Epigenetic Modification in Human Male Germ Line

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DECLARATION

I hereby declare that the work embodied in this text is the result of original research and that this thesis does not contain any material that has been submitted for any degree or diploma to any university or institution. Also, to the best of my knowledge, it does not contain any material published by anyone except where due reference made.

(Signed): ........ Jaleh Barzideh ........

Jaleh Barzideh

Endorsement by supervisors

Professor Rodney J. Scott.................................

Professor R. John Aitken.................................
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I dedicate this droplet of knowledge to a family who may it be useful to relieve a pain or to make a smile on their lips or continuing love in their hearts.
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<tr>
<td>5-m cytosine</td>
<td>5- methyl cytosine</td>
</tr>
<tr>
<td>5mdc</td>
<td>5- methyl-2’ deoxycitadine</td>
</tr>
<tr>
<td>dc</td>
<td>2’- deoxycitidine</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary DNA</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
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<td>DMSO</td>
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<tr>
<td>Dnmt</td>
<td>DNA methyl cytosine transferase</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiothreitol</td>
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<td>ECL</td>
<td>enhanced chemiluminescence</td>
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<tr>
<td>HDAT</td>
<td>histone Deacetyltransferase</td>
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<td>HPLC</td>
<td>High performance liquid chromatography</td>
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<td>PCNA</td>
<td>proliferating cell nuclear antigen</td>
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<td>platelet-derived growth factor</td>
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<td>protease inhibitor</td>
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<td>rotation per minute</td>
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<td>SDS</td>
<td>sodium dodecyl sulfate</td>
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<tr>
<td>SNP</td>
<td>single nucleotide polymorphism</td>
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<tr>
<td>TAE</td>
<td>TRIs –acetate- EDTA</td>
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<td>Tween-20</td>
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<td>tris (hydroxymethyl) aminomethane</td>
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## List Of Abbreviations

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<td>A</td>
<td>Amperes</td>
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<td>Bp</td>
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<td>volume per volume</td>
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<tr>
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<td>weight per volume</td>
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### Prefixes

- K: kilo
- M: mill
- µ: micro
- n: nano
ABSTRACT

The purpose of this study was to examine the methylation status of human sperm DNA in relation to the functional competence of these cells. In order to achieve this aim discontinuous Percoll gradient density centrifugation was used to generate sperm populations that were either normal (high-density Percoll fraction) or functionally impaired (low density Percoll fraction). The methylation status of these cells was then examined using HPLC, immunocytochemistry or flow cytometry and ultimately correlated with additional markers reflecting the tendency of these cells to default to an intrinsic apoptotic pathway.

The results of this study suggest that the mitochondrial genome is heavily methylated during spermatogenesis possibly as a means of suppressing expression of the paternal mitochondrial genome following fertilization. Extensive methylation of the mitochondrial genome appeared to be a ubiquitous, consistent feature of these cells and was present in both the high and low quality sperm populations. By contrast, the methylation status of the nuclear genome appeared to change dramatically in relation to the functional competence of the spermatozoa, such that defective cells exhibited a statistically significant increase in nuclear DNA methylation. Such hypermethylation of defective cells was confirmed by all 3 of the techniques used in this study (HPLC, immunocytochemistry and flow cytometry).

Different patterns of 5-methylcytosine expression were observed sperm nuclei by immunocytochemistry and possible interpretations offered in terms of the packaging of chromosomes into the nucleus during sperm differentiation. Furthermore, the methylation status of these cells was negatively correlated with various aspects of sperm function including sperm motility and the tendency of these cells to become apoptotic as reflected by the expression of activated caspases and Annexin-V binding. In addition the hypermethylation of human spermatozoa was highly correlated with their capacity to bind chromomycin3A, a marker that reflects the efficiency of sperm chromatin protamination.
These results clearly suggest that defective human spermatozoa are associated with hypermethylation of their nuclear genome, possibly as a consequence of the defective control of DNA methyltransferase activity during spermiogenesis. These hypermethylated cells are functionally defective and exhibit a tendency to default to an apoptotic cascade that features activation of endogenous caspases, phosphatidylserine exteriorization and DNA damage. These results have important implications for the safety of assisted conception procedures that frequently involve the forced fertilization of oocytes with defective spermatozoa.
CHAPTER 1

Introduction and Literature Review

1.1 Introduction

The aetiology of infertility in men still is a mystery. This condition is relatively common and affects approximately one in twenty of the male population (McLachlan and de Kretser, 2001). Increased DNA damage, associated with the spermatozoa of infertile men, could transfer genetic damage to the offspring via assisted reproductive technology especially when defective spermatozoa are used to induce conception via Intra-Cytoplasmic Sperm Injection (ICSI). Sertoli cells control the proliferation and development of germ cells, limiting the number of spermatozoa generated and eradicating defective cells via apoptosis. Occasionally differentiating germ cells may escape the apoptotic machinery, with the result that the semen of sub fertile men may possess mature spermatozoa carrying DNA strand breaks as a legacy of this abortive apoptotic process, (Taylor et. al., 2004).

In addition, spermatozoa from infertile males may also exhibit increased signs of immaturity. DNA damage could occur at any stage of spermatogenesis from the pre-meiotic replication of stem cells to chromatin remodelling during the DNA packaging stage of spermiogenesis. DNA methylation and histone modification are two important signs of epigenetic modification in human sperm genome. These events highlight the need for a better understanding the process of cell divisions, apoptosis and DNA susceptibility to damage, gene expression, and epigenetic regulation during spermatogenesis.
1.2 The cell cycle during spermatogenesis

After puberty, the male germ cell passes through the different stages of mitosis and meiosis and produces haploid spermatocytes in the seminiferous tubules of the testes, whereas in the female, meiosis begins immediately after primordial germ cell migration to the embryonic ovary. A retinoic acid activated signal transduction pathway stimulates the oogonal germ line to enter meiosis whereas, there is no such stimulatory effect in male germ line. It is believed that retinoic acid degrades in the differentiating male reproductive tract and, as a result, meiotic division is suppressed until puberty when Sertoli cells start secretion of endogenous retinoic acid (Suzuki and Saga 2008).

Sertoli cells in the germinal epithelium of the seminiferous tubules play an essential role in the proliferation and differentiation of germ cells. The process of sperm production in the seminiferous tubules continues throughout the entire lifespan of most mammals except for a minor decline in the quantity and quality of sperm production because of ageing. Spermatozoa are produced at a steady rate during spermatogenesis with a daily output of generally around $8.5 \times 10^6$ sperm/g testicular tissue in the human which is about half as efficient as the rat testes, which produces $17.4 \times 10^6$ sperm/g testicular tissue/per day (Johnson et al., 1980). Male germ cells proliferate and differentiate to form mature spermatozoa during a spermatogenesis cycle that lasts around 64 days (Misell et al., 2006).

1.2.1 Proliferative processes of spermatogenesis

Male germ cells develop through the process of spermatogenesis continuously. Renewal and proliferation happens at the same time. Therefore, each segment of seminiferous tubule contains four or five different products of spermatogonia in different stages of proliferation and differentiation. Proliferation of germ cells occurs at the basal membrane, which is helpful for nourishment of spermatogonia by Sertoli cells, but the differentiation stage occurs in the luminal part of the seminiferous tubules. This is protected from the immune system. Replicating germ cells in the periphery of the seminiferous tubules therefore pass across the blood-testes barrier before entering the meiotic phase of development, which ultimately results in the production of haploid...
gametes. The proliferation process in the mouse embryo takes 35 days (Clermont and Trott M., 1969; Clermont Y., 1966a).

