isolation of sperm DNA.

1.4 Spermatogenesis and Epigenetics

Whereas an organism's genotype is relatively static throughout life, the epigenome is highly dynamic. Epigenetic alteration is defined as any heritable change in gene expression potential without a change in DNA sequence [42]. These alterations occur in response to the internal and external environment, and include DNA methylation, non-coding RNA, and histone modifications. Because of its role in the regulation of gene expression, it is critical to development and disease. The epigenome is reprogrammed in the gametes and embryo from generation to generation, allowing for totipotency and preventing the transmission of epigenetic error [43]. However, because not all regions of the epigenome are reprogrammed, the transmission of epigenetic information from parents to offspring may occur [9]. Therefore, epigenetic information passed on by gametes may provide information on parental environmental exposures. To date, the mechanisms underlying epigenetic inheritance are largely unknown, and there is a lack of evidence to support epigenetic transmission via the sperm.

DNA methylation is the most characterized epigenetic modification and, in mammals, occurs almost exclusively at the 5-position of cytosine residues within CpG dinucleotides [44]. DNA methylation is catalyzed by DNA methyltransferases and coordinates with other epigenetic modifications to suppress gene expression [45]. Briefly, methyl-CpG-binding protein 2 binds methylated CpGs and recruits chromatin-remodeling

complexes and histone deacetylases [46]. This leads to chromatin condensation which results in limited accessibility of the transcriptional machinery to promoter regions, suppressing gene expression.

DNA methylation is essential for allele-specific imprinting, a non-Mendelian "parent-of-origin" form of mono-allelic inheritance. For an imprinted gene, either the paternal (if the gene is maternally-expressed) or maternal (if the gene is paternally-expressed) allele is heavily methylated (imprinted). Imprinting differs from bi-allelic expression, the traditional, Mendelian form of inheritance where both parental alleles are equally expressed for a given gene. For imprinted genes present in somatic cells, about 50% methylation is expected because only one of the alleles is imprinted. For gametes, however, only one allele is present per gene after meiosis, therefore either complete (100%) or no (0%) methylation is expected, depending on whether the gamete is male or female. Therefore, for human sperm, complete and no methylation would be expected for maternally-expressed and paternally-expressed imprinted genes, respectively.

In primordial germ cells, during gonadal sex determination, parental methylation marks are reset and subsequently re-established in a sex-specific manner during gametogenesis, where haploidization occurs [43]. Methylation marks in imprinted genes and repeat regions are then maintained through fertilization into adulthood and other marks undergo de-methylation. This process of epigenetic erasure and subsequent reprogramming is essential for sperm maturation and represents a critical window of susceptibility during which environmental agents may adversely influence epigenetic regulation [9].

Previous studies of DNA methylation in sperm revealed unique patterns compared with somatic cells [34,47,48]. Overall, the sperm genome has been shown to be hypomethylated compared to differentiated somatic cells, particularly at histone-enriched promoters of developmental genes involved in spermatogenesis, cell cycle, cell metabolism, and embryogenesis. These patterns are necessary to package and poise the sperm genome for spermatogenesis and future embryonic development [35]. If these patterns and imprinting of genes are disturbed during spermatogenesis, gene expression essential for proper development may be compromised in the resulting offspring [49]. Alterations in the sperm epigenome have also been associated with male fertility issues such as low sperm count, motility, and morphology [9-11] as well as overall male infertility [12,13].

1.5 Summary

Previous literature has indicated that sperm possess key biomarkers for proper spermatogenesis as well as successful embryonic development after fertilization. In particular, assessments of developmental players during spermatogenesis from both a genetics and epigenetics standpoint, through polymorphisms and mutations as well as DNA methylation, may be utilized as clinical markers of male reproductive health. However, nuclear compaction due to the replacement of histones with protamines precludes the extraction of high quality DNA using traditional somatic cell techniques. Therefore, the need for an efficient DNA extraction protocol for human sperm suitable for clinical research is required.

CHAPTER 2

LITERATURE REVIEW OF DNA EXTRACTION METHODS

A wide array of techniques exists to isolate high quality and molecular weight DNA from mammalian somatic cells. However, these techniques are ineffective for mammalian sperm [50,51]. This is because unlike in somatic cells, in sperm cells nearly all histones are replaced by protamines held together by disulfide bonds, compacting the sperm nucleus and thus rendering it resistant to conventional lysis procedures [38].

2.1 Sperm DNA Extraction

The development of efficient methods for isolating DNA from mammalian sperm has been a gradual process. Bahnak et al. reported a protocol using guanidine thiocyanate in a lysis buffer made with sodium citrate, sodium lauroyl sarcosinate (Sarkosyl), and β-mercaptoethanol (reducing agent) to isolate high quality mammalian sperm DNA[50]. The DNA extracted was then successfully visualized during Southern blot analysis. However, the procedure was tedious, requiring lengthy steps such as CsCl ultracentrifugation for 20 hours and dialyzing the banded DNA for 24 hours against Tris-HCl and ethylenediaminetetraacetic acid (EDTA).

The inclusion of proteinase K in subsequent methods to enhance the activity of chaotropic agents such as guanidine thiocyanate by digesting nucleoproteins eliminated the need for lengthy ultracentrifugation and dialyzing steps. In a method developed by Pacheco et al. [52], sperm pellets were lysed for 16 hours in a solution containing Tris-HCl, dithiothreitol (DTT; another commonly used reducing agent), sodium chloride, EDTA, sodium dodecyl sulfate (SDS), proteinase K, and β-mercaptoethanol. DNA was

then extracted using a phenol/chloroform protocol, and DNA was ethanol precipitated. While the authors were able to extract DNA used for subsequent DNA methylation analyses, the method still required at least an overnight incubation. The method also employed the use of harmful organic solvents (phenol and chloroform) that are undesirable for simple laboratory procedures.

The method developed by Hossain et al.[53] was one of the first to eliminate overnight procedures from the sperm DNA extraction protocol. Hossain et al. modified the original protocol by Bahnak et al. for the preparation of human sperm DNA by including proteinase K in the lysis buffer (containing guanidinium thiocyanate) to digest nucleoproteins, and isopropanol to precipitate DNA. This modification eliminated the need to mechanically homogenize the cells, use organic solvents for extraction, and use ultracentrifugation for DNA precipitation. Therefore, the degradation of DNA through mechanical homogenization and organic solvents was minimized, and the overall procedure could be performed in ordinary laboratory facilities in a reduced amount of time (only an incubation period of 3 hours for lysis was required). However, incomplete protein digestion and removal of chaotropic salts persisted, limiting the quality of the DNA yield [33].

2.1.1 Griffin's DNA Extraction Method

A recent method by Griffin is worth highlighting because modifications were made to Hossain's protocol to increase the quality and yield of mammalian sperm DNA by eliminating incomplete protein digestion and removal of chaotropic salts that may

coprecipitate with DNA [33]. Descriptions of the lysis and extraction components as well as the steps of and modifications to Hossain's protocol were provided in detail [33].

Guanidine thiocyanate is one variety of chaotropic agent employed during DNA extractions. Its functions include 1) disruption of the hydrate shell of DNA, rendering it insoluble in aqueous solutions, 2) irreversible inactivation of RNases and DNases, 3) disruption of the hydrophobic structures of proteins, metabolites, and other contaminants so that they become soluble in aqueous solutions, and 4) disintegration of cellular membranes. Guanidine thiocyanate enhances the activity of proteinase K, an enzyme that aids in protein solubilization and lysis. Creating hydrophobic and hydrophilic environments for DNA and proteins respectively is necessary to 1) help bind the DNA to the hydrophobic silica membrane of spin columns in commercial extraction kits and 2) remove proteins and other contaminants during subsequent washing steps.

The replacement of histones with protamines, which are held together by disulfide bonds, compacts the sperm nucleus and renders it resistant to DNA extraction by traditional somatic cell methods. Therefore, the use of reducing agents to dissociate protamines from DNA using strong reducing agents is required. DTT and β-mercaptoethanol are examples of such reducing agents which cleave disulfide bonds and allow proteins to unfold [33]. Griffin employed DTT in the lysis buffer because it is more effective and less toxic, works more efficiently, and has a milder odor compared to β-mercaptoethanol. Furthermore, even though SDS has previously been used in DNA extractions, it has a very low solubility in high-salt chaotropic solutions. Griffin employed Sarkosyl in the lysis buffer because it is soluble in high-salt chaotropic solutions and, like SDS, is used to denature proteins and disrupt cellular membranes [33].

Overall, lysis was completed within 2 hours, whereas 3 hours was used in Hossain's protocol. After lysis, the addition of isopropanol allowed precipitation of DNA, and two subsequent washes with alcohol and sodium citrate removed any chaotropic salts into solution.

The extraction method resulted in high quality, high molecular weight genomic DNA, with a yield of approximately 80%, an A260/280 ratio ranging between 1.8 and 2.0, and an A260/230 ratio of 2.0 and greater (as expected for pure DNA) [33]. The DNA was also efficiently digested with restriction enzymes and amplified by PCR [33].

Despite these desirable results, a few issues do not make Griffin's sperm DNA extraction protocol ideal for clinical research: 1) The lack of a protocol to stabilize DNA, 2) The lengthy period taken for lysis (2 hours), 3) The unpleasant sulfur odor of DTT [54] and 4) DTT becomes unstable in solution and must be prepared fresh for every extraction [54]. The lack of a means to prevent the degradation of DNA yield and quality may potentially increase the cost of clinical research because degraded sperm DNA would need to be constantly replaced with new samples obtained from volunteers. In addition, the lengthy period for lysis coupled with the need to prepare fresh DTT for each sperm DNA extraction would make Griffin's methods inefficient for studies requiring large sample sizes. Therefore, Griffin's methods may only be suitable for applications where few patients or volunteers are required to be tested over a short period such as in fertility clinics. Because clinical research typically involves large sample sizes over potentially large time periods, a more efficient method is required where large numbers of sperm samples can be collected and stored for long durations until DNA extraction and analysis begins.

2.2 Summary

Table 1 presents a summary of the methods developed for extracting sperm DNA. The most recent method by Griffin for DNA extraction provides a promising approach for consistently attaining high-quality yields of DNA [33,55]. Furthermore, this approach has been validated by quality control methods that demonstrate 1) the ability to visualize sperm genomic DNA on an agarose gel, 2) the ability to perform restriction enzyme analysis, and 3) amplification of target regions using qPCR, However, the methods involve a lengthy lysis procedure (2 hours) and lack a suitable storage procedure for maintaining stable yields of DNA. These limitations may hamper efficient clinical research for investigating male reproductive health. In addition, even though several studies have been able to obtain DNA for downstream epigenetic profiling [9,34,35,39,56], none have incorporated the recent methods by Griffin.

Table 1: Summary of the development of methods for the extraction of DNA from human sperm.

Author, year	Lysis Buffer	Reducing Agent	Procedure Time	Advantages	Disadvantages
Bahnak et al., 1988	Guanidine thiocyanate, β-mercaptoethanol, Sarkosyl, sodium citrate	β-mercaptoethanol	20+ hours ultracentrifugation and dialysis.	One of the first to isolate high quality sperm DNA.	Lengthy ultracentrifugation (20 hrs) and dialyzing steps (24 hrs).
Hossain et al., 1997	Guanidine thiocyanate, β-mercaptoethanol, Sarkosyl, sodium citrate and Proteinase K	β-mercaptoethanol	3 hours	Eliminated need for mechanical homogenization, use of organic solvents, and ultracentrifugation; could be performed in ordinary laboratories; Lysis was completed in 3 hours.	Incomplete protein digestion and removal of chaotropic salts.
Pacheco et al., 2011	Tris, DTT, sodium chloride, EDTA, SDS, proteinase K, β-mercaptoethanol	DTT and β-mercaptoethanol	16 hours	Lysis could be performed at room temperature in ordinary laboratory facilities.	A lengthy overnight incubation is required; Use of harmful organic solvents (chloroform) required.
Griffin, 2013	Guanidine thiocyanate, DTT, Sarkosyl, sodium chloride, and Proteinase K	DTT	2 hours	Eliminated incomplete protein digestion and removal of chaotropic salts. Lysis completed in 2 hours.	Lack methods to stabilize sperm DNA; lysis time not ideal for clinical research.

For the present study, we have optimized a rapid, simple method of extracting high quality, high molecular weight genomic DNA from human sperm. Because we were able to stabilize sperm DNA for 4 weeks and reduce the duration of lysis procedures to five minutes at room temperature, our methods may be preferred to existing approaches for clinical research where procedure time and viable sample storage duration are important criteria. In addition, we have performed several downstream quality control procedures: 1) PCR amplification of genomic DNA using *ALU* primers, 2) *Hinf1* restriction enzyme digestion and analysis, and 3) DNA methylation analysis using selected maternally and paternally imprinted genes.