1.2.2 Differentiative stages of spermatogenesis

At puberty the diploid secondary spermatocytes produced by meiosis I undergo a second meiotic division to produce round spermatids. Following the second meiotic division the haploid spermatid undergoes a differentiation process, known as spermiogenesis, to produce spermatozoa that then separate from the germinal epithelium during another complex process referred to as spermiation. Meiosis I takes 3 weeks to complete and comprises four major stages: leptotene, zygotene, pachytene and diplotene (Freeman, 2008). In leptotene chromosome, condensation is initiated and in zygotene, condensed homologous chromosomes come together in pairs to form tetrads. At this stage synaptonemal complexes (SCs) form between homologous chromosomes in order, mediate chromosome pairing, synapsis and recombination. During pachytene, recombination between homologous sister chromatids occur and as this stage ends; the SCs are no longer visible.

During the second meiotic division, the secondary spermatocytes, each containing a single set of chromosomes, rapidly undergoes chromosomal condensation and enters the cell division cycle passing through metaphase, anaphase and telophase on the way to producing haploid round spermatids (Dekretser and Kerr, 1994). During metaphase, the spindle fibres attach to the centrosomes of homologous chromatids and independent chromosomal alignment occurs. At anaphase, chromatid separation takes place as these structures transit to opposite poles of cell. Then during telophase, cytoplasm and nuclei begin dividing. Finally, at the end of meiosis; four round spermatids come out of every secondary spermatocyte. Gene transcription occurs throughout the above-mentioned stages until the middle of post meiotic phase.

1.3 Apoptosis in Spermatogenesis

Apoptosis is important in different biological processes, including development, differentiation, proliferation/homoeostasis as well as the regulation of germ line. Although, the process of apoptosis in ejaculated sperm is not completely clear, it is
suggested that to be a response to DNA damage in defective spermatozoa, similar to programmed death cell in somatic cells. In addition to its potential role in the eradication of defective spermatozoa, apoptosis is also thought to be important for the establishment of normal spermatogenesis and in the testicular response to stress created. For instance, by the exposure of these cells to environmental toxicant or a failure of gonadotrophic support apoptosis would eradicate the defected spermatozoa (Sakkas et al., 1996).

1.3.1 Role of Apoptosis during spermatogenesis and ejaculated spermatozoa

Apoptosis is an active process at all stages of spermatogenesis in male germ line from proliferation of the spermatogonial stem cell populations through germ differentiation in the germinal epithelium to the survival of the mature spermatozoon. In the mammalian testis, apoptosis plays a key physiological role during development in controlling germ cell numbers so that the ratio of germ cells to Sertoli cells is optimal for spermatogenesis (Bartke., 1995, Allan et al., 1992, Billig et al., 1995). Impairment of apoptosis through the functional deletion of key mediators of this process generates a male infertility phenotype due to an imbalance in germ- and Sertoli- cell numbers (Rodriguez et al., 1997). In the adult testes, apoptosis is also thought to play a key role in regulating the quality of the gametes.

Thus in the human testes, one spermatogonium produces about 100 spermatozoa, which is significantly less than the expected value of 4096 (Woolveridge et al., 2000), indicating the involvement of apoptosis in this process. Testicular germ cell apoptosis happens constantly throughout life and Fas mediates this process. However, in subfertile men this apoptotic process is accentuated with the result that Fas positivity and DNA damage are highly represented in the ejaculated spermatozoa (Richburg, 1996).

Currently it is understood that germ cell apoptosis is a significant mechanism for normal spermatogenesis since biopsies from human testicular tissue show clear signs of spontaneous apoptosis (Brinkworth et al., 1997). In addition, several independent reports have emphasized the importance of apoptosis in the etiology of male infertility because cases of azoospermia or severe oligozoo spermia are associated with a
heightened incidence of apoptotic germ cells in testicular tissue. Although the cause of this increased incidence of apoptosis is unknown, we do know that heavy metals like cadmium and some reproductive toxicants (e.g., insecticides or herbicides) are able to induce germ cell apoptosis. In addition, transgenic animal exhibiting a Bax deficiency have also showed high level of apoptosis in germ cells and demonstrated infertility (Rodriguez, 1997).

The characteristic signs of apoptosis in germ cells are similar to somatic cells although the progress of this phenomenon shows some interesting differences, particularly when spermatozoa are considered. At all stages of spermatogenesis both phosphatylserine externalisation and caspase activation are clear signs that apoptosis is taking place. During the early stages of spermatogenesis, the factor that mediates apoptosis in the male germ line is Fas. The latter is a protein from the tumour necrosis family (Suda et al., 1993) which interacts with another factor known as FasLigand. When FasLigand binds to a Fas positive cell, cell death is induced because of apoptosis.

In the normal situation, Sertoli cells release FasL, which triggers cell death in Fas positive germ cells and restricts the germ cell population to a size that can be supported by the Sertoli cells (Rodriguez et al., 1997). Mature spermatozoa from normal men rarely show any sign of DNA damage or Fas positivity. As a result, in men with normal sperm parameters value the percentage of Fas positive spermatozoa is low (Sakkas et al., 1995).

However, in men with abnormal sperm parameters, high levels of DNA damage are often observed possibly because germ cells that have been marked for apoptosis fail to complete this process (Sakkas et al., 1995). As a result, testicular attempts to remove defective germ cells via apoptosis do not efficiently eradicate these cells and they continue their differentiation to spermatozoa that carrying DNA strand breaks as a legacy of the abortive apoptotic process that failed to eliminate them.

The relationship between apoptosis and defective sperm function has been largely clarified in the past decade, with many researches demonstrating the expression of
different aspects of this process such as DNA strand breaks, phosphatidylserine (PS) externalization and caspases expression in ejaculated human spermatozoa. Although the role of apoptosis in somatic cells is completely clear, according to some authorities, the existence and function of this process in ejaculated sperm it is still the subject of controversy (Gadella and Harrison, 2002) In brief germ cell apoptosis has, a critical role in spermatogenesis process (Sakkas et al., 1999) is activated in sperm as a mechanism of eradication of abnormal spermatozoa or in response to environmental stress or injuries. Ejaculated spermatozoa in human have showed somatic cells apoptotic characteristics and mechanism (Gorczyca, 1993).

1.3.2 Molecular Mechanisms of Apoptosis signalling pathways

The role of apoptosis in the mediation of germ cell death during normal spermatogenesis in various mammalian species, including man, has been demonstrated (Bartke, 1995; Sinha Hikim et al., 1998; Print and Loveland, 2000). This phenomenon has been investigated during spermatogenesis and various apoptotic factors have been identified (Tapanainen et al., 1993; Callard et al., 1995; Billig et al., 1996; Hadziselimovic et al., 1997; Rodriguez et al., 1997; Fan and Robaine, 1998; Sinha Hikim and Swerdloff, 1999; Jurisicova et al., 1999; Pentikainen et al., 2000), but these investigations have resulted in conflicting data on apoptosis in ejaculated spermatozoa. According to these sources a variety of stimuli are capable of inducing the process of apoptosis. Some chemical products are universally active and induce apoptosis in all cells, while other apoptosis-inducing agents are more selective in their targets (Rich et al. 2000).

Rossi and Aitken (1997) have reviewed that there is enormous potential for interactions between leukocytes and the male reproductive system. Also, it has been shown that Annexin-V binds to negatively charged asymmetric phospholipids following exposure of phosphatidylserine on the outside of the plasma membrane (Koopman et al., 1994; Martin et al., 1995; van Heerde et al., 1995). Moreover, conflicting signals can induce apoptosis in normal cells (Wyllie, 1997b.). Cytokines induce apoptosis during physiological cell turnover in response to death factors, such as Fas ligand (FasL) (reviewed in Nagata 1994). Apoptosis could be triggered by pathological insult rather
than being a physiological mechanism for the control of cell turnover or it could even be induced by agents that might damage DNA, such as radiation, cytostatic drugs or genotoxic compounds (Bratton & Cohen 2001). Some drugs such as staurosporin activate apoptosis via an unknown pathway (Ojeda et al. 1995). Furthermore some chemical agents, such as hydrogen peroxide, can also trigger the apoptotic pathway in several cell types (Madesh & Hajnoczky 2001, Gorman et al. 1997). DNA damage can be detected by p53, that will either lead to an arrest of the cell cycle or death through the activation of apoptosis (reviewed in Balint & Vousden 2001).

In brief, the general process of apoptosis can be initiated by stimuli from outside the cell (extrinsic apoptotic pathway type I) or inside the cell (intrinsic apoptosis pathways type II) in response to DNA damage or oxidative stress. Apoptotic mediators are present as catalytically inactive pro enzymes. The most important of these factors is Caspases, which include two groups: initiator caspases and executioner caspases. The Initiator Caspases stimulate the Executioner Caspases. In the extrinsic pathway, translocation of the phospholipid phosphatidylserine (PS) from the inner to the outer layer of the plasma membrane is an early sign of apoptosis. Perforin granzyme-dependent engulfing is another pathway that involves cytotoxic T-cells. The extrinsic, intrinsic, and granzyme B pathways come together to execute the same fundamental process (Martinvalet et al., 2005). Biochemical features of apoptosis and different pathways of that have been reviewed in the following paragraphs.

1.3.3 Biochemical features of Apoptosis

Apoptosis involves very precise biochemical pathways that enable eukaryotes to delete or arrest the production of unwanted cells. The chemical pathways associated with this phenomenon have been well characterized and studied in somatic cells. Caspases exist as inactive proenzymes in most cells and require the stimulation of a proteolytic cascade to activate them. Some procaspases are self-activated. This attribute of the apoptotic signalling pathway, in which one caspase activates other caspases, leads to rapid progression of apoptosis and, ultimately, cell death.
1.3.4 Caspases

Caspase stands for cysteine-dependent aspartate-specific protease (Alnemri et al., 1996). This family of enzymes possesses proteolytic activity and is able to cleave proteins at aspartic acid residues, although different caspases have different specificities dependent on the recognition of neighbouring amino acids. Once caspases are initially activated, it looks to be an irreversible pathway towards cell death. To date, 14 members of caspases have been identified. They can be classified to initiators (caspase-2,-8,-9,-10), executioners (caspase-3,-6,-7) and inflammatory caspases (caspase-1,-4,-5) (Cohen, 1997).

1.3.4.1 Initiator Caspases

The apoptotic pathway starts with the initiator caspases. Activated Caspase 2, mediates stress-induced apoptotic death. Some protein complexes including the death domain protein PIDD and the adaptor protein RAIDD (Tinel Tschopp, 2004) FADD (Fas-associated death domain), activator of caspase 8, in addition TNF_R1 are needed to induce apoptosis via Fas, although they are not necessary for other death pathways (Valfolomeev et al., 1998, Zhang et al., 1998). However, caspases-9 and its adaptor Apaf-1 are needed for DNA damage, corticosteroid and staurosporine-induced cell death. In Fas mediated apoptosis of human T-cells, caspases 8 and 10 play essential roles; also, both of them contain death effector domains (Wang et al., 2001).

1.3.4.2 Executioner Caspases

Extrinsic and intrinsic pathways of apoptosis converge in the final ‘executioner phase’ of apoptosis. The enzymatic activity of executioner caspases including caspase-3, caspase-6, and caspase-7 is essential for cleaving different substrates during this phase of the apoptotic cascade. Cellular substrates such as cytokeratins, PARP, the plasma membrane cytoskeletal protein alphafodrin, and others, have shown microscopical and biochemical changes in apoptotic cells in presence of executioner caspases (Slee et al., 2001). Caspase-3, the most important of the executioner caspases, can be activated by the presence of any of the initiator caspases (caspase-8, caspase-9, or caspase-10). Caspase 3 is specifically necessary for activation of the endonuclease CAD (Caspase Activated DNase). In normal proliferating cells, this nuclease exists in association with
an inhibitor of CAD activation known as ICAD. Activated caspase-3 cleaves and inactivates ICAD to release active CAD. CAD then cleaves chromosomal DNA within the nuclei, which ultimately leads chromatin condensation typical of pyknotic nuclei. In the next step of apoptosis cytoskeleton reorganization leads to the formation of apoptotic bodies in the cell. In final stage, dead, apoptotic cells are taken up by phagocytosis.

1.3.5 Extrinsic Pathway

The extrinsic signalling pathway initiates apoptosis when the death receptors on the cell surface make contact with pro-apoptotic ligands such as Apo2L/TRAIL and CD95L/FasL, which, in turn, activate death receptors that come from TNF receptor gene family. The TNF receptor family share the same cysteine-rich extracellular domains and a cytoplasmic domain of about 80 amino acids called the “death domain” (Ashkenazi et., 1998). Activation of this death domain causes the death signal to be transmitted from the cell surface to the caspase -8. Activated initiator caspases then stimulate the executioner caspases to initiate the cellular processes that lead to cell death. The FasL/FasR and TNF-α/TNFR1 models best characterize the sequences of events that define the extrinsic phase of apoptosis. In these models, homologous trimeric ligands bind to clusters of receptors.

The binding of Fas ligand to Fas receptor, results in the binding of the adapter protein FADD while the binding of TNF ligand to TNF receptor results in the binding of the adapter protein TRADD with recruitment of FADD and receptor-interacting protein (RIP) (Grimm et al., 1996). FADD then associates with procaspase-8 via dimerization of the death effectors domain (DED). This process forms a death-inducing signalling complex (DISC) which leads to auto-catalytic activation of procaspase-8. The execution phase of apoptosis is triggered when caspase-8 is activated. Death receptor-mediated apoptosis can be inhibited by a protein called c-FLIP which will bind to FADD and caspase-8 making them ineffective (Scaffidi, 1999).
1.3.6 Intrinsic Pathway

The intrinsic signalling pathways initiate apoptosis without any receptor-mediated stimuli. Intracellular signals act directly on targets within the cell and are mitochondria-initiated events. The stimuli that initiate the intrinsic pathway produce intracellular signals that may act in either a positive or negative fashion. Negative signals involve a lack of growth factors, hormones and cytokines that can lead to apoptosis. In other words, absence of the apoptotic suppressor factors cause activation of apoptosis. Radiation, toxins, hypoxia, hyperthermia, viral infections, and free radicals are other stimuli that activate apoptosis in a positive fashion.

All of these stimuli cause changes in the inner leaf of mitochondrial membrane that result in opening of the mitochondrial permeability transition (MPT) pore, loss of mitochondrial trans membrane potential and release of two main groups’ of pro-apoptotic proteins from the inter membrane space into the cytosol. The first group consists of cytochrome c, Smac/DIABLO, and the serine protease HtrA2/Omi. These proteins activate the caspase-dependent mitochondrial pathway. Cytochrome c binds Apaf-1 as well as procaspase-9 to produce an “apoptosome”. The clustering of procaspase-9 in this way leads to caspase-9 activation. Smac/DIABLO and HtrA2/Omi are reported as inhibitor of IAP (Inhibitor of Apoptosis) activity.