CHAPTER 3

METHODS

3.1 Sperm Cell Isolation

To isolate and purify sperm cells and prepare them for DNA extraction, fresh semen samples were collected from three healthy volunteers (n=3) who were required to have a period of at least 48 hours of abstinence. Sperm cells were then isolated using a modified Continuous One-Step PureCeption Gradient (SAGE Form #81804) protocol. The process of isolating human sperm is summarized in **Figure 4**. This study was approved by the Institutional Review Board of the University of Massachusetts.

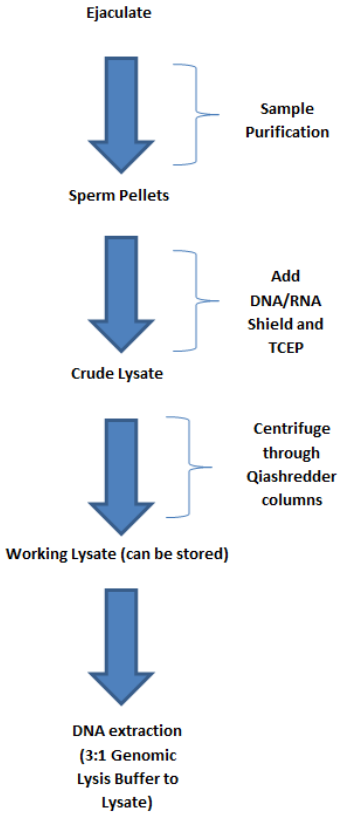

Figure 4: Summary flow diagram illustrating the steps of sperm cell isolation, purification, and storage for DNA extraction.

First, PureCeption 100% Isotonic Solution (SAGE Ref #ART-2100) and Quinn's Sperm Washing Medium (SAGE Ref #ART-1006) were brought to 37^{O}C. A 90% PureCeption solution was made by adding 1 volume of Quinn's Sperm Washing Medium to 9 volumes of PureCeption 100% Isotonic Solution. In a conical centrifuge tube, 1.5-2.0mL of fresh liquefied semen was gently layered on top of 1.0mL of the prepared 90% PureCeption. If the semen volume was greater than 2.0mL, more than one tube of 90% PureCeption was used. The tube was then centrifuged for 30 minutes at 500 *x g*. The 90% PureCeption and seminal fluid were carefully removed without disturbing the sperm pellet, leaving a small amount of 90% PureCeption over the sperm pellet.

After transferring the sperm pellet in residual 90% PureCeption solution to a clean conical centrifuge tube, the pellet was resuspended in 4mL of Quinn's Sperm Washing Medium. The tube was then centrifuged at 500 *x g* for five minutes to wash away residual 90% PureCeption. One mL of washed sample was transferred to a new 1.5mL microcentrifuge tube of which 20µL of the washed sample was transferred to a 0.6mL microcentrifuge tube for cell counting. The 20µL cell counting aliquot was immediately stored at -30^{O}C. The sample was centrifuged for 1 minute at maximum speed and the supernatant was removed from the sperm pellet. The sperm pellet was stored in an appropriate volume (see methods below) of DNA/RNA Shield (Zymo Research Cat # R1100-1-50) and was then lysed and homogenized for future DNA extractions (see "Sperm Lysis and Homogenization" and "DNA Extraction" methods below). If the sperm sample in DNA/RNA Shield was not used immediately, it was stored at 4^{O}C.

3.2 Testing the Utility of Proteinase K Treatment

DNA/RNA Shield contains a high concentration of chaotropic guanidine thiocyanate such that all nucleoproteins may dissolve in solution. If all nucleoproteins dissolve in solution, the need for proteinase K to digest these proteins may be eliminated. We therefore tested the hypothesis that the addition of proteinase K in lysis steps involving DNA/RNA Shield has no discernable effect on sperm DNA concentrations. After removing the supernatant from the 1mL washed sample, the sperm pellet was resuspended in 300µL of DNA/RNA Shield. Two separate mixtures made up to a total volume of 300µL were made with and without 25mg/mL proteinase K (Promega Part # 9PIV302) to make a 1:1 dilution of DNA/RNA Shield with nuclease-free water as follows: 1) Mix with proteinase K (150µL sperm cells in DNA/RNA Shield, 144µL of nuclease-free water, 3µL (0.01M) of 1M DTT (Promega, Cat # V3151), 3µL (75µg) of proteinase K) or 2) Mix without proteinase K (150µL of sperm cells in DNA/RNA Shield, 147µL of nuclease-free water, 3µL (0.01M) of DTT). The mixtures were incubated for 1 hour at 56^{O}C and a modified protocol from the Quick-gDNA MiniPrep Kit (Zymo Research Cat # D3025) was used to extract the sperm DNA, starting with the addition of Genomic Lysis Buffer from the extraction kit in a 3:1 ratio (see "DNA Extraction" method below). DNA yields and quality were then determined using the Nanodrop 2000 Spectrophotometer (Thermo Scientific, Listing # E112352).

3.3 Comparing the Effectiveness of Different Reducing Agents

3.3.1 Sperm Cell Counting for Time-Course Experiment

Tris(2-carboxyethyl)phosphine (TCEP) has been demonstrated to be an odorless reducing agent, more stable and more powerful than the commonly used reducing agent, DTT [54]. Therefore, we compared the effectiveness of TCEP to DTT as reducing agents for sperm DNA extraction using a time-course experiment. We hypothesized that treatment of sperm cells with TCEP would result in more efficient sperm cell lysis than DTT. Isolated sperm pellets that were stored at -20^{O}C were treated with duplicates of 25mM Bond-Breaker TCEP Solution Neutral pH, 0.5M (Thermo Scientific Prod #77720), and 100mM of DTT. As a negative control, we also treated sperm pellets with 200µL of DNA/RNA Shield. Sperm cells were counted at regular time intervals for a period of 20 minutes under each counting square of a Bright-Line Hemocytometer (AO Scientific Instruments Cat # 1483) using an inverted light microscope (Donsanto Corporation Model TMS-F No. 210744) at 40X magnification. The mean number of cells remaining per mL of cell suspension was calculated using the following formula:

$$Mean\ Number\ of\ cells\ per\ mL\ cell\ suspension =$$

$$\frac{Total\ cell\ count}{Number\ of\ squares\ counted} \times \frac{250000}{2} \times\ dilution\ factor$$

The value 250000/2 represents the dimensions of a single counting square and the dilution factor represents the factor used to dilute the original cell suspension.

3.3.2 Comparison of DNA Yields Obtained Using DTT and TCEP

We tested the hypothesis that treatment of sperm cells with TCEP would result in greater sperm DNA concentrations than DTT. To the 1mL washed sample obtained from sperm cell isolation, 100µL of DNA/RNA Shield and either 5µL of 1M DTT or 10µL of 500mM TCEP were added to make 50mM solutions of DTT and TCEP. The samples were then pulse vortexed for 15 minutes using the Pulse Vortex Mixer (Fisher Scientific Cat # 02215375) and a modified protocol from the Quick-gDNA MiniPrep Kit was used to extract the sperm DNA, starting with the addition of Genomic Lysis Buffer from the extraction kit in a 3:1 ratio (see "DNA Extraction" method below). DNA yields and quality were then determined using the Nanodrop 2000 Spectrophotometer.

3.4 Testing the Utility of QIAshredder Columns for Sperm Cell Homogenization

We tested whether QIAshredder columns (Qiagen Cat # 79656), when used together with TCEP to homogenize and lyse sperm cells, would increase DNA yield compared to using TCEP alone. We hypothesized that QIAshredder homogenization following sperm lysis steps would increase sperm DNA concentrations. To an isolated sperm pellet, 360µL of DNA/RNA Shield and 40µL (50mM) of TCEP were added. The sperm sample was then pulse vortexed for 5 minutes using the Pulse Vortex Mixer. Using the QIAshredder kit, 200µL of the sperm sample were centrifuged for 2 minutes at maximum speed through QIAshredder columns and collected in collecting tubes. A modified protocol from the Quick-gDNA MiniPrep Kit was used to extract sperm DNA from the 200µL sperm samples that did and did not undergo QIAshredder homogenization, starting with the addition of Genomic Lysis Buffer from the extraction

kit in a 3:1 ratio (see "DNA Extraction" method below). DNA yields and quality were then determined using the Nanodrop 2000 Spectrophotometer.

3.5 Optimized Sperm Cell Lysis and Homogenization

After removing the supernatant from the 1mL washed sample during sperm cell isolation, the sperm pellet was resuspended in 900µL of DNA/RNA Shield and 100µL (50mM) of 500mM TCEP to lyse the cells. The sample was then incubated at room temperature for 5 minutes, with occasional pulse vortexing using the Pulse Vortex Mixer. After incubation, the sample was centrifuged in a QIAshredder column for 2 minutes at maximum speed. If the sperm cells were not used immediately, they were stored at $4^{O}C$ for 1 month.

3.6 DNA Extraction

Guanidine thiocyanate is a chaotropic agent that removes nucleoproteins into solution by destabilizing hydrophobic interactions and hydrogen bonds. Because DNA/RNA Shield contains a high concentration of guanidine thiocyanate, it would be expected to remove nucleoproteins that degrade DNA such as DNAses into solution, stabilizing any DNA in suspended cells. Therefore, using our fully optimized sperm isolation and DNA extraction protocol (**Appendix A**), we compared the stability of fresh and 4-week-old sperm samples stored at $4^{O}C$ in DNA/RNA Shield, hypothesizing that the samples would yield similar DNA concentrations. A modified protocol from the Quick-gDNA MiniPrep Kit was used to isolate and purify genomic DNA from fresh and 4-week-old sperm samples. Genomic Lysis Buffer from the kit was added to the sperm pellet suspended in DNA/RNA Shield and TCEP in a 3:1 ratio. The mixture was then

vortexed for 4-6 seconds and transferred to the kit's spin column in a collection tube. The tube was centrifuged at 10,000 *x g* for 1 minute and the collection tube was discarded.

After transferring the spin column to a new collection tube, 200µL of the kit's DNA Pre-Wash Buffer was added to the column and centrifuged at 10,000 *x g* for 1 minute. Two washes were then performed by adding 500µL of the kit's g-DNA Wash Buffer to the spin column and centrifuging at 10,000 *x g* for 1 minute. The spin column was incubated at room temperature for 5 minutes and transferred to a clean microcentrifuge tube.

To elute the DNA, 100µL of the kit's DNA Elution Buffer was added to the spin column. The spin column was then incubated for 3 minutes at room temperature and centrifuged at maximum speed for 30 seconds to elute the DNA. The yield and quality of the DNA were then determined using Nanodrop 2000 Spectrophotometer. If the DNA was not used immediately, it was stored at 4^{O}C for future use.

3.7 PCR and Restriction Digest of Sperm DNA

As a quality control we tested the hypothesis that fresh and 4-week-old sperm samples would perform with equal efficacy during *ALU* PCR and *Hinf1* digest. To perform PCR of the isolated sperm DNA, individual 10µL PCR reactions were prepared using 5.0µL(1X) of 2X GoTaq Hot Start Colorless Master Mix (Promega Ref # M513B), 1.0µL (0.1µM) of 1µM *ALU* (446bp product) forward and reverse primer mix (Integrated DNA Technologies), 2.0µL of nuclease-free water, and 2.0µL (46ng) of genomic sperm DNA. The PCR reaction conditions were as follows: 1) Hot start (94^{O}C) for 2 minutes, 2) 30 cycles of denaturing (94^{O}C), annealing (68^{O}C), and extension (72^{O}C), each for 30

seconds, and 3) final extension (72^{O}C) for 5 minutes. **Supplementary Table B.1** presents a summary of the properties of the *ALU* primers.

To perform restriction digests of the isolated sperm DNA, individual *Hinf1* restriction digest reactions were prepared using 17.00µL (561ng) of genomic sperm DNA, 2.0µL(1X) of 10X CutSmart buffer (New England BioLabs Cat # B7204S), and 1.0µL (100U) of 10,000U/mL *Hinf1* restriction enzyme (New England BioLabs Cat # R0155S). The restriction digests were performed by incubating the reactions at 37^{O}C for 1 hour and then heating at 80^{O}C for 20 minutes to inactivate the enzyme.

A 0.7% agarose gel was prepared for electrophoresis and samples were added to wells in duplicates as follows: 3µL (69ng) genomic sperm DNA, 5µL *ALU* PCR product, and 7µL (196ng) of *Hinf1* digestion products. After electrophoresis was performed at 300V for 20 minutes, the gel was stained with 0.5µg/mL ethidium bromide solution for 15 minutes, destained for 15 minutes with deionized water, and visualized using the Benchtop Variable Transilluminator (UVP Cat # M-26XV) and BioDoc-It Imaging System (UVP Cat # M-26X).

3.8 Preparing Sperm DNA Samples for DNA Methylation Analysis

As an additional quality control, we tested the hypothesis that there would be no discernable differences in DNA methylation between fresh and 4-week-old sperm samples across *SNURF*, *PEG10*, and *H19* imprinted loci. The protocol for preparing genomic DNA for DNA methylation analysis using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) is

summarized in a scheme by van den Boom and Ehrich in **Figure 5** [57]. The protocol was performed by two different experimenters.

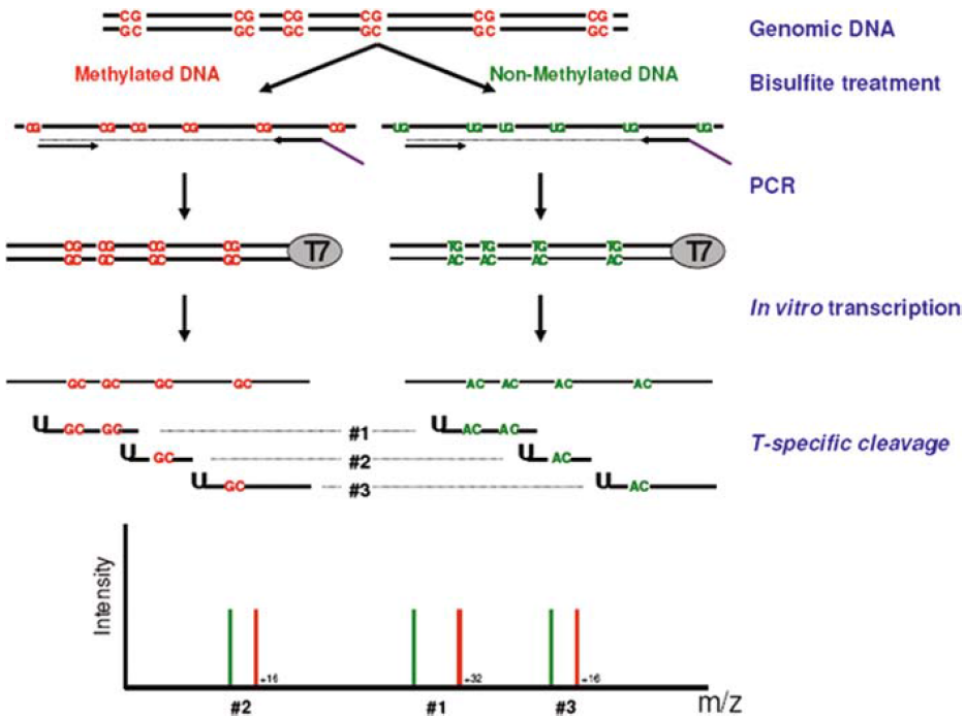

Figure 5: Summary scheme for preparation of bisulfite converted DNA samples for DNA methylation analysis using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS). Genomic DNA is bisulfite treated to introduce methylation-dependent sequence changes and then amplified by PCR. After amplification by PCR, the PCR product is treated with shrimp alkaline phosphatase (SAP) to remove unincorporated dNTPs that may interfere with the subsequent cleavage reaction and methylation analyses. The reverse strands of the PCR products are then transcribed into a single-stranded RNA. The methylation dependent C/T changes introduced during bisulfite treatment should be represented as G/A changes in the RNA transcript. The transcript is then cleaved base specifically by RNase A, which cleaves at every U, and the cleavage products are analyzed by MALDI-TOF MS. Mass signals representing methylated and non-methylated DNA build signal pairs, each representative of the CpG site within the analyzed sequence substring. G/A changes in the RNA transcript lead to corresponding mass signals that shift 16Da for a methylation event. If two or more CpG sites are embedded within a cleavage product, mass signals may shift by multiples of 16Da. This diagram was taken from van den Boom and Ehrich [57].

3.8.1 Bisulfite Treatment of Sperm DNA Samples

The EZ DNA Methylation Kit (Zymo Research) was used for bisulfite treatment of 500ng of each genomic sperm DNA sample. Adding 100µL of M-Elution Buffer

(Zymo Research) resulted in a mean yield of 235ng of bisulfite-converted DNA for DNA methylation analysis. Bisulfite treatment introduces methylation dependent cytosine/uracil (C/U) sequence changes and each uracil is subsequently converted to thymidine (T) after PCR [57].

3.8.2 PCR Amplification of Imprinted Genes

Following bisulfite treatment, each of the three imprinted loci was amplified using imprinted primers (Integrated DNA Technologies) for the paternally-expressed imprinted genes *PEG10* and *SNURF* and the maternally-expressed imprinted gene *H19* (**Supplementary Table B.1**) through qPCR. Individual 5µL PCR reactions were prepared using 2.5µL (1X) of 2X GoTaq Hot Start Colorless Master Mix, 1.0µL (0.2µM) of 1µM forward and reverse primer mix (Integrated DNA Technologies), and 1.5µL (3.5ng) of bisulfite-converted sperm DNA. The PCR reaction conditions were as follows: 1) Hot start (95^{O}C) for 2 minutes, and 2) 40 cycles of denaturing (95^{O}C), annealing (58^{O}C), and extension (72^{O}C), each for 30 seconds.

3.8.3 Shrimp Alkaline Phosphatase (SAP) Treatment

PCR products were then treated with shrimp alkaline phosphatase (SAP; Sequenom Item No. 10002.1) to remove unincorporated dNTPs. Keeping SAP enzyme on ice, SAP enzyme solution was prepared for each PCR product using 1.70µL nuclease-free water and 0.30µL of SAP enzyme. After the PCR reactions were completed, 2µL of SAP enzyme solution were added to each sample. The samples were centrifuged at 3,000 *x g* for 1 minute and incubated at 37^{O}C for 20 minutes. The SAP enzyme was then inactivated at 85^{O}C for 5 minutes before the samples were cooled and held at 4^{O}C.

3.8.4 T7 Cleavage Transcription Protocol

A T Cleavage Transcription (T7) reaction was then performed to generate a single-stranded RNA molecule from the PCR product. The RNA strand is cleaved base specifically by RNase A after each uracil. Any C/T sequence changes introduced by bisulfite treatment are reflected as guanidine/adenine (G/A) changes on the reverse RNA strand, resulting in a mass difference of 16 Da for each CpG site enclosed in the cleavage products generated from the RNA transcript [57]. Briefly, a 5μL T Cleavage Transcription/RNase A mix was prepared for each reaction using 3.15μL of nuclease-free water, 0.89μL of 5X T7 Polymerase buffer (Sequenom Item No. 10059), 0.24μL of T Cleavage Transcription Mix (Sequenom Item No. 08051), 0.22μL of 100mM DTT (Sequenom Item No. 10062), 0.44μL of T7 RNA & DNA Polymerase (Sequenom Item No. 08050), and 0.06μL of RNase A (Sequenom Item No. 10061). After SAP reactions were completed, the SAP treated PCR samples were centrifuged at 540 *x g* for 1 minute before 2μL of T Cleavage Transcription/RNaseA mix was added to each sample. The samples were then centrifuged at 540 *x g* for 1 minute before they were incubated for 3 hours at 37^{O}C and held at 4^{O}C overnight.

3.8.5 Conditioning with Clean Resin and MassARRAY Analysis

After the T7 cleavage transcription reaction was completed, 20μL of nuclease-free water was added to each sample and the samples were centrifuged at 540 *x g* for 1 minute. To each sample, 6mg of clean resin (Sequenom Item No. 08040) were then added to each sample. The samples were then taped to a rotator and allowed to rotate for 30 minutes. After rotation was completed, the samples were centrifuged at 3,200 *x* g for 5

minutes and 10nL of each sample was dispensed onto a SpectroCHIP (Sequenom Item No. 01509) using the MassARRAY Nanodispenser (Sequenom Model # RS1000). Identification of methylated sites and determination of the degree of methylation for each imprinted locus was then assessed in the cleavage products containing the imprinted genes (*PEG10*, *SNURF*, and *H19*) using the MassARRAY Analyzer 4 (Sequenom Typ. PHX-1) matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) platform. The mass signals representing nonmethylated and methylated DNA form signal pairs, each representative of the CpG sites within the analyzed sequence substring. The relative amount of methylated DNA for each CpG site is then calculated from the ratio of the signal intensities for each pair [57]. The MassARRAY platform generates quantitative methylation results for each sequence-defined analytical unit (either one individual CpG site or an aggregate of subsequent CpG sites), each referred to as a "CpG unit."

3.9 Statistical Analysis

Given the large error variances and imprecise estimates of measured parameters that would be produced from the small number of participants in our study (n = 3), we expected that the power to observe statistical differences in our results between treatment groups would be low. Therefore, we found it inappropriate to perform tests for statistical significance. Rather, we interpreted our results based on the consistency and magnitude of differences between treatment groups.

CHAPTER 4

RESULTS

4.1 Testing the Utility of Proteinase K Treatment

Figure 6 presents the comparison between lysing sperm cells in DNA/RNA Shield with 0.01M DTT and 0.01M DTT plus 75µg proteinase K for sperm samples obtained from two volunteers. The DNA concentration obtained after treating sperm sample 1 with 0.01M DTT plus 75µg proteinase K (mean = 33.4$\pm$0.21ng/µL) was marginally greater than that obtained after treatment with 0.01M DTT alone (mean = 30.9$\pm$0.85ng/µL). On the other hand, the DNA concentration obtained after treating sperm sample 2 with 0.01M DTT plus 75µg proteinase K (mean = 28.9$\pm$0.00ng/µL) was marginally lower than that obtained after treatment with 0.01M DTT alone (30.1$\pm$0.28ng/µL).

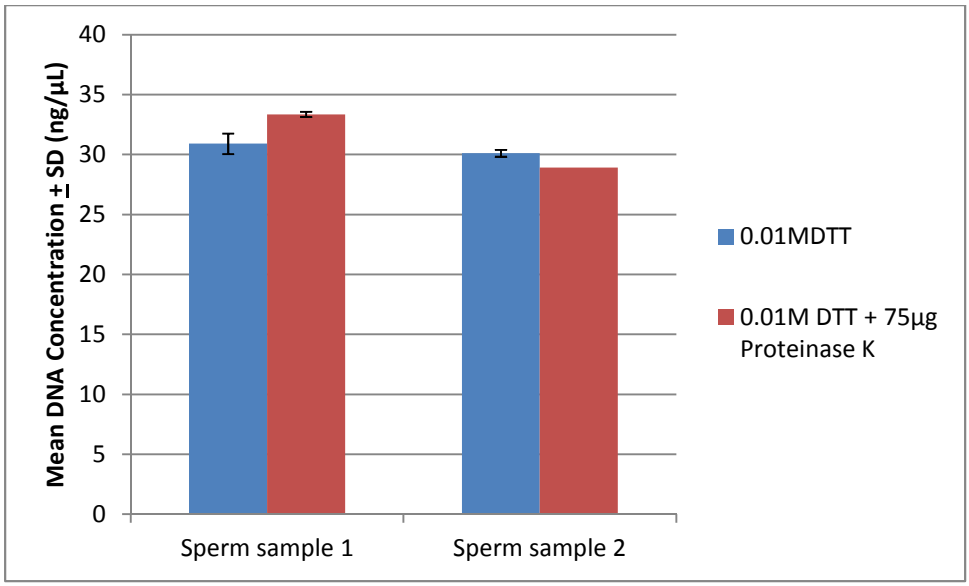

Figure 6: Comparison of sperm DNA concentrations obtained from sperm samples from two healthy volunteers through DNA extraction after lysis in DNA/RNA Shield with either 0.01M dithiothreitol (DTT) or 0.01M DTT and 75µg proteinase K.

4.2 Testing the Effectiveness of Different Reducing Agents

4.2.1 Sperm Cell Counting for Time-Course Experiment

Figure 7 presents the mean percentage of sperm cells remaining per mL cell suspension in DNA/RNA Shield over time after treatment with TCEP and DTT. Treatment of sperm cells with 100mM DTT resulted in a gradual decrease in the percentage of sperm cells/mL cell suspension observed over a period of 20 minutes, after which no sperm cells were visible. In comparison to DTT, there was a sharper decline in the percentage of sperm cells/mL cell suspension after treatment with 25mM TCEP over 1 minute, after which no sperm cells were visible. There was no visible decline in the percentage of sperm cells after treatment with DNA/RNA Shield only over a period of 20 minutes.