A family of proteins called Bcl-2 (Cory et al., 2002) controls the mitochondrial apoptotic pathway. P53 is a tumour suppressor protein that plays a critical role in regulation of the Bcl-2 family (Schuler and Green, 2001). The Bcl-2 family of proteins control mitochondrial membrane permeability. To date, 25 genes have been identified in the Bcl-2 family. The anti-apoptotic proteins comprise for example, Bcl-2 and Bcl-x, and some of the pro-apoptotic proteins comprise Bax, Bak, Bid and Bad. These proteins have special significance since they can determine if the cell commits to apoptosis or aborts the process. It has been shown that the main mechanism of action of the Bcl-2 family of proteins is the control of cytochrome c release from the mitochondria via a change in mitochondrial membrane permeability.
Two members of the Bcl2 family, Puma and Noxa, are also involved in pro-apoptosis. Puma plays an important role in p53-mediated apoptosis. It has been shown that overexpression of Puma \textit{in vitro} is accompanied by increased BAX expression, BAX conformational change, translocation to the mitochondria, cytochrome c release and reduction in the mitochondrial membrane potential (Liu et al., 2003). Noxa is also a candidate mediator of p53-induced apoptosis. Studies show that this protein can localize to the mitochondria and interact with anti-apoptotic Bcl-2 family members (Fig1.1) resulting in the activation of caspase-9 (Oda et al., 2000). Since both Puma and Noxa are induced by p53, they might mediate the apoptosis that is initiated by genotoxic damage or cancerous activation (Boya et al., 2001).

**Figure 1.1 Schematic diagram of major apoptotic pathways.**

Phases of the apoptotic process with respect to MMP. In the left part of the figure, pro-apoptotic pathways converging on mitochondria are shown. Different apoptogenic molecules act on a variety of tentatively identified mitochondrial receptors (arrows), which in turn regulate MMP. The degradation pathways triggered by MMP are depicted on the right. Apoptosis regulatory proteins encoded by pathogens can either target the signal-transducing pathways upstream of mitochondria or the mitochondrion itself. This has functional consequences. Thus, apoptosis inhibitors acting at the mitochondrial level are likely to have a broader spectrum of cytoprotective action than inhibitors acting on upstream signal. GSH, glutathione; ROS, reactive oxygen species. From: Boya, et al (2001) Viral and bacterial proteins regulating apoptosis at the mitochondrial level The EMBO Journal \textbf{20}, 4325 - 4331 doi:10.1093/emboj/20.16.4325
1.4 Epigenetic regulation during cell life cycle

Two crucial factors involved in gene silencing are DNA methylation and histone modifications that influence patterns of gene expression and hence differentiation. The biochemical reactions that control the sites and rates of DNA transcription are under investigation and still not well recognised. One of the most important such factors are DNA methylation, which is established and maintained on genomes by DNA methyltransferase (Dnmt1) (Lei et al., 1996, Okano et al., 1999). DNA methylation occurs in the presence of a complex of enzymatic and non enzymatic mediators. We review DNA methyl transferases as the members of an enzymatic complex that interacts with proliferating cell nuclear antigen (PCNA) and enhances methylation efficiency (Schermelleh et al., 2007, Spada et al., 2007). In addition, the SRA domain of the UHRF1 protein that plays a central regulatory role for DNA methylation and also mediate activity of all three DNA methyltransferases, histone modifiers in a non enzymatic manner to DNA (Meilinger et al., 2009).

1.4.1 DNA methylation

Methyl transferase enzymes methylate DNA on cytosine residues. Methylation is focused on cytosine-rich CpG islands, which lie in the 5’ regulatory regions of many genes; in the human 70%-80% of all CPG regions are methylated (Ehrlich., 1982). In different species, the levels of methylation are different, which may indicate a different role for methylation in these organisms. Primordial germ cell shows a decrease in DNA methylation of primordial germ cells that is seen during the proliferative phase of spermatogonial and oogonial differentiation (Tada et al., 1997, Reik et al., 2001). Methylation on carbon 5 of cytosine is regulated by different groups of proteins. The protein Np95 has mentioned as non enzymatic (Hashimotto et al., 2008) and methyltransferases proteins have enzymatic roles in the CH3 agent transferring to the (cytosine-5), DNA methyltransferase member’s characteristics as well as mediator protein UHRF1 interactions on 5-methyl cytosine base are part of this review.

1.4.2 Mammalians DNA methyl transferases

The fundamental characteristic of DNA methylation in mammals is the transfer of a methyl group from S-adenosyl methionine to the C5 of cytosine (Lan et al., 2010). The
following discussion includes a brief description of the biological and biochemical behaviour of the DNA nucleotide methyltransferases proteins.

1.4.3 Biology of mammalian DNA methyltransferases

In mammals and other vertebrates, CpG dinucleotides methylation at the C5 position of cytosine (5mC) is more prevalence than any other kind of methylation (Figure 1.2A). Methylation processes occur in two phases; de novo methylation establishes the methylation state and, this is followed by maintenance of the methylation information in the new duplicated DNA strands after DNA replication. Hemi-methylated CpG sites on replicated DNA detect by Dnmt3 family select cytosine on the new strand for establishing new de novo methylation pattern, whereas Dnmt1 maintains the pattern of methylation during chromosome replication (Chen and Li, 2006) (Figure 1.2C) which may cause to heritable information.

All the known mammalian Dnmtases have a common catalytic domain which domain, which is similar in prokaryotic enzymes and composed of ten conserved amino acid motifs engaged in the catalytic activity (D’Aiuto et al., 2010). In addition, the Dnmt1 and Dnmt3 enzymes contain a large N-terminal regulatory domain that may affect the activity of the enzyme (Hermann et al 2004). Dnmt1, Dnmt3a, Dnmt3b and Dnmt2 are the most important methyl transferases and play different biological roles for the establishment or maintenance of methylation (Fig 1.2B).
Figure 1.2: 5-methylcytosine situations on double stranded DNA

A) DNA cytosine methylation at ring carbon C5. See the text for a summary of the mechanism. The question mark indicates possible activity of DNA demethylases (Kress et al., 2006). Members of the DNMT family. Schematic representation of Dnmt1 and Dnmt3. Dnmt2 is a tRNA\textsuperscript{\text{Asp}} MTase (Goll et al., 2006).

B) Roman numerals refer to conserved motifs of DNA MTases (Kumar et al., 1994); motif IV includes the Cys nucleophile that forms a transient covalent bond to C6 of the target cytosine. Other details are explained in the text or in work by Goll and Bestor (2005).

C) Maintenance versus de novo methylation. As described in the text, the roles of the Dnmts are not completely distinct in this respect. The pale-blue segments are substrate sequences (usually CpG), and the turquoise shapes represent methyl groups on the cytosines. After replication or repair, the duplex is methylated on one strand only (Cheng 2008).

1.4.4 The Dnmt1 members

The DNA methyltransferase1 family has the highest level of expression in cells and mediates maintenance of methylation patterns. However, in human cancer cells, Dnmt1 may play a role in both the de novo methylation and maintenance methylation of tumor suppressor genes (Wing et al., 2006, Ting et al. 2006). The members of this family are Dnmt10, which is a germ-cell-specific variant shorter than Dnmt1 (Howell et al.,
Dnmt1b has 48 amino acid more than Dnmt1 (1620 amino acid) and its function still is unknown (Fig. 1.3). Dnmt1 possesses a large N-terminal domain which possesses a regulatory function and a smaller C-terminal catalytic domain. The catalytic domain of Dnmt1 can only show enzymatic activity in the presence of the N-terminal region (Zimmermann et al., 1997, Margot et al., 2000, Fatemi et al 2001). These flexible termini also interact with histone modification (Bhaumik et al 2007, Shilatifard. 2006). DNA methyl transferase domains show different functions and interactions with the other enzymes or the own family members (Hermann et al 2004).