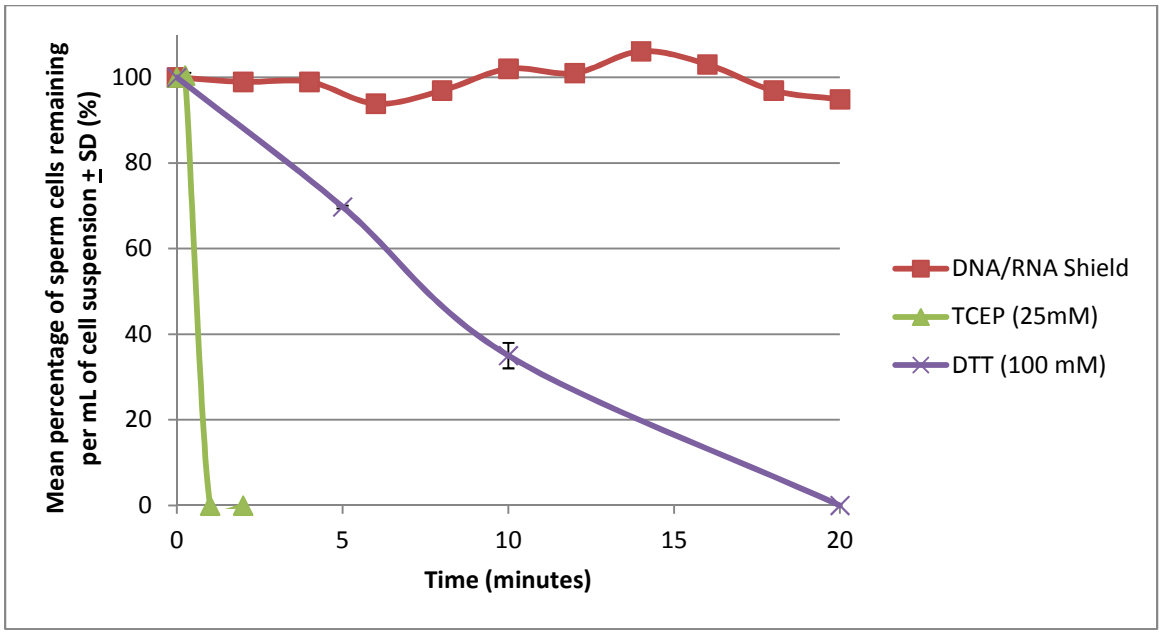

Figure 7: The mean percentage of sperm cells remaining per mL of cell suspension in DNA/RNA Shield over a period of 20 minutes after treatment with 25mM tris(2-carboxyethyl)phosphine (TCEP) and 100mM dithiothreitol (DTT).

4.2.2 Comparison of DNA Concentrations Obtained Using DTT and TCEP

Figure 8 presents the comparison of DNA concentrations obtained from sperm samples from two volunteers through DNA extraction after treatment with either 50mM DTT or TCEP reducing agents. There was an increase in the concentration of DNA obtained from both sperm samples after treatment with 50mM TCEP compared to 50mM DTT. Treatment with 50mM TCEP in sperm sample 1 resulted in a mean DNA concentration of 17.2±0.50ng/µL compared to a mean DNA concentration of 12.6±0.28ng/µL after treatment with 50mM DTT. There was also an increase in the mean DNA concentration of sperm sample 2 after treatment with 50mM TCEP (21.3±0.71ng/µL) compared to 50mM DTT (12.3±0.35ng/µL).

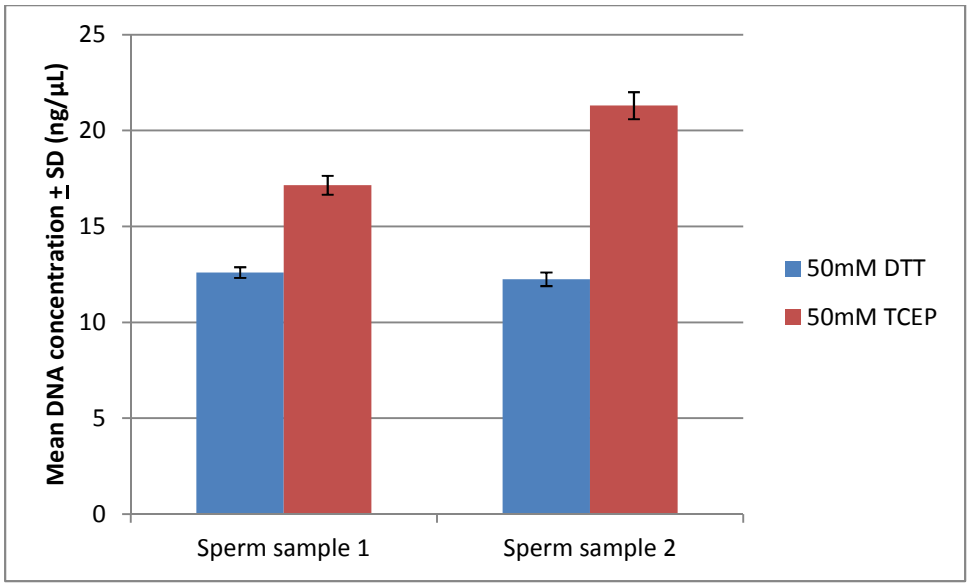

Figure 8: Comparison of sperm DNA concentrations obtained from sperm samples from two healthy volunteers through DNA extraction after lysis with either 50mM dithiothreitol (DTT) or tris(2-carboxyethyl)phosphine (TCEP)reducing agents.

4.3 Homogenization of Sperm Cells via QIAshredder

Figure 9 presents the comparison of sperm DNA concentrations obtained from sperm samples from two volunteers through DNA extraction after lysis with 50mM TCEP only or homogenization with QIAshredder columns followed by lysis with 50mM TCEP. There was an increase in the concentration of DNA obtained from both sperm samples after homogenizing with QIAshredder columns followed by lysis with 50mM TCEP compared to lysis with 50mM TCEP alone. QIAshredder coupled with TCEP in sperm sample 1 resulted in a mean DNA concentration of 25.9$\pm$0.35ng/µL compared to a mean DNA concentration of 18.6$\pm$0.99ng/µL after lysis with 50mM TCEP only. There was also a marked increase in the mean DNA concentration of sperm sample 2 after coupled treatment with QIAshredder and TCEP (21.7$\pm$0.49ng/µL) compared to 50mM TCEP only (12.3$\pm$0.35ng/µL).

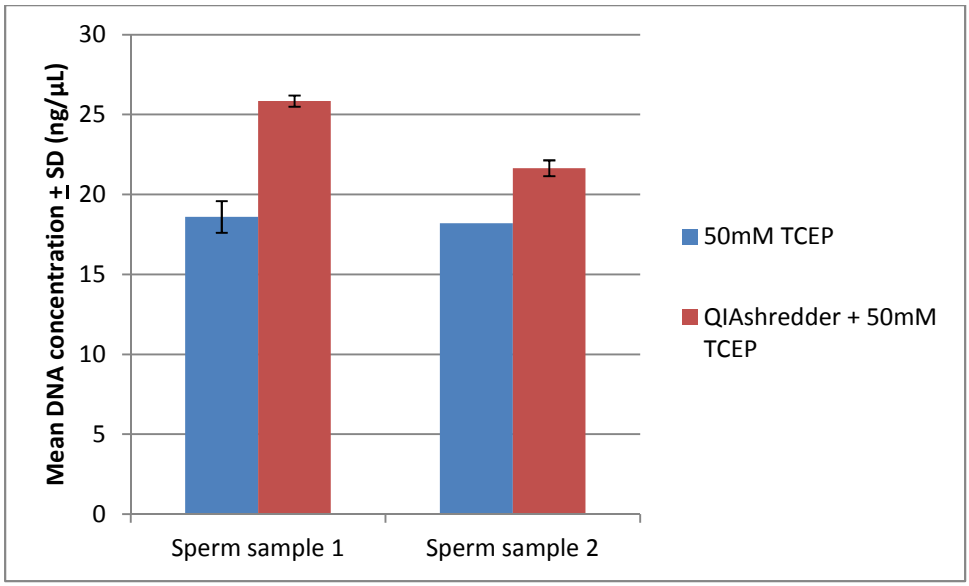

Figure 9: Comparison of sperm DNA concentrations obtained from sperm samples from two healthy volunteers through DNA extraction after lysis with 50mM tris(2-carboxyethyl)phosphine (TCEP) only or homogenization with QIAshredder followed by lysis with 50mM TCEP.

4.4 Stability of Sperm DNA

4.4.1 Sperm DNA Concentrations and Quality

Figure 10 compares the DNA concentrations attained from baseline and 4-week-old sperm samples stored in DNA/RNA Shield at 4^{O}C obtained from three volunteers after performing DNA extraction procedures involving QIAshredder and 50mM TCEP. The mean DNA concentration appeared to increase after 4 weeks of storage for sperm samples 1 (36.2$\pm$2.75 ng/µL to 40.0$\pm$2.98ng/µL) and 3 (32.2$\pm$1.38ng/µL to 37.6$\pm$1.38ng/µL) but decrease for sperm sample 2 (44.3$\pm$3.93ng/µL to 38.7$\pm$3.93ng/µL) although these changes were minor. Overall, the amount of time sperm samples were stored in DNA/RNA Shield did not appear to show an effect on mean DNA concentrations attained.

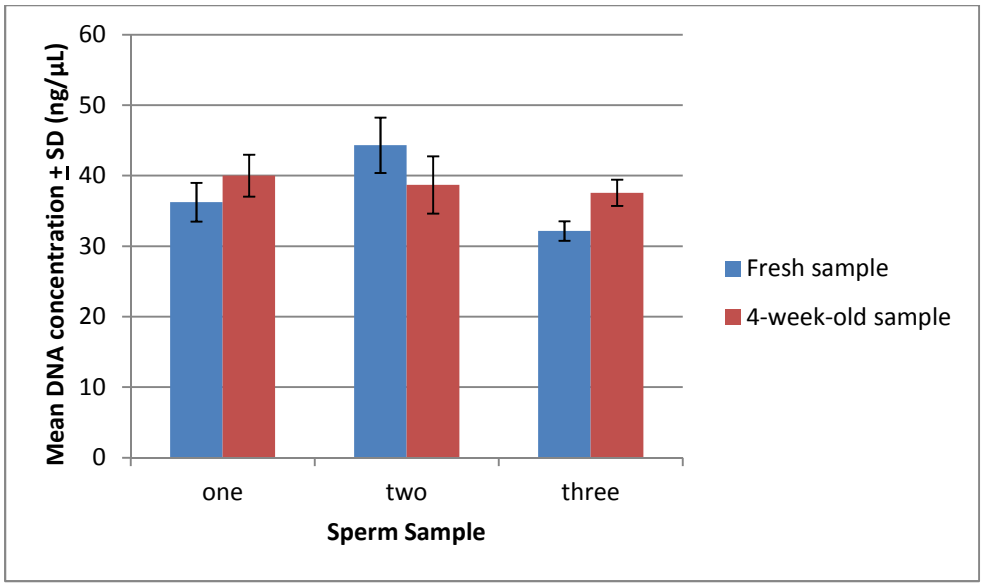

Figure 10: Comparison of DNA concentrations attained from fresh and 4-week-old sperm samples obtained from three healthy volunteers stored in DNA/RNA Shield after performing DNA extraction procedures involving QIAshredder and 50mM tris(2-carboxyethyl)phosphine (TCEP).

Table 2 shows the mean A260/A280 and A260/A230 ratios obtained for sperm samples stored in DNA/RNA shield at baseline after 4 weeks. A260/A280 ratios indicate possible contamination from proteins or phenols, and are expected to be in the range 1.8-1.9 for pure DNA. The mean A260/A280 ratios for fresh and 4-week-old sperm samples ranged from 1.83 to 1.89. These ratios were similar between fresh and 4-week-old sperm samples. A260/A230 ratios are used as secondary measures of DNA purity and indicate possible contamination from ethylenediaminetetraacetic acid (EDTA), carbohydrates, or phenols. These ratios are expected to range from 2.0-2.2 for pure DNA. The mean A260/A230 ratios ranged from 0.67 to 1.76 and were also similar between fresh and 4-week-old sperm samples.

Table 2: Mean A260/A280 and A260/A230 ratios for DNA extracted from fresh and 4-week-old sperm samples obtained from three healthy volunteers. Sperm samples were suspended in DNA/RNA Shield and 50mM tris(2-carboxyethyl)phosphine (TCEP) before being homogenized using QIAshredder columns. Sperm DNA was then extracted from fresh and 4-week-old sperm samples using modified protocol from the Quick-gDNA MiniPrep Kit and quantified using Nanodrop 2000 Spectrophotometer.

	Fresh	4 weeks	Fresh	4 weeks
Sample	Mean 260/280 (SD)	Mean 260/280 (SD)	Mean 260/230 (SD)	Mean 260/230 (SD)
1	1.88 (0.01)	1.87 (0.01)	1.25 (0.43)	1.03 (0.61)
2	1.84 (0.02)	1.87 (0.02)	0.67 (0.15)	0.67 (0.81)
3	1.89 (0.02)	1.83 (0.02)	1.76 (0.17)	1.27 (0.21)

4.4.2 Restriction Digest and PCR of Sperm DNA

As a quality control procedure, we assessed the ability to perform downstream genetic analyses using DNA extracted from baseline and 4-week-old samples from one of the volunteers. **Figure 11** presents a 0.7% agarose gel image of undigested genomic sperm DNA, *ALU* PCR product (446bp), and restriction digests of genomic sperm DNA using *Hinf1* restriction enzyme for fresh and 4-week-old sperm DNA samples. The gel indicates that 1) genomic sperm DNA was visualized with equal band intensity from both fresh and 4-week-old sperm samples, indicated by the bands appearing greater than 24kb, 2) *ALU* PCR product size was equal to that expected (446bp) and at equal band intensity for both fresh and 4-week-old sperm samples, and 3) equal and efficient digestion of sperm genomic DNA by *Hinf1* enzymes, indicated by the equal intensity of streaks starting halfway down the gel.

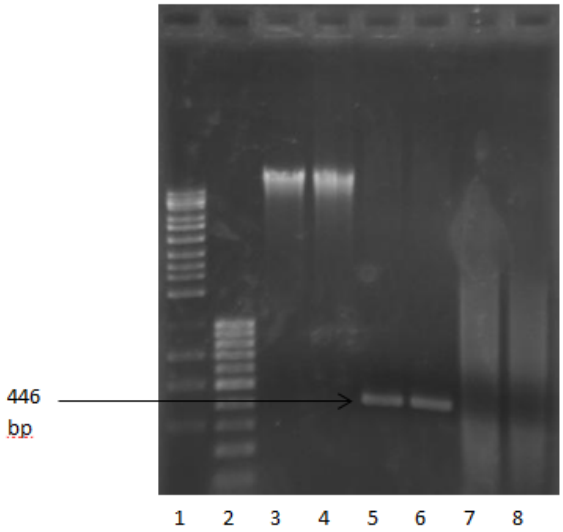

Figure 11: Electrophoresis of fresh and 4-week-old sperm DNA samples on 0.7% agarose gel. Legend: Lane 1 = 24kb DNA ladder, Lane 2= 100 bp DNA ladder, Lane 3 = undigested fresh genomic DNA, Lane 4 = undigested 4-week genomic DNA, Lane 5 = fresh *ALU* PCR product (446 bp), Lane 6 = 4-week *ALU* PCR product (446bp), Lane 7 = fresh *Hinf1* digest, Lane 8 = 4-week *Hinf1* digest.

4.4.3 DNA Methylation of Imprinted Genes

To evaluate the feasibility of DNA methylation analyses in sperm genomic DNA, and to rule out DNA contamination from somatic cells, we chose three imprinted genes for our analyses: two paternally-expressed imprinted genes (*PEG10* and *SNURF*) and one maternally-expressed imprinted gene (*H19*). Somatic cells possess two alleles, each inherited from a different parent. Therefore, for imprinted genes, we would expect to find a mean methylation percentage of about 50% in somatic cells. Because we were investigating male gametes, each containing one allele for a given gene, we expected the allele of paternally-expressed imprinted genes to be unmethylated, and the allele of maternally-expressed imprinted genes to be fully methylated.

Bisulfite conversion of 500ng of genomic sperm DNA from the EZ DNA Methylation Kit resulted in a mean yield of 235ng of bisulfite-converted DNA used for

DNA methylation analysis. The degree of methylation at each CpG site for each sample in a given imprinted gene is summarized in an epigram (**Figure 12**). The mean percentage of methylation across the imprinted loci for each sample is summarized in **Table 3**. As expected, there was little to no methylation in *SNURF* and *PEG10* while *H19* was heavily methylated for each sample (**Figure 12**; **Table 3**). We also found that the mean percentages of methylation across all CpG sites were similar between fresh and 4-week-old sperm samples for *SNURF* (1.43$\pm$1.02% and 1.55$\pm$0.95% respectively), *PEG10* (3.69$\pm$0.66% and 4.28$\pm$1.52% respectively), and *H19* (88.93$\pm$3.24% and 91.78$\pm$2.00% respectively).

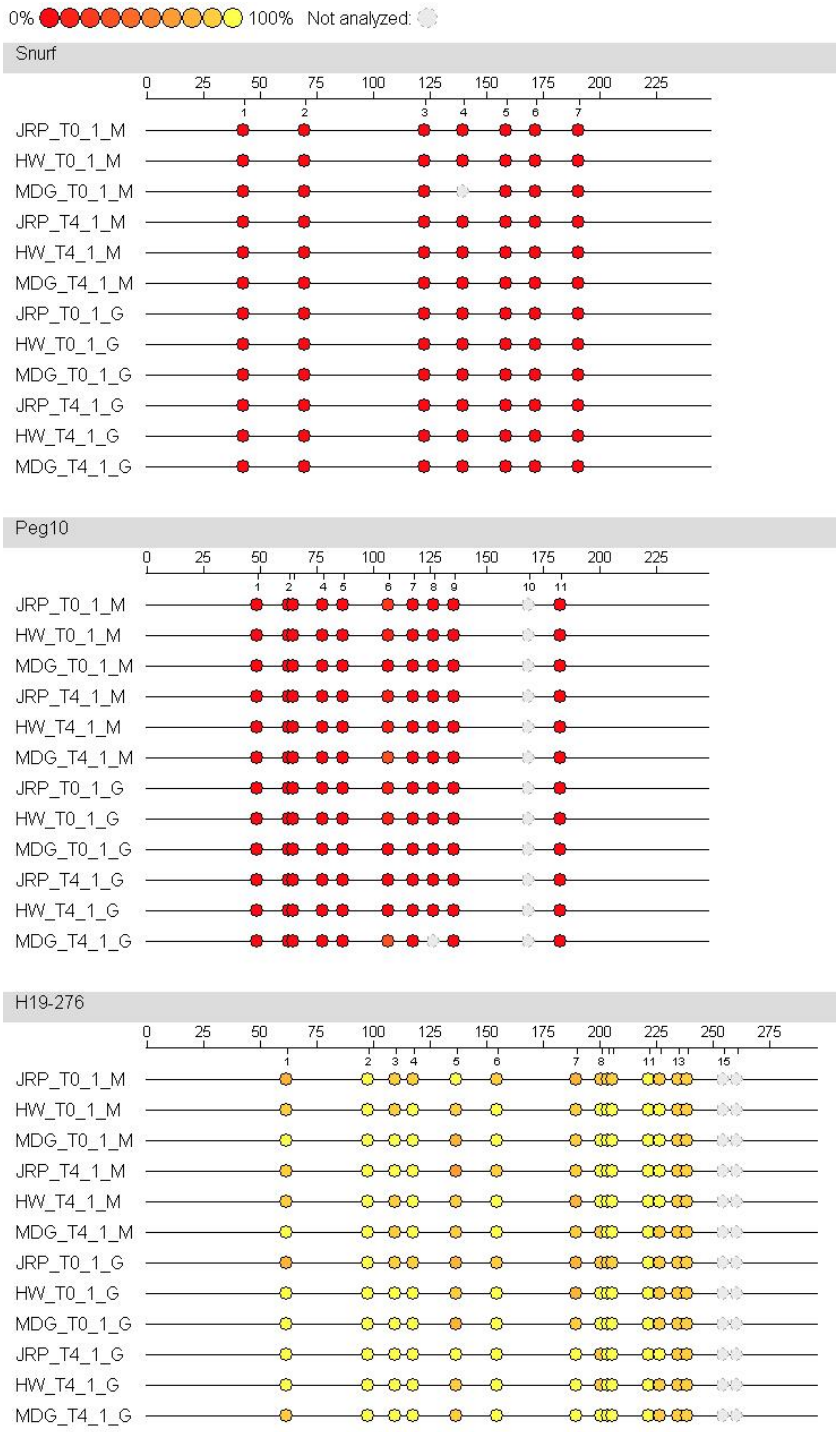

Figure 12: The degree of methylation at each CpG site for two paternally-expressed (*SNURF* and *PEG10*) and one maternally-expressed (*H19*) imprinted loci for DNA extracted from fresh (T0) and 4-week-old (T4) human sperm samples obtained from three healthy volunteers. Different letters (M or G) attached to the end of each sample indicate that the sample was run by a different individual. Red circles indicate no methylation, yellow circles indicate heavy methylation, and white circles indicate no analysis at their respective CpG sites.

Table 3: The mean percentage of methylation occurring across paternally-expressed imprinted loci (*SNURF* and *PEG10*) and maternally-expressed-imprinted loci (*H19*) for fresh and 4-week-old sperm samples obtained from three healthy volunteers.

	Mean Percentage (%) Methylation across *SNURF* Locus (SD)		Mean Percentage (%) Methylation across *PEG10* Locus (SD)		Mean Percentage (%) Methylation across *H19* Locus (SD)	
Sample	**Fresh**	**4 weeks**	**Fresh**	**4 weeks**	**Fresh**	**4 weeks**
1	1.21 (0.30)	1.07 (0.10)	3.94 (0.86)	3.00 (0.79)	84.92 (1.89)	92.13 (2.53)
2	2.29 (1.21)	1.50 (1.11)	4.06 (0.39)	3.83 (0.24)	90.67 (0.47)	91.50 (3.06)
3	0.79 (1.11)	2.07 (1.52)	3.06 (0.24)	6.01 (1.08)	91.21 (0.41)	91.71 (1.94)
Mean	1.43 (1.02)	1.55 (0.95)	3.69 (0.66)	4.28 (1.52)	88.93 (3.24)	91.78 (2.00)

CHAPTER 5

DISCUSSION AND CONCLUSIONS

5.1 Optimization of Methods for Extraction of Human Sperm DNA

5.1.1 Sperm Homogenization and Lysis Methods

We have optimized a rapid (5 minutes of cell lysis), cost-effective protocol for sperm cell lysis during DNA extractions. First, we eliminated the need for proteinase K during lysis of sperm cells involving high concentrations of guanidine thiocyanate in DNA/RNA Shield by demonstrating that there was no discernable change in DNA concentrations obtained after lysis treatment with proteinase K and DTT compared to DTT alone. Therefore, because proteinase K is unnecessary in the presence of high concentrations of guanidine thiocyanate in DNA/RNA Shield for the effective lysis of sperm cells, the costs of having to use proteinase K during DNA extractions are eliminated. Secondly, our results also suggest that TCEP is more effective at lysing sperm cells and results in greater yields of sperm DNA after 5 minutes of lysis at room temperature compared to DTT. This has desirable implications for clinical research because 1) the need for lengthy incubation steps (2 hours or more) involving heat is eliminated and 2) TCEP is odorless and 3) TCEP is more stable at room temperature compared to DTT [54], eliminating the need to prepare fresh aliquots of reducing agents for each DNA extraction and improving cost-efficiency of research. Finally, we optimized our lysis methods by demonstrating that using QIAshredder columns coupled with TCEP for homogenizing and lysing sperm cells produced greater yields of DNA compared to using TCEP alone.

5.1.2 Stability of Sperm DNA after 4 Weeks of Storage

Our optimized DNA extraction protocol produced comparable yields of DNA for fresh and 4-week-old sperm samples stored in DNA/RNA shield at 4^{O}C. In addition, the A260/A280 ratios for fresh and 4-week-old sperm samples were similar and in the range between 1.8-1.9 expected for pure DNA, suggesting comparable DNA quality between these samples. A260/A230 ratios for fresh and 4-week-old sperm samples were also similar, but not in the expected range between 2.0-2.2 for pure DNA, suggesting the presence of residual EDTA during extraction procedures. However, because EDTA is used in commercial elution buffers to elute DNA used in many successful downstream analyses, it would not be expected to affect the quality of the DNA.

Our quality control analyses also revealed equal feasibility of downstream genetic and epigenetic analyses in fresh and 4-week-old sperm DNA samples. We found that genetic analyses using restriction digestion and PCR may be performed equally efficiently on sperm DNA extracted from both fresh and 4-week-old sperm samples. The ability to perform these analyses has wide ranging applications in assessing clinical markers of male reproductive health because reduced fidelity and efficiency of DNA repair mechanisms during spermatogenesis, due to age or environmental factors, may lead to harmful mutations in genes that are associated with adverse male reproductive health outcomes [22] as well as disease and autism spectrum disorders in the next generation [4-8].

We were also able to measure methylation in bisulfite-converted sperm DNA for epigenetic analyses. The degree of DNA methylation was similar between fresh and 4-

week-old sperm samples, indicating equal efficacy for DNA methylation analyses from fresh and 4-week-old sperm samples. In addition, we found little to no methylation in the two paternally-expressed imprinted genes (*SNURF* and *PEG10*), and heavy methylation in the maternally-expressed imprinted gene (*H19*). The magnitudes of methylation for the imprinted loci were also consistent with those found in previous literature for male gametes [58]. Therefore, we were able to rule out any contamination from somatic cells, where we would have expected to find approximately 50% methylation for either maternally-expressed or paternally-expressed imprinted genes.