![Figure 1.3. Schematic representation of Dnmt1 family](image)

Charge-rich region contains several translation start points, and the interaction site with DMAP1. PCNA: PCNA-interaction site; NLS: nuclear localization signal; P: major phosphorylation site at Ser 514, Cys-rich-region: cysteine-rich zinc binding motif (ATRX type zinc finger); Pb-region: polybromo-1 protein homologous region containing two BAH domains; GK-repeats: glycine-lysine-repeats. In the central part of the figure the structures of the BAH domain in the N-terminal region of S. cerevisiae Orc1p (PDB code: 1m4z) and of a CXXC type zinc finger in the RecQ helicase catalytic core (1O0Y) are shown. Two helices and a connecting loop that are not part of the conserved BAH structure are coloured violet. Note that the Cys-rich domain of Dnmt1 contains two complete CXXC motifs and therefore most likely is equivalent to two of the RecQ zinc fingers. The proteins are represented by their Ca-ribbon, α-helical parts are coloured red, β-strands cyan (Adapted from Hermann et al 2004).
The basic function of Dnmt1 in mammalian cells has been discovered using mice in which this gene has been knocked out. Mid-gestation death with significantly decreased levels of DNA methylation was observed in deficient mice, demonstrating the critical role played by this enzyme in the cell cycle (Li et al., 1992). It interacts with the factors that control the replication fork, PCNA, and p21WAF1, as well as inhibitors of cyclin-dependent kinases (CDKs) (Chuang et al., 1997). Dnmt1 inhibition has detected as an agent of DNA replication (Knox et al., 2000), and the expression of Dnmt1 is intimately involved with DNA replication (Araujo et al., 1998), histone modifying enzymes such as histone methyltransferase SUV39H1 (Fuks et al., 2003) and the histone deacetylases HDAC1 and HDAC2 (Rountree et al., Robertson et al., 2000, Pradhan et al., 2002). It also binds with methyl CpG binding proteins UHRF1 (Kimura et al., 2003) and heterochromatin binding protein HP1 (Fuks et al., 2003) which interact with H3K9 methylated chromatin via its chromodomains (Fig1.4).

All these interactions lead to gene silencing and transcriptional repression and stabilization of the methylation status on the cytosine. Furthermore, Dnmt1 has interaction with other family members, the Dnmt3a and Dnmt3b (Kim et al., 2002). Gene regulation and epigenetic modification via methylation occur in a complicated interaction between Dnmt1 and mediator proteins, which result in phosphorylation and other post transcriptional modifications (Glickman et al., 1997). In vitro experiments have shown that Dnmt1 exhibits a 15- to 40-fold greater affinity for hemimethylated compared with unmethylated DNA (Fatemi et al., 2001, Zucker et al., 1985, Pradhan et al., 1999) leading to the suggestion that this enzyme acts to maintain the methylation status of cells. While in normal tissues the CpG islands are mostly unmethylated, in cancer tissues CpG islands are hypermethylated that causes an elevation in all DNA methyl transferas in these tissues (Kuerbitz et al., 1996).
Figure 1.4. Schematic presentation of Dnmt1 and UHRF1 interaction. Proposed mechanism of heterochromatin formation through UHRF1 at DNA replication fork or DNA repair site. (1) UHRF1 binds to PCNA and the SRA domain of UHRF1, recognizing hemi-methylated CpG on newly synthesized DNA. Then histones are reassembled. (2) UHRF1 recruits DNMT1 to methylate both DNA strands to transfer methyl. UHRF1 also recruits G9a to methylate histone H3K9. Methylated histone H3K9 binds to the PHD domain of UHRF1. (3) UHRF1 recruits HDAC1 to the site and deacetylates histones. As a consequence these histones become positively charged and bind tightly to the negatively charged DNA, causing heterochromatin formation. MBD3 does not possess binding affinity to methyl-CpG, thus, it is omitted from this figure. Abbreviations are; MBD, methylbinding domain (Adapted from Unoki et al 2009).

1.4.5 The Dnmt3 members

When Dnmt3 was discovered in mouse, it was found; to possess two different sub units Dnmt3a and Dnmt3b. These subunits are very similar to one another but encoded by separate genes. The general structures of both Dnmt3 enzymes are similar to Dnmt1 with a C-terminal catalytic domain that contains all the Dnamtses motifs (Fig1.2). It has been found that there is no preference for hemimethylated DNA in Dnmt3 subunits.
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(Okano et al., 1998, Yoder et al., 1998, Gowher et al., 2001) and for this reason, this family is thought to be involved in de novo methylation. The de novo methylation activity of Dnmt3a and 3b has been observed in vivo and in vitro (Gray et al., 2010).

Dnmt3a and Dnmt3b undergo post translational modifications (Kang et al., 2001, Ling et al., 2004) and interact with a number of proteins including Dnmt1(Kim et al., 2002) H3K9 methyltransferase Suv39 (Fuks et al., 2003) and HDACs (Fuks et al., 2001). Dnmt3b is over expressed in tumours, while the expression level of Dnmt3a is only moderately elevated in some tumours (Okano et al., 1999, Robertson, et al. 1999). These results suggest that Dnmt3b plays an important role in the tumorigenesis area. These results indicate that Dnmt3b is a special factor for *denovo* methylation rather than Dnmt3a.

The other member of the Dnmt3 family is Dnmt3L, which possesses a similar structure to the Dnmt3a and 3b enzymes (Aapola et al., 2000) (Fig1.2B). Its N-terminal part contains just the PHD domain and the C-terminal part has extended up to motif VIII. Dnmt3L does not possess any catalytic activity itself but has been observed to exert a stimulatory action on Dnmt3a methylation activity (Chedin, et al., 2002). Experiments have shown an interaction between Dnmt3L and Dnmt3a, Dnmt3b (Hata et al., 2002) and histone deacetylase (Deplus, et al., 2002). The structure and function of the Dnmt3L, Dnmt3a and Dnmt3b enzymes show similarities during gametogenesis also embryonic process (Hata et al., 2002, Bourc et al., 2001).

1.4.6 Dnmt2

The Dnmt2 is represented among eukaryotes in both those organisms that show methylation and those in which methylation has not yet been detected. Dnmt2 has been shortened in the major part of the N-terminal regulatory domain (Fig 1.2B). The structure of Dnmt2 shows a structural similarity with prokaryotic DNA-(cytosine-C5)-MTases in S-adenosyl-L homocysteine complexes (Dong et al., 2001) (Fig.1.2B).

Dnmt2 was discovered in 1998; however, no particular biochemical activity has been assigned to this molecule yet (Yoder et al., 1998, Okano et al., 1998). Thus the activity and biological function of this enzyme is not yet understood and may not be significant.
given that its presence in mouse embryonic stem cells as well as most human and mouse adult tissues is low (Okano et al., 1998).

1.4.7 Regulation of DNA methyl transferases in mammals

Methylation involves the mediation of methyltransferases to catalyse the transfer methyl groups to target DNA sequences. Little is known about the regulatory mechanisms by which particular sequences are designated for de novo methylation in either the germ line or in somatic tissues. While Surani and his colleagues (Tada et al. 1997) have declared demethylation to be the dominant property of primordial germ cells, two distinct groups of enzymes has been reported that regulate initiation and maintenance of methylation in the germ line. i) de novo enzymes methylate specific sequences at particular stages of gametogenesis and (ii) maintenance enzymes that can only perpetuate patterns created by the de novo methylation enzymes. Dnmt1 is the predominant DNA methyltransferase in mammalian cells (Leonhardt et al. 1992) and, in maintenance mode, methylates hemimethylated DNA. It also has predominant de novo methylation activity and is omni present in all cell types (Yoder et al. 1997a). Furthermore, Dnmt1 presentation at high levels in post-meiotic germ cells could indicate to de novo methylation occurrence in these cells (Trasler et al. 1990). However Lei et al. (1996) has reported another DNA methyltransferase that may have a role in the silencing of newly integrated retroviral DNA. However, there is little evidence of a family of sequence-specific DNA methyltransferases active during gametogenesis. Dnmt1 has demonstrated a major regulatory role between DNA methyltransferase in both de novo and maintenance methylation (Yoder et al. 1997a).

Dnmt1 is regulated by alternative splicing and protein sequestration in post-meiotic germ cells. The enzyme is reported in post-mitotic spermatocytes and is segregated into into the nuclei of differentiating germ cells during the leptotene/zygotene stage of meiosis (Jue et al. 1995). This stage of spermatogenesis may be the site of de novo methylation of the paternal genome. At the pachytene stage, the Dnmt1 protein disappears while the mRNA remains abundant. All of the Dnmt1 mRNA population has a pachytene spermatocyte-specific 5’ exon (Mertineit et al. 1998). This exon includes some short open reading frames that normally take part in translation and as a result this
pachytene-specific mRNA cannot be translated. Bestor and Tycko (1996) have reported that recombination intermediates might be deregulated by de novo methylation. Therefore, post-transcriptional down-regulation of Dnmt1 production at the pachytene stage of spermatogenesis might be a method of protecting meiotic chromosomes against de novo methylation.

pachytene spermatocyte-specific mRNA or the mRNA found in all cycling somatic cells (Carlson et al. 1992; Li et al. 1993). During oocyte maturation, after meiotic Furthermore, the amounts of Dnmt1 protein in mature oocytes is reported to be 30,000-times more than a somatic cell (Carlson et al. 1992). However, this protein is encoded by an mRNA that contains a 5’ exon that is different from that present in either the recombination, maternally imprinted genes may undergo de novo methylation whereas the cytological data suggest that paternally imprinted genes may undergo de novo methylation during the leptotene/zygotene stage of spermatogenesis. These predictions have been researched directly by evaluation of methylation changes in DNA from purified populations of germ cells (Bestor, 1998).

Protein localization also regulates the de novo methyltransferases’ access to the DNA. It has been determined that during germ cell development, when the germ cells colonize the gonad and imprints need to be erased, Dnmt3b is active in nuclei while Dnmt3a is undetectable (Hajkova et al., 2002). Further, Dnmt3a is observed in the male germ cells beginning at e15.5 (Lees-Murdock et al., 2005; Sakai et al., 2004), when methylation is occurring. Studies on the homozygous deletion of the Dnmt3a gene has revealed a lack of de novo methylation in both the prospermatogonia and the growing oocyte (Kaneda et al., 2004). This knockout study has not detected which isoform, Dnmt3a or Dnmt3a2, is more important in germ cells; however more recent research has reported that Dnmt3a2 is the only form observed in the male germ cells during the period of de novo methylation (Sakai et al., 2004). Another report from Kaneda et al., 2004 has declared that Dnmt3b deletion has not change the methylation status of germ cells. Additional studies are clearly needed to determine the regulatory role of methyltransfrases during different stages of spermatogenesis.
Various theories have been put forward to explain how the catalytic activity of cytosine methyltransferases is regulated. The theory of base flipping, in which the base is recognised and pushed out of the DNA helix by residues on the enzyme is the most accepted. Methyltransferases embrace the flipped base, invade to DNA, and finally trap the cytosine (Cheng, 1996). Analysis of the biochemical characteristics of methyltransferase motifs in the catalytic domains of eukaryotes and prokaryotic enzymes has revealed some interesting differences. In mammals, special proteins are needed to stimulate these enzymes; for instance, the catalytic domain of Dnmt1 is active and the enzyme can display its role just in the presence of the N-terminal part of the enzyme. The catalytic domains of Dnmt3 can also exhibit enzymatic activity in isolation (Reither et al., 2003). Such activities help to target the DNA methyltransferase activity to the right regions of DNA in need of being methylated.

These methylated regions are highly conserved in mice and humans; so the methylation pattern in mice is a model to explain a wide variety of different target sequences, such as endogenous retroviruses and retrotransposons (Bourc’his and Bestor, 2004; Walsh et al., 1998), imprinted genes and diseases (Li et al., 1993) and genes on the inactive X chromosome (Csankovszki et al., 2001). Once methylation is established on the inactive X chromosome and the imprinted genes have been effectively silenced via this mechanism, they remain stably shut down in each cell lineage during the lifetime of the organism, due to the faithful copying of methylation patterns during mitosis.

1.4.8 Maintenance of methylation patterns

The evidence shows that methylation occurs in euchromatin at the early S phase of the cell cycle and in heterochromatin at later stages. Gene knock out studies show that Dnmt1 can maintain the methylation pattern in euchromatic regions without Dnmt3 family. However, the latter are required for maintenance of methylation at heterochromatic regions. Therefore, euchromatin carries a detailed pattern of DNA methylation and because of a preference for hemimethylated CG sites; Dnmt1 is the only enzyme in the cell that is able to copy this pattern. There is a theory that Dnmt1 is a part of the replication fork and it is associated with PCNA and its localization at replication foci. Consequently, methylation can happen immediately after replication,
which would explain the short time gap between DNA replication and methylation (Gruenbaum et al., 1983, Araujo et al., 1998, Leonhardt et al., 1992).

1.4.9 Preservation of methylation levels
The process of establishing a pattern of DNA methylation is not fully understood. Evidence shows that global de novo methylation of the genome occurs during embryogenesis; however certain parts of the DNA are tightly bound to proteins or transcription factors and are protected against this process (Brandeis et al., 1994). The methylation in DNA happens in different stage of S-phase replication and DNA methyl transfrases take part in different stages specifically. Euchromatic DNA contains detailed genetic information but the heterochromatic part is methylated dominantly and does not contain the all the information for methylation. Therefore, these regions does not need to ability of reading the pattern of CG methylation, but need to active methyl transfrases enzymes. In late replication phase Dnmt3a and Dnmt3b take part in the methylation activity also, Dnmt1 contribute to the process with a preference for hemimethylated DNA (Goyal et al., 2006).

In addition, methylation could be established at heterochromatic area during embryogenesis, and it has been showed the establishment mechanism of new methylation and de novo methylation is very similar (Liang et al., 2002., Leonhardt et al., 1992, Gruenbaum et al., 1983). The methylation pattern of the DNA during the gene transcription in embryonic cells is associated with heavily presentation of Dnmt3a and 3B result in an increased level of global remethylation of the DNA (Lucifero et al., 2004, Hata et al., 2002). Evidence show Dnmt3 and Dnmt1 are requirements of methylation in DNA plus the other factors such as H3K9 methylation can lead to methylation in heterochromatic DNA (Lehnertz. et al. 2003). An UHRF1 protein coordinates all factors, Dnmt1, Dnmt3a and Dnmt3b and other mediators (Kim et al., 2002, Margot, 2003).

1.4.10 Biochemistry of mammalian DNA methyltransferases
Basic structures of C5-MTases in both prokaryote and eukaryote are similar and all methyl transfrases are common in their structures and possess 10 conserved motifs
(Goll et al., 2005, Roberts, et al., 2007, Hermann, 2004, Kumar, et al., 1994). The typical structure of methyltransferases contains two domains; a large N-terminal regulatory domain observed in Dnmt1 and Dnmt3 enzymes (Fig.1.5) and another catalytic domain which is common among all DNA-(cytosine-C5)-mtases and contains a central parallel six-stranded beta-sheet connected to each other by alpha-helices. This domain contains of two sub domains both similar in structure, the smaller sub domain recognizes the 5-methyl cytosine and causes the cytosine residue to flip out of the DNA helix (Klimasauskas et al,1994, Sankpal, et al 2002); it may also contribute in the binding pocket for the flipped-out base. The other domain is responsible for S-adenosyl-L-methionine (AdoMet) binding cofactor. The chemical strategy of base flipping and usage of S-adenosyl-L-methionine as a source of methyl groups is common to all methyl transferases (Gowher et al., 2006, Tomasz et al., 2008, Klimasauskas et al., 1994, Reinisch et al, 1995)

Figure 1.5. Domain organization of the mammalian Dnmtses.
The mammalian methyltransferases are divided into an N-terminal regulatory part and a C-terminal catalytic part. The C-terminal part shows strong amino acid sequence homology to prokaryotic DNA-(cytosine-C5)-MTase and contains all the conserved catalytic amino acid motifs (abbreviated by Roman numerals) defined for the prokaryotic enzymes. The boxes shown in the MTase sequence indicate the various domains, structural and sequence motifs identified in these proteins. (Adapted from Hermann et al 2004)
1.4.11 Recruiting S-adenosyl-L-methionine