Erasure of imprinting and methylation marks during gonadal sex determination and subsequent reestablishment of these marks during spermatogenesis represent critical windows of susceptibility during which environmental agents may adversely influence sperm epigenetic regulation [9]. A wide range of animal and epidemiological studies has linked exposure to endocrine disrupting chemicals during these susceptibility windows to adverse male reproductive health outcomes [59,60]. For example, exposure to phthalate metabolites at different doses in gestating F0 generation female rats during fetal gonadal sex determination has been shown to promote epigenetic inheritance of adverse health outcomes in male offspring such as pubertal abnormalities, testis disease, and obesity[10]. Analysis of the male F3 generation sperm epigenome revealed that aberrant methylation patterns were correlated with the pathologies identified. In addition, several epidemiological studies have linked exposure to phthalates to male infertility outcomes such as low sperm count, motility, and morphology [61-66], as well as reduction in fecundity specific to males [67]. Because alterations in the sperm epigenome have been associated with adverse male reproductive health outcomes [35], DNA methylation

marks in sperm may provide part of the mechanistic pathway between environmental exposures and male reproductive health and inform more targeted treatment and intervention strategies to reduce the risk of these outcomes.

5.2 Limitations

5.2.1 Measurement Error

Variation in human measurements between sperm samples when performing DNA extraction protocol may have reduced the accuracy of resulting sperm DNA concentrations. However, because DNA extraction protocol was performed by the same experimenter under controlled conditions, any variation due to human error would be minimal.

In addition, because DNA methylation quality control preparations for PCR, SAP, and T7 reactions were conducted by two different experimenters, human error and variation in human measurements may have reduced the accuracy of methylation results. However, all reaction preparations were conducted by the experimenters at the same time, and reactions from both experimenters were run at the same time on the same instruments, thus minimizing the potential impact of this source of error.

Measurement error can also occur due to the imprecision inherent to the DNA methylation assays performed. However, because these assays follow a standardized protocol, any variation occurring in fresh sperm samples would cancel out the same variation occurring for 4-week-old samples.

5.2.2 Small Sample Size

All sperm samples were obtained from three healthy volunteers and used for our analyses. This small sample size may result in inaccurate estimates of differences in DNA yields, DNA quality, and degree of DNA methylation in imprinted genes between our fresh and 4-week-old sperm samples. This inaccuracy produces large standard errors so that the power to detect statistical differences between fresh and 4-week-old sperm samples would be reduced compared to larger sample sizes. Therefore, we found it inappropriate to perform any tests for statistical significance because the results of such tests would be virtually meaningless given our lack of statistical power. Future studies that utilize our optimized protocol using sufficiently large sample sizes to perform appropriate statistical tests would help to validate our results.

5.2.3 Missing DNA Methylation Data

The MassARRAY (Sequenom) MALDI-TOF platform was unable to provide DNA methylation data for certain CpG sites in the imprinted genes we assessed. This typically occurs if the mass of a T7 cleavage product containing one or more CpG sites is too high or low to fall within the mass window of detection used by the MassARRAY platform. The lack of DNA methylation data could pose problems for analysis if the actual methylation levels for CpG sites in the undetected T7 cleavage products differ from those that were detected, impacting our quality control assessment. However, because only one imprinted allele is present for a given gamete, it is unlikely that the degree of methylation would vary by the number of T7 cleavage products detected for a given imprinted locus.

5.3 Future Assessments of Sperm Profiles for Clinical Research

5.3.1 Importance of RNA in Sperm

A wide range of RNAs exist in mature human spermatozoa, from large messenger RNAs (mRNAs) [68] to small noncoding RNAs (sncRNAs) [69]. Mature spermatozoa are transcriptionally and translationally quiescent because they are devoid of intact ribosomal RNAs (rRNAs), including 28S and 18S transcripts [70]. Failure to observe rRNAs in sperm was previously attributed to the large reduction in cytoplasmic volume during spermiogenesis which expelled translational machinery [71,72]. However, recent studies using Next Generation Sequencing revealed that rRNA fragments abound, suggesting that cleavage (not expulsion of rRNA) is responsible for preventing spurious translation following spermiogenesis[73,74].

Until recently, the observations of RNA in mature sperm were met with skepticism because of the view that the highly condensed sperm nucleus is transcriptionally inactive[70] and contamination from mitochondria or cytoplasmic residues could not be ruled out [75]. Advances in RNA extraction technologies in the late 1990s using reverse transcriptase polymerase chain reaction (RT-PCR), *in situ* hybridization, and microarrays lacked any residual cytoplasm, mitochondria or somatic contaminants during preparations, thus ruling out any possible contamination [68,76-78]. The validity of all three methods was subsequently confirmed in a single study [79].

Both mammalian and plant studies have identified many RNAs in sperm [80,81] and these RNAs have been associated with a wide range of biological processes [68,69]. These processes continue to be subject to investigation, but provide the first clues to

understanding the early events of post-fertilization and development. One generally accepted hypothesis is that paternally derived mRNAs remain translationally inactive in mature sperm, and some mRNAs are selectively retained until delivery to the oocyte [73]. The mechanism by which this occurs is the subject of much debate, but one possibility has been well characterized. During the final transcriptional period of spermiogenesis, several mRNAs are produced and then sequestered for storage as inactive messenger ribonucleoprotein particles (mRNPs) [82]. This sequestration is necessary for preservation of intact mRNAs before the sperm is delivered to the oocyte at fertilization. A study that compared transcripts retained in sperm from pooled and individual human ejaculates found the existence of a common spermatozoal mRNA fingerprint [68]. The RNA profile found included transcripts implicated in fertilization and development. These RNAs have since been independently observed in zygotes following fertilization [83]. The findings suggest that the RNAs retained in sperm and delivered to the oocyte at fertilization are not solely remnants of transcription during spermatogenesis, but may be essential for future embryonic development.

sncRNAs have also been suggested to play a role in regulating gene expression during spermatogenesis and future embryonic development, influencing offspring phenotype [84,85]. sncRNAs are approximately between 18 and 39 nucleotides in size and classified according to their biogenesis [86]. In somatic cells, these molecules function in post-transcriptional gene regulation, chromatin structure, and inhibiting transposition [73]. Small interfering RNAs (siRNA) and miRNAs are two of the most characterized classes of sncRNAs. Ranging in length from 20-24 nucleotides, these molecules are processed from loops in single-stranded DNA known as hairpins in

pathways involving the RNA-induced silencing complex (RISC) and DICER [73]. Data pertaining to sncRNAs in mature sperm have been largely uncharacterized to date.

A recent review highlighted that although miRNAs were the first class of sncRNAs observed in mammalian sperm, they account for only a small percentage (3%) of known sncRNAs aligned to the sperm genome [73]. Because post-transcriptional regulation of early embryonic development is strongly down-regulated during oocyte maturation and not required for preimplantation development [87,88], it is possible that paternal miRNAs and other sncRNAs delivered to the zygote bypass this regulatory pathway altogether. In somatic cells, sncRNAs bind to complimentary promoter regions, silencing gene transcription through the recruitment of PcG proteins and repressive histone marks [89]. The majority of miRNAs identified in sperm originate from promoter regions [73], suggesting that these transcripts may bind to paternal DNA during nuclear remodeling, influencing sperm chromatin structure, before being delivered to the oocyte.

In addition to siRNAs and miRNAs, the presence of piwi-interacting RNAs (piRNAs) has been demonstrated in spermatogenic cells [90]. Ranging in size from 26-30 nucleotides, these sncRNAs are produced independent of DICER and RISC, not requiring double-stranded RNA folding [73]. Their function is essential to spermatogenesis because, complementary to transposons, these RNAs repress the rate of transposition, protecting the paternal genome from mobile elements [73]. Though assumed to be absent from mature sperm because of their function, a restricted set of piRNAs may be retained [73].

The potential applications of sperm RNAs have been summarized in a review [75]. The presence of RNAs in mature sperm indicates that the male gamete does not only serve as a vehicle for paternal DNA to the oocyte, but also carries key molecular markers in RNA, chromatin, and the nuclear matrix that are essential for proper embryonic development [73]. The presence or absence of the various stage-specific transcripts in mature sperm may provide a means to assess the fidelity of each stage of spermatogenesis. For example, PRM transcripts have been applied in clinics as their absence from ejaculate samples has been used to 1) confirm vasectomy and 2) diagnose male-factor infertility [75]. Overall, all RNAs reflect the transcriptional history of spermatogenic differentiation and their applications show great promise as a diagnostic tools.

5.3.2 Extraction of Sperm RNA

The identification of RNA in mature spermatozoa, together with evidence linking its function to male fertility and future embryonic development [73,75,83], necessitated the development of reliable protocol to extract high-quality, high molecular weight RNA for downstream applications such as microarray profiling or PCR. The heterogeneous population of cells present in ejaculate and the small quantity of RNA present in cells (50fg of RNA/cell and 0.3fg of sncRNA/cell[55]) has represented some of the main challenges to the successful development of this protocol over several years[55,91,92]. Furthermore, because mature spermatozoa are transcriptionally and translationally quiescent, rRNA markers are virtually absent, hindering quality assessment. These challenges have necessitated 1) a purifying step to isolate only spermatozoa from a pool of somatic cell-containing ejaculate, 2) optimization of RNA extraction protocol to

maximize yield, and 3) the identification of new markers for sperm RNA quality assessment.

In the most recent protocol described by Goodrich et al. [55], a guanidinium thiocyanate-phenol-chloroform method to remove contaminating DNA was combined with Qiagen's column-based RNeasy Mini and Minielute kits to obtain high-quality fractions of spermatozoal sncRNAs and mRNAs. The protocol incorporated β-mercaptoethanol for cell lysis, nuclease-free stainless steel beads for homogenization using Disruptor Genie (Scientific Industries), and RNase block (Stratagene) to inhibit degradation from residual RNases.

Quality control was performed through Turbo DNase buffer (Ambion) treatment, reverse transcription, and PCR amplification with intron-spanning primers to verify the absence of genomic contamination and mRNA integrity[55]. Real-time PCR with PRM1 primers showed that DNase-treated samples were void of amplification while only human genomic controls amplified, indicating that DNA contaminants were successfully removed. In addition, RNA integrity was assessed through two methods. First, reverse-transcription of spermatozoal RNAs and subsequent real-time PCR on the cDNA products using PRM1 primers were performed. Products from cDNA amplification were smaller than products from DNA amplification because the PRM1 primers chosen were intron spanning [55]. Secondly, Bioanalyzer (Agilent) kits for total RNA and small RNA were used to verify 1) the absence of intact 18S and 28S rRNAs and 2) the presence of sncRNA respectively. The profiles showed that 18S and 28S rRNAs were undetectable, confirming their degradation, as well as the presence of sncRNAs as a rise in fluorescent units above background between 6 and 30 nucleotides in length [55].

While the recent methods published by Goodrich et al. [55] demonstrate the ability to yield high quality, high molecular weight RNA, these methods lack a means to stabilize the RNA for periods suitable for clinical research. We are currently developing a method for rapidly attaining high quality RNA from human sperm using similar stabilizing protocol from our DNA extraction methods.

5.3.3 Sperm Histone Retention and Histone Modifications

A major subject of debate is whether selective post-meiotic retention of histones poises specific genomic regions of the sperm for early use during embryonic development. Initial evidence supporting this notion came from findings that showed histones bound to DNA in a sequence-specific manner around gene regulatory regions [93,94]. Isolation and interrogation of histone-associated sequences indicated that these regions include imprinted regions [95], telomeres [96,97], retroposon DNA [96], and specific gene loci [94,96,98]. In comparison to these regions, centromeric and pericentromeric regions of mammalian sperm have been found to lack histones, presenting a mix of histones and protamines [94].

Recent advances in genome-wide analysis techniques now allow detection of histone-enriched regions at the primary sequence level. For example, CGH tiling arrays have associated histone-bound DNA with gene-dense regions, developmentally regulated promoters, and CTCF binding sites [99]. Next generation sequencing (NGS) exhibited even higher resolution analysis, revealing enrichment of histone-associated sequences at developmentally important genes such as spermatogenesis genes, embryonic transcription factors, and signaling machinery, as well as microRNA (miRNA) and imprinted gene

regions [34]. Further analyses revealed that internal exons also show significantly greater histone enrichment than intronic sequences, and histones were found to be distributed at relatively low levels outside of promoter regions [100]. It has also been proposed that histone-bound DNA retained in mature sperm mark sites of nuclear matrix attachment at scaffold/matrix attachment regions (S/MARs) anchoring decondensed DNA loops of prior cell types [36]. These markers may serve to deliver further information on paternal nuclear architecture to the zygote [36].

The promoters of developmental genes and certain noncoding RNAs in sperm have been associated with H3 Lysine 4 methylation (H3K4me3), a gene-activating histone modification, while lacking H3 Lysine 27 methylation (H3K27me3), a repressive histone modification [34]. On the other hand, promoters of genes encoding transcription factors important for embryonic development and morphogenesis bear two histone modifications with antagonistic roles: H3K4me3 and H3K27me3-together known as 'bivalent' chromatin [34]. At these sites, large regions of H3K27me3 overlap with smaller regions of H3K4me3, potentially poising genes for either activation or repression later in development [101].

Overall, these findings suggest that the sperm genome may be packaged and poised for two important processes: 1) spermatogenesis through active chromatin marks, and 2) future embryonic development through bivalent chromatin domains. These possibilities open new questions about whether various environmental and lifestyle factors may influence sperm histone modifications in a manner that impacts male fertility or future embryo development. The ability to perform sperm histone modification analysis has been demonstrated using standard chromatin immunoprecipitation (ChIP)

assays [34,101]. Whether our sperm stabilization methods are compatible with these assays for clinical research warrants further study.

5.4 Conclusions

We have optimized methods to extract human sperm DNA rapidly under simple conditions, eliminating the need for costly, inefficient alternative protocol not ideal for clinical research. Furthermore, storing sperm samples in DNA/RNA shield at $4^{O}C$ stabilized sperm DNA mass and quality over a period of 4 weeks. This stability increases the cost-efficiency of clinical research because it minimizes the need to produce fresh sperm DNA samples for each downstream genetic and epigenetic application.

APPENDIX A

HUMAN SPERM CELL ISOLATION AND DNA EXTRACTION

A.1 Materials

PureCeption 100% Isotonic Solution	SAGE Ref # ART-2100
Quinn's Sperm Washing Medium	SAGE Ref # ART-1006
DNA/RNA Shield	Zymo Research Cat # R1100-1-50
Bond-Breaker TCEP Solution Neutral pH, 0.5M	Thermo Scientific Prod # 77720
Pulse Vortex Mixer	Fisher Scientific Cat # 02215375
QIAshredder	Qiagen Cat # 7965
Quick-gDNA MiniPrep Kit	Zymo Research Cat # D3025

A.2 Sperm Cell Isolation Protocol

1. Bring PureCeption 100% Isotonic Solution and Quinn's Sperm Washing Medium to 37^{O}C before use. Make a 90% PureCeption solution by adding 1 volume of Quinn's Sperm Washing Medium to 9 volumes of PureCeption 100% Isotonic Solution.

2. Add 1.0mL of 90% PureCeption to a 15mL conical centrifuge tube.

3. Gently layer 1.5-2.0mL of fresh liquefied semen on top of the 90% PureCeption using a transfer pipette. There should be no mixing of the sample and the 90% PureCeption. If the semen volume is more than 2.0mL, use more than one tube of 90% PureCeption.

4. Centrifuge at 500 *x g* for 30 minutes.

5. Using a pipette, carefully remove the 90% PureCeption and seminal fluid without disturbing the sperm pellet, leaving a small amount of 90% PureCeption over the sperm pellet. Aspirate from the top downward, always keeping the pipette tip just below the fluid surface. If no sperm pellet is clearly visible, remove all but 0.5mL

of the 90% PureCeption layer. This will allow for the collection of sperm suspended in the 90% PureCeption. Transfer the sperm pellet in this residual medium to a clean conical centrifuge tube for further washing.

6. Using a pipette, add 4mL of Quinn's Sperm Washing Medium and resuspend the pellet by gently tapping with your fingers.

7. Centrifuge the mixture at 500 *x g* for five minutes to wash away residual 90% PureCeption solution.

8. Placing the pipette to the bottom of the tube, remove 1mL of washed sample and place in a new 1.5mL microcentrifuge tube.

9. Centrifuge the tube for 1 minute at maximum speed and then carefully remove the supernatant.

10. Resuspend the sperm pellet in 900µL of DNA/RNA shield and 100µL (50mM) of 0.5M TCEP. Mix and incubate at room temperature for 5 minutes with occasional vortexing using the Pulse Vortex Mixer.

11. Centrifuge half the volume of mixture in a QIAshredder column twice for 2 minutes at maximum speed. If the sperm cells will not be used immediately, cells in DNA/RNA shield can be stored at 4^{O}C for up to a month.

A.3 Sperm DNA Isolation Protocol

1. Add Genomic Lysis Buffer from the Quick-gDNA MiniPrep Kit to the mixture in a 3:1 ratio.

2. Transfer the mixture to a Zymo-Spin Column in a Collection Tube. Centrifuge at 10,000 *x g* for one minute. Discard the Collection Tube with the flow through.

3. Transfer the Zymo-Spin Column to a new Collection Tube. Add 200µL of DNA Pre-Wash Buffer to the spin column. Centrifuge at 10,000 *x g* for one minute.

4. Add 500µL of g-DNA Wash Buffer to the spin column. Centrifuge at 10,000 *x g* for one minute. Repeat this step once and then incubate at room temperature for five minutes.

5. Transfer the spin column to a clean microcentrifuge tube. Add 100µL DNA Elution Buffer to the spin column. Incubate for 3 minutes at room temperature and then centrifuge at maximum speed for 30 seconds to elute the DNA. If the DNA will not be used immediately, store at 4^{O}C.

APPENDIX B

SUPPLEMENTARY TABLES AND FIGURES

Table B.1: Primers used to amplify *ALU*, *SNURF*, *PEG10*, and *H19* loci by PCR from DNA extracted from human sperm samples. *ALU* was amplified from non-bisulfite-converted DNA for assessment of PCR amplification and the imprinted loci *SNURF, PEG10*, and *H19* were amplified from bisulfite converted DNA for DNA methylation analysis. Underlined sequences in lowercase letters represent the T7-promoter tagged reverse primer for in vitro transcription and a 10mer-tag sequence added to the forward primer to balance the PCR primer length.

Genomic Locus	Primer	Size (bp)	Tm (^{O}C)	GC%	Product size (bp)	Sequence
SNURF	Forward	35	70.6	48.6	270	5'-aggaagagagAGGGGGTATTAGAAGGGGTAGTAGT-3'
	Reverse	57	73.2	38.6		5'-cagtaatacgactcactatagggagaaggctATAAATTTTATATATTATTTTAATTT-3'
PEG10	Forward	35	69.4	40.0	269	5'-aggaagagagGTTTGGTTTAGGTGTGGGATTTTAT-3'
	Reverse	56	73.1	39.3		5'-cagtaatacgactcactatagggagaaggctTTTAAATTTTTAAAATTTAATTTTT-3'
H19	Forward	37	63.8	40.5	276	5'-aggaagagagGGAAAATGTAAGATTTTGGTGGAATAT-3'
	Reverse	24	69.7	58.3		5'-cagtaatacgactcactatagggagaaggctTAGTGTAGGTTTATATATTATAGT-3'
ALU	Forward	25	65.0	60.0	446	5'-GGATCTCAGGGTGGGTGGCAATGCT-3'
	Reverse	26	63.1	53.8		5'-GAAAGGCAAGCTACCAGAAGCCCCAA-3'

BIBLIOGRAPHY

1. CDC (2013) Infertility and Impaired Fecundity in the United States, 1982–2010: Data From the National Survey of Family Growth.

2. Anawalt BD (2013) Approach to male infertility and induction of spermatogenesis. J Clin Endocrinol Metab 98: 3532-3542.

3. Swan SH, Elkin EP, Fenster L (2000) The question of declining sperm density revisited: an analysis of 101 studies published 1934-1996. Environ Health Perspect 108: 961-966.

4. Crow JF (2000) The origins, patterns and implications of human spontaneous mutation. Nat Rev Genet 1: 40-47.

5. Kong A, Frigge ML, Masson G, Besenbacher S, Sulem P, et al. (2012) Rate of de novo mutations and the importance of father's age to disease risk. Nature 488: 471-475.

6. Sanders SJ, Murtha MT, Gupta AR, Murdoch JD, Raubeson MJ, et al. (2012) De novo mutations revealed by whole-exome sequencing are strongly associated with autism. Nature 485: 237-241.

7. Neale BM, Kou Y, Liu L, Ma'ayan A, Samocha KE, et al. (2012) Patterns and rates of exonic de novo mutations in autism spectrum disorders. Nature 485: 242-245.

8. O'Roak BJ, Vives L, Girirajan S, Karakoc E, Krumm N, et al. (2012) Sporadic autism exomes reveal a highly interconnected protein network of de novo mutations. Nature 485: 246-250.

9. Godmann M, Lambrot R, Kimmins S (2009) The dynamic epigenetic program in male germ cells: Its role in spermatogenesis, testis cancer, and its response to the environment. Microsc Res Tech 72: 603-619.

10. Manikkam M, Tracey R, Guerrero-Bosagna C, Skinner MK (2013) Plastics derived endocrine disruptors (BPA, DEHP and DBP) induce epigenetic transgenerational inheritance of obesity, reproductive disease and sperm epimutations. PLoS One 8: e55387.

11. Schutte B, El Hajj N, Kuhtz J, Nanda I, Gromoll J, et al. (2013) Broad DNA methylation changes of spermatogenesis, inflammation and immune response-related genes in a subgroup of sperm samples for assisted reproduction. Andrology 1: 822-829.

12. Poplinski A, Tuttelmann F, Kanber D, Horsthemke B, Gromoll J (2010) Idiopathic male infertility is strongly associated with aberrant methylation of MEST and IGF2/H19 ICR1. Int J Androl 33: 642-649.

13. Li B, Li JB, Xiao XF, Ma YF, Wang J, et al. (2013) Altered DNA methylation patterns of the H19 differentially methylated region and the DAZL gene promoter are associated with defective human sperm. PLoS One 8: e71215.

14. Hess RA, Renato de Franca L (2008) Spermatogenesis and cycle of the seminiferous epithelium. Adv Exp Med Biol 636: 1-15.

15. Meistrich ML, Hess RA (2013) Assessment of spermatogenesis through staging of seminiferous tubules. Methods Mol Biol 927: 299-307.

16. Leblond CP, Clermont Y (1952) Definition of the stages of the cycle of the seminiferous epithelium in the rat. Ann N Y Acad Sci 55: 548-573.

17. Oakberg EF (1956) A description of spermiogenesis in the mouse and its use in analysis of the cycle of the seminiferous epithelium and germ cell renewal. Am J Anat 99: 391-413.

18. College O (2013) Anatomy and Physiology of the Male Reproductive System. In: College O, editor. Anatomy & Physiology. Rice University: Houston; Texas. pp. 1206-1216.

19. Shaw W, Padubidri VG, Daftary SN, Howkins J, Bourne G (2010). Shaw's Textbook of Gynaecology. Noida: Elsevier. pp. 201.

20. Gilbert S (2000) Spermatogenesis. Developmental Biology. 6 ed. Sunderland (MA): Sinauer Associates.

21. Dym M (1994) Spermatogonial stem cells of the testis. Proc Natl Acad Sci U S A 91: 11287-11289.

22. Schwab KR, Smith GD, Dressler GR (2013) Arrested spermatogenesis and evidence for DNA damage in PTIP mutant testes. Dev Biol 373: 64-71.

23. Carrell DT (2012) Epigenetics of the male gamete. Fertil Steril 97: 267-274.

24. Yanagimachi R (2005) Male gamete contributions to the embryo. Ann N Y Acad Sci 1061: 203-207.

25. Oliva R (2006) Protamines and male infertility. Hum Reprod Update 12: 417-435.

26. Carrell DT, Emery BR, Hammoud S (2007) Altered protamine expression and diminished spermatogenesis: what is the link? Hum Reprod Update 13: 313-327.

27. Churikov D, Siino J, Svetlova M, Zhang K, Gineitis A, et al. (2004) Novel human testis-specific histone H2B encoded by the interrupted gene on the X chromosome. Genomics 84: 745-756.

28. Rousseaux S, Boussouar F, Gaucher J, Reynoird N, Montellier E, et al. (2011) Molecular models for post-meiotic male genome reprogramming. Syst Biol Reprod Med 57: 50-53.

29. Song N, Liu J, An S, Nishino T, Hishikawa Y, et al. (2011) Immunohistochemical Analysis of Histone H3 Modifications in Germ Cells during Mouse Spermatogenesis. Acta Histochem Cytochem 44: 183-190.

30. Meistrich ML, Mohapatra B, Shirley CR, Zhao M (2003) Roles of transition nuclear proteins in spermiogenesis. Chromosoma 111: 483-488.

31. Corzett M, Mazrimas J, Balhorn R (2002) Protamine 1: protamine 2 stoichiometry in the sperm of eutherian mammals. Mol Reprod Dev 61: 519-527.

32. Cree LH, Balhorn R, Brewer LR (2011) Single molecule studies of DNA-protamine interactions. Protein Pept Lett 18: 802-810.

33. Griffin J (2013) Methods of sperm DNA extraction for genetic and epigenetic studies. Methods Mol Biol 927: 379-384.

34. Hammoud SS, Nix DA, Zhang H, Purwar J, Carrell DT, et al. (2009) Distinctive chromatin in human sperm packages genes for embryo development. Nature 460: 473-478.