As reviewed above, methyltransferases methylate DNA on the C5 position of cytosine after replication, using an enzymatic process. The catalytic component of this protein takes part in a chemical reaction with S-adenosyl-L-methionine (AdoMet) as the essential source of the methyl groups for transference to cytosine bases (Gowher et al., 2006). All DNA methyltransferases transfer methyl group to the cytosine via recruiting S-adenosyl-L-methionine (AdoMet) (Tomasz et al., 2008). In this process adenosyl-L-methionine (AdoMet) is attached to a sulphonium atom (Fig 1.6A), which causes the molecule to become thermodynamically unstable and induces the methyl thiol of the methionine to exhibit chemical reactivity towards nucleophilic complexes such as nitrogen, oxygen and activated C atoms (carbanions) (Klimašauskas and Weinhold, 2007). The enzyme’s effect on the substrate involves a nucleophilic catalytic attack on the carbon-6 of the targeted cytosine. This attack happens via the thiol group of the cysteine residue on the active site of cytosine-C5-MTases (Fig1.6B) (Klimasauskas et al., 1994, Reinisch et al, 1995).
Figure 1.6. Chemical features of cytosine methylation on C5 cytosine.

**Figure A:** mechanism of cytosine methylation in presence of S-adenosyl-L-methionine and DNA methyl transferases (adapted from Bird, 2002).

**Figure B:** nucleophilic attack of the enzyme into the carbon-6 of the targeted cytosine performed by the thiol group of the cysteine residue in a PCQ motif in the active site of cytosine-C5-MTases (motif IV) in Covalent reaction between DNA and the active site cysteine, has been shown for many DNA methyl transferases (Klimasauskas et al., 1994, Reinisch et al., 1995). (Adapted from Hermann et al, 2004)

### 1.4.12 Base flipping

The base flipping is an ancient process that may be involved in many enzymes engaged in DNA repair and all DNA methyltransferases (Klimasauskas et al., 1994, Reinisch et al., 1995, Goedecke et al., 2001). Smith (1994) obtained data suggesting that human methyltransferase is a protein that recognises mismatches based in DNA damage. Then Huang et al (2003) reported that DNA-protein interaction enhances the process of base flipping by 5-methylcytosin transferases. Recently, the regulatory effect of non
enzymatic mediator proteins known as UHRF1 has been illustrated by crystallography (Hashimoto et al., 2008).

Base flipping causes more exposure of the miss match cytosine to the DNA methyl transferase enzymes and/or other mediators, allowing for chemical interaction. When cytosine is flipped out, the hydrogen bonds between two bases C-G are broken and cytosine is not any more engaged in the double helix but rotated about its flanking sugar-phosphate bonds and surrounded in the catalytic enzyme pocket. Dnamtases, motifs IV and VIII, interact in detecting the cytosine and make a pocket around the flipped out cytosine residue (Klimasauskas et al., 1994, Sankpal et al., 2002). While the other domain in the C-terminal part of the enzyme comprising motifs I, II, and X is responsible for S-adenosyl-L-methionine (AdoMet) binding (Hermann et al 2004).

Although base flipping is well defined in terms, the details of this process are confusing and not well characterised. Bheemanaik et al in 2006 posed an important question with regard to the mechanism of base flipping, which is whether the enzyme itself induces the process actively, or whether DNA methyltransferase just uses pre-existing flipped-out bases on the DNA helix. Then Liebert et al (2004) suggested that the enzyme promoted base flipping. In addition, this group have shown that base flipping is very fast but binding of the flipped-out base into the active site pocket of the enzyme is slow. Base flipping occurs in the absence of AdoMet, but binding of the target base in the active-site pocket requires AdoMet. Base flipping analysis of non-canonical sites has shown that target recognition occurs before the base is flipped out. All of these observations indicate that DNA recognition occurs first, followed by cofactor binding and base flipping (Liebert et al in 2004).

1.5 Non enzymatic protein interaction in the process of DNA methylation

As it reviewed DNA methylation happens in carbon 5 of cytosine in CPG islands. The enzymatic activity of DNA methyl transfrases maintains the methylation pattern and causes inheritance changes during replication in new strand daughter on the CpG sequences (Tajima et al., 1998, Herman et al 2004). The SRA domain of the protein
UHRF1 in human, known as ICBP90, recognises miss-match CpG in hemi-methylated sites and via insertion of its loops makes contact with the DNA. This non enzymatic protein mediates Dnmt1 reactions at the miss-match sites (Bostic et al., 2007, Sharif et al., 2007). The methylcytosine base at the hemi-methylated site is flipped out of the DNA helix in the SRA–DNA complex and placed into a protein pocket. Crystallography of the structural complex shows that non-methylated cytosines are flipped out of the DNA helix and then targeted for methylation by coordination of DNA methyl transferases and UHRF1. These researches have been revealed the structure and function of the protein UHRF1 and specificity of SRA domain in base flipping reaction.

1.5.1 The Structure of UHRF1 protein

Two proteins, UHRF1 and UHRF2, are responsible for ubiquitination, acetylation and methylation reactions involved in transcriptional regulation, DNA repair and epigenetic modification of the genome. UHRF1 recognizes hemimethylate CPG islands on DNA during replication and organizes the three major epigenetic activities, including E3 ligases, histone modification and DNA methylation, via a ring domain with contributions from histone acetylases and methyl transfrases during cell division and DNA repair. The UHRF1 is a 95 kDa nuclear protein in mouse, homologous to 90kD ICBP90 in human (Unoki, 2008). In the structure of UHRF1 five functional domains has been recognized the ubiquitin-like domain (UBL) at the N terminus connected by a tandem Tudor domain to the plant homeodomain (PHD), really interesting new gene (RING) domain at the C terminus and SET and RING associated (SRA) domain which is of interest in the context of this thesis (Fig. 1.7).

![Figure 1.7 UHRF1—a multi-domaine protéine.](image)

1.5.2 UHRF1 protein binds to hemi methylated CpG

Crystallographic analysis of UHRF1 revealed that the SET and RING-associated (SRA) domains of the protein recognize the hemimethylated CpG region and brings Dnamts1 to this site (Fig1.8) (Sharif et al., 2007). The SRA domain of mouse UHRF1 is a prominent protein, which causes miss match bases to flip out during interaction with DNA (Hashimoto et al., 2008).

![Figure1.8. Schematic representation of SRA domain of UHRF1 between hemimethylated CPG and Dnmt1.](image)

**UHRF1 structure shows how the SET and RING-associated (SRA) domain of the protein recognizes the hemimethylated CpG region and loads Dnamts1 to the site**

*Adapted From the following article:
Kyohei Arita, Mariko Ariyoshi, Hidehito Tochio, Yusuke Nakamura & Masahiro Shirakawa (2008)*

**Recognition of hemi-methylated DNA by the SRA protein UHRF1 by a base-flipping mechanism**

*Nature 455, 818-821 doi:10.1038/nature07249*

1.5.3 Mechanism of UHRF1 (SRA domain) binding to hemi methylated CpG

The SRA domain has two packed twisted beta-sheets, which create a structure like a moon’s crescent. The inner surface of the crescent binds to the DNA (Hashimoto et al., 2008). The crescent possesses three loops (Fig 1.7B) at inner side, which contain a region of 50 amino acid residues. The specialised loop for recognising miss match CpG contains Asn494 and Arg496, which penetrate into DNA through the major groove
while another loop, responsible for the promotion of base flipping and including His450 and Val451 in red (Fig1.10), approaches DNA via the opposing minor groove. A third loop possessing Tyr 483–Ser486 and Tyr471–Asp474 forms a 5mC-binding pocket. Two tips R496 and V451 approach to the DNA from opposite grooves of the DNA helix, bind to each other and establish Vander Waals contacts (Fig1-9) (Sharif et al., 2007, Arita 2008, Hashimoto et al., 2009).