35. Hammoud SS, Nix DA, Hammoud AO, Gibson M, Cairns BR, et al. (2011) Genome-wide analysis identifies changes in histone retention and epigenetic modifications at developmental and imprinted gene loci in the sperm of infertile men. Hum Reprod 26: 2558-2569.

36. Ward WS (2010) Function of sperm chromatin structural elements in fertilization and development. Mol Hum Reprod 16: 30-36.

37. Yamauchi Y, Shaman JA, Ward WS (2011) Non-genetic contributions of the sperm nucleus to embryonic development. Asian J Androl 13: 31-35.

38. Dominguez K, Arca CD (2011) The relationship between chromatin structure and DNA damage in mammalian spermatozoa. Sperm Chromatin: Springer. pp. 61-68.

39. Hammoud SS, Purwar J, Pflueger C, Cairns BR, Carrell DT (2010) Alterations in sperm DNA methylation patterns at imprinted loci in two classes of infertility. Fertil Steril 94: 1728-1733.

40. Aston KI, Punj V, Liu L, Carrell DT (2012) Genome-wide sperm deoxyribonucleic acid methylation is altered in some men with abnormal chromatin packaging or poor in vitro fertilization embryogenesis. Fertil Steril 97: 285-292.

41. Risch N, Reich EW, Wishnick MM, McCarthy JG (1987) Spontaneous mutation and parental age in humans. Am J Hum Genet 41: 218-248.

42. Jaenisch R, Bird A (2003) Epigenetic regulation of gene expression: how the genome integrates intrinsic and environmental signals. Nat Genet 33 Suppl: 245-254.

43. Smallwood SA, Kelsey G (2012) De novo DNA methylation: a germ cell perspective. Trends Genet 28: 33-42.

44. Singal R, Ginder GD (1999) DNA methylation. Blood 93: 4059-4070.

45. Ng HH, Bird A (1999) DNA methylation and chromatin modification. Curr Opin Genet Dev 9: 158-163.

46. Bienvenu T, Chelly J (2006) Molecular genetics of Rett syndrome: when DNA methylation goes unrecognized. Nat Rev Genet 7: 415-426.

47. Oakes CC, La Salle S, Smiraglia DJ, Robaire B, Trasler JM (2007) A unique configuration of genome-wide DNA methylation patterns in the testis. Proc Natl Acad Sci U S A 104: 228-233.

48. Weber M, Hellmann I, Stadler MB, Ramos L, Paabo S, et al. (2007) Distribution, silencing potential and evolutionary impact of promoter DNA methylation in the human genome. Nat Genet 39: 457-466.

49. Li E, Beard C, Jaenisch R (1993) Role for DNA methylation in genomic imprinting. Nature 366: 362-365.

50. Bahnak BR, Wu QY, Coulombel L, Drouet L, Kerbiriou-Nabias D, et al. (1988) A simple and efficient method for isolating high molecular weight DNA from mammalian sperm. Nucleic Acids Res 16: 1208.

51. Shiurba R, Nandi S (1979) Isolation and characterization of germ line DNA from mouse sperm. Proc Natl Acad Sci U S A 76: 3947-3951.

52. Pacheco SE, Houseman EA, Christensen BC, Marsit CJ, Kelsey KT, et al. (2011) Integrative DNA methylation and gene expression analyses identify DNA packaging and epigenetic regulatory genes associated with low motility sperm. PLoS One 6: e20280.

53. Hossain AM, Rizk B, Behzadian A, Thorneycroft IH (1997) Modified guanidinium thiocyanate method for human sperm DNA isolation. Mol Hum Reprod 3: 953-956.

54. Han JC, Han GY (1994) A procedure for quantitative determination of tris(2-carboxyethyl)phosphine, an odorless reducing agent more stable and effective than dithiothreitol. Anal Biochem 220: 5-10.

55. Goodrich RJ, Anton E, Krawetz SA (2013) Isolating mRNA and small noncoding RNAs from human sperm. Methods Mol Biol 927: 385-396.

56. Lambrot R, Xu C, Saint-Phar S, Chountalos G, Cohen T, et al. (2013) Low paternal dietary folate alters the mouse sperm epigenome and is associated with negative pregnancy outcomes. Nat Commun 4: 2889.

57. van den Boom D, Ehrich M (2009) Mass spectrometric analysis of cytosine methylation by base-specific cleavage and primer extension methods. Methods Mol Biol 507: 207-227.

58. Marques CJ, Joao Pinho M, Carvalho F, Bieche I, Barros A, et al. (2011) DNA methylation imprinting marks and DNA methyltransferase expression in human spermatogenic cell stages. Epigenetics 6: 1354-1361.

59. Jurewicz J, Hanke W (2011) Exposure to phthalates: reproductive outcome and children health. A review of epidemiological studies. Int J Occup Med Environ Health 24: 115-141.

60. Yeung BH, Wan HT, Law AY, Wong CK (2011) Endocrine disrupting chemicals: Multiple effects on testicular signaling and spermatogenesis. Spermatogenesis 1: 231-239.

61. Duty SM, Silva MJ, Barr DB, Brock JW, Ryan L, et al. (2003) Phthalate exposure and human semen parameters. Epidemiology 14: 269-277.

62. Jonsson BA, Richthoff J, Rylander L, Giwercman A, Hagmar L (2005) Urinary phthalate metabolites and biomarkers of reproductive function in young men. Epidemiology 16: 487-493.

63. Hauser R, Meeker JD, Duty S, Silva MJ, Calafat AM (2006) Altered semen quality in relation to urinary concentrations of phthalate monoester and oxidative metabolites. Epidemiology 17: 682-691.

64. Rozati R, Reddy PP, Reddanna P, Mujtaba R (2002) Role of environmental estrogens in the deterioration of male factor fertility. Fertil Steril 78: 1187-1194.

65. Pant N, Shukla M, Kumar Patel D, Shukla Y, Mathur N, et al. (2008) Correlation of phthalate exposures with semen quality. Toxicol Appl Pharmacol 231: 112-116.

66. Huang LP, Lee CC, Hsu PC, Shih TS (2011) The association between semen quality in workers and the concentration of di(2-ethylhexyl) phthalate in polyvinyl chloride pellet plant air. Fertil Steril 96: 90-94.

67. Buck Louis GM, Sundaram R, Sweeney AM, Schisterman EF, Maisog J, et al. (2014) Urinary bisphenol A, phthalates, and couple fecundity: the Longitudinal Investigation of Fertility and the Environment (LIFE) Study. Fertil Steril.

68. Ostermeier GC, Dix DJ, Miller D, Khatri P, Krawetz SA (2002) Spermatozoal RNA profiles of normal fertile men. Lancet 360: 772-777.

69. Ostermeier GC, Goodrich RJ, Moldenhauer JS, Diamond MP, Krawetz SA (2005) A suite of novel human spermatozoal RNAs. J Androl 26: 70-74.

70. Kierszenbaum AL, Tres LL (1975) Structural and transcriptional features of the mouse spermatid genome. J Cell Biol 65: 258-270.

71. Braun RE (2001) Packaging paternal chromosomes with protamine. Nat Genet 28: 10-12.

72. Sassone-Corsi P (2002) Unique chromatin remodeling and transcriptional regulation in spermatogenesis. Science 296: 2176-2178.

73. Johnson GD, Lalancette C, Linnemann AK, Leduc F, Boissonneault G, et al. (2011) The sperm nucleus: chromatin, RNA, and the nuclear matrix. Reproduction 141: 21-36.

74. Johnson GD, Sendler E, Lalancette C, Hauser R, Diamond MP, et al. (2011) Cleavage of rRNA ensures translational cessation in sperm at fertilization. Mol Hum Reprod 17: 721-726.

75. Krawetz SA (2005) Paternal contribution: new insights and future challenges. Nat Rev Genet 6: 633-642.

76. Wykes SM, Visscher DW, Krawetz SA (1997) Haploid transcripts persist in mature human spermatozoa. Mol Hum Reprod 3: 15-19.

77. Miller D, Briggs D, Snowden H, Hamlington J, Rollinson S, et al. (1999) A complex population of RNAs exists in human ejaculate spermatozoa: implications for understanding molecular aspects of spermiogenesis. Gene 237: 385-392.

78. Miller D, Tang PZ, Skinner C, Lilford R (1994) Differential RNA fingerprinting as a tool in the analysis of spermatozoal gene expression. Hum Reprod 9: 864-869.

79. Dadoune JP, Pawlak A, Alfonsi MF, Siffroi JP (2005) Identification of transcripts by macroarrays, RT-PCR and in situ hybridization in human ejaculate spermatozoa. Mol Hum Reprod 11: 133-140.

80. Dadoune JP, Siffroi JP, Alfonsi MF (2004) Transcription in haploid male germ cells. Int Rev Cytol 237: 1-56.

81. Engel ML, Chaboud A, Dumas C, McCormick S (2003) Sperm cells of Zea mays have a complex complement of mRNAs. Plant J 34: 697-707.

82. Kleene KC (2003) Patterns, mechanisms, and functions of translation regulation in mammalian spermatogenic cells. Cytogenet Genome Res 103: 217-224.

83. Ostermeier GC, Miller D, Huntriss JD, Diamond MP, Krawetz SA (2004) Reproductive biology: delivering spermatozoan RNA to the oocyte. Nature 429: 154.

84. Rassoulzadegan M, Grandjean V, Gounon P, Vincent S, Gillot I, et al. (2006) RNA-mediated non-mendelian inheritance of an epigenetic change in the mouse. Nature 441: 469-474.

85. Grandjean V, Gounon P, Wagner N, Martin L, Wagner KD, et al. (2009) The miR-124-Sox9 paramutation: RNA-mediated epigenetic control of embryonic and adult growth. Development 136: 3647-3655.

86. Moazed D (2009) Small RNAs in transcriptional gene silencing and genome defence. Nature 457: 413-420.

87. Ma J, Flemr M, Stein P, Berninger P, Malik R, et al. (2010) MicroRNA activity is suppressed in mouse oocytes. Curr Biol 20: 265-270.

88. Suh N, Baehner L, Moltzahn F, Melton C, Shenoy A, et al. (2010) MicroRNA function is globally suppressed in mouse oocytes and early embryos. Curr Biol 20: 271-277.

89. Kim DH, Saetrom P, Snove O, Jr., Rossi JJ (2008) MicroRNA-directed transcriptional gene silencing in mammalian cells. Proc Natl Acad Sci U S A 105: 16230-16235.

90. Lau NC (2010) Small RNAs in the animal gonad: guarding genomes and guiding development. Int J Biochem Cell Biol 42: 1334-1347.

91. Cappallo-Obermann H, Schulze W, Jastrow H, Baukloh V, Spiess AN (2011) Highly purified spermatozoal RNA obtained by a novel method indicates an unusual 28S/18S rRNA ratio and suggests impaired ribosome assembly. Mol Hum Reprod 17: 669-678.

92. Goodrich R, Johnson G, Krawetz SA (2007) The preparation of human spermatozoal RNA for clinical analysis. Arch Androl 53: 161-167.

93. Gatewood JM, Cook GR, Balhorn R, Bradbury EM, Schmid CW (1987) Sequence-specific packaging of DNA in human sperm chromatin. Science 236: 962-964.

94. Wykes SM, Krawetz SA (2003) The structural organization of sperm chromatin. J Biol Chem 278: 29471-29477.

95. Banerjee S, Smallwood A (1998) Chromatin modification of imprinted H19 gene in mammalian spermatozoa. Mol Reprod Dev 50: 474-484.

96. Pittoggi C, Renzi L, Zaccagnini G, Cimini D, Degrassi F, et al. (1999) A fraction of mouse sperm chromatin is organized in nucleosomal hypersensitive domains enriched in retroposon DNA. J Cell Sci 112 (Pt 20): 3537-3548.

97. Zalenskaya IA, Bradbury EM, Zalensky AO (2000) Chromatin structure of telomere domain in human sperm. Biochem Biophys Res Commun 279: 213-218.

98. Gardiner-Garden M, Ballesteros M, Gordon M, Tam PP (1998) Histone- and protamine-DNA association: conservation of different patterns within the beta-globin domain in human sperm. Mol Cell Biol 18: 3350-3356.

99. Arpanahi A, Brinkworth M, Iles D, Krawetz SA, Paradowska A, et al. (2009) Endonuclease-sensitive regions of human spermatozoal chromatin are highly enriched in promoter and CTCF binding sequences. Genome Res 19: 1338-1349.

100. Nahkuri S, Taft RJ, Mattick JS (2009) Nucleosomes are preferentially positioned at exons in somatic and sperm cells. Cell Cycle 8: 3420-3424.

101. Bernstein BE, Mikkelsen TS, Xie X, Kamal M, Huebert DJ, et al. (2006) A bivalent chromatin structure marks key developmental genes in embryonic stem cells. Cell 125: 315-326.