![Figure1.9](image)

**Figure1.9. The schematic drawing of SRA domain of UHRF1.**

*The side chains of V451 of the base flipping loop and R496 of the CpG recognition loop are in direct van der Waals contact. These two loops CpG recognition and base flipping—penetrate into the DNA helix from opposite directions (Adapted from Hashimoto et al 2008).*

As demonstrated in Fig1.9, two amino acids, V451 of the base flipping loop and R496 of the CpG recognition loop are bound to each other by VanderWalls forces. The latter amino acid, R 496, binds with orphan Guanine too. The whole of these activities flip the base completely out of the DNA helix and locates it in a binding pocket that is in contact with DNA with hydrogen bonds and Van der Waals forces (Sharif et al., 2007, Hashimoto et al., 2009). The contact between the methyl group and C5 position in 5mC is in Vander Waals contact with Cα and Cβ atoms of Serine 486 (Fig.1-10). The extra helical 5mC binds with two tyrosines (Tyr 471 and Tyr 483), stacking to DNA in between a hydrophobic formed cage (Fig 1.10).
Figure 1.10 Schematic structure of SRA domain of UHRF1 and flipped out 5-methyl cytosine

Summary of the SRA–DNA interactions; mc, main-chain-atom-mediated contacts; w, water-mediated hydrogen bonds. Black boxes represent CpG recognition sequence. The side chains of V451 of the base flipping loop and R496 of the CpG recognition loop are in direct van der Waals contact. The 5mC flips out and binds in a cage-like pocket (Adapted from Hashimoto et al 2008).

1.5.4 PHD domain of UHRF1 binds to H3K4me3

The PHD finger is another domain of the UHRF1 protein (Fig 1.11). This zinc finger-like domain includes nearly 60 amino acids. The definition of PHD fingers originates from conserved plant homeo domain proteins. It has been reported that this finger is connected with Lysine methylation of histones (Wysockais et al., 2006). This phenomenon is recognized as an important component of the epigenetic process that causes activation or inactivation of chromatin domains. However, this process has not been subject of our study, so I shall only review the fundamental nature of histone modification and chromatin remodelling during spermatogenesis.
Figure 1.11 Hypothetical model of the PHD domain of UHRF1 as the regulator within human DNA.

Dnmt1-mediated replication-coupled cross talk between DNA methylation and histone modifications. The existence of both silencing mark readers recognizing DNA (via the SRA) and histone (via the Tudor and/or PHD domain) facilitates the idea of maintenance and conversion of epigenetic silencing marks on both DNA and histone modifications (Adapted from Cheng and Blumenthal 2010. Coordinated chromatin control: structural and functional linkage of DNA and histone methylation (Biochem. 49:2999-3008)

1.6 Histone modification

DNA is super coiled around octameric protein complexes called nucleosomes, which are comprised of histones (Fig1.12). These proteins, like DNA, undergo modification. The tendency to associate with negatively charged DNA is due to positive charges associated with key regions of the histones (Gilbert, 2003). This octamer undergoes modification by such processes as methylation, acetylation, phosphorylation, ubiquitination and sumoylation. Gene expression levels are related to the nature and permanence of these post-translational modifications. Methylation is not only the most common type of modification in DNA but also one of the more stable modifications to occur in histones.
This process is mediated by histone methyltransferases (HMTases) which methylate the Arginine in the histone tail and may cause gene activation or silencing (Berger, 2002). Lysine residues go under mono, di and tri methylation, which may also indicate silencing or activation of genes during spermatogenesis (Jenuwein et al., 2001). Usually DNA methylation and histone deacetylation together cause gene silencing. They are signal for DNA to be condensed and exhibit minimum gene expression. Acetylation of H4 in spermatogenesis is associated with histone protamine replacement (Meistrich et al., 1992).

1.6.1 Chromatin Remodelling

Most information about the regulation of gene transcription is from study of somatic cells; however, recent data has suggested that the process of gene regulation in the germ line is different. The mechanism of chromatin reorganisation in haploid cells during spermatogenesis is quite specialised in terms of the proteins involved and the way in which they are regulated (Meistrich et al., 2003, Shirley et al., 2004). Chromatin remodelling in mammalian sperm exhibits two major characteristics. First, the process of post meiotic transcription in male cells occurs in a way that is different from somatic cells. Second, many of the genes expressed are specific to the male germ line.
Figure 1.12 Histones are the major structural proteins of chromosomes. The DNA molecule is wrapped twice around a histone octamer to make a Nucleosome. Six nucleosomes are assembled into a solenoid in association with H1 histones. The solenoids are, in turn, coiled onto a scaffold, which is further coiled to make the chromosomal matrix (Adapted from Figure Griffiths et al (2004); text annotation by Steven M. Carr (2007)

http://www.mun.ca/biology/scarr/Histone_Protein_Structure.html

1.6.2 Chromatin in spermatogenesis

Chromatin remodelling in somatic cells is limited to activation and silencing events in relation to gene transcription. However during spermatogenesis, spermatogonis experience massive reorganisation of their chromatin, they progress through meiosis, and the chromatin compaction associated with spermiogenesis during which nuclear histones become replaced by protamines.

Post meiotically, specific molecular mechanisms control the increased process of transcription and expression of specific genes in male germ cells. After meiosis, chromatin is compacted into the head of each spermatozoon in a nuclear volume that is one twentieth that of a somatic cell. This remarkable contraction of nuclear volume is facilitated by the histone to protamine transition. Arginine and cysteine are two amino acids in the structure of protamine that according to Mills et al., 1977 generate the unique toroid architecture of the chromatin in the sperm. In mammals, replacement of
histones with protamines involves transition proteins. Transition proteins 1 and 2 are two small proteins that replace nuclear histones when chromatin condensation starts (Mills et al., 1977).

Mice with mutant transition protein1 (TP1) and transition protein 2 (TP2) have normal fertility and reveal only minor abnormalities in spermiogenesis, suggesting that these two proteins do not have an essential role in histone replacement and chromatin condensation. The processes of phosphorylation and dephosphorylation control the displacement of transition proteins. TP2 is phosphorylated by protein kinase A, that is reduce the chromatin condensation at the site of TP2’s DNA (Meetei et al., 2002). The process of histone to protamine transition requires orchestration by specific signalling mechanisms and accurate timing. Ubiquitination is a form of post translational modification to proteins that plays an essential role in the signalling processes that drive the histone-protamine transition by controlling the half life, stability, refolding and translocation of these proteins and post meiotic phosphorylates protamine 2 happens by protein kinase CAM kinase 4(Wu et al., 2000).

The protamine replacement of histone during spermiogenesis starts with the silencing of gene transcription (Fig1.13). Heterochromatin protein 1 (HP1) recognizes a methyl residue on the tails of histones when the chromatin is condensing during this silencing period. When chromatin undergoes reorganisation, HP1 becomes associated with male–specific heterochromatin. This change can be used for detecting male embryos as HP1 appears during the onset of heterochromatization in male embryos, while it is distributed across the chromosomes in the female embryo (Bongiorni, 2001). It has been suggested that some regions of the chromosomes characterized by heterochromatin, do not alter their condensation status during the cell cycle as other parts of chromosome do (Heitz, 1928). It is believed HP1 might target methylated protamines or residual histones that participate in the Chromocenter structure.
Figure 1.13 Spermatogenesis follows a carefully orchestrated differentiation program that depends on transcriptional regulation.

The transcription phase begins in spermatogonia. Meiotic cells have a high level of transcription that is promoted by histone acetylation, making DNA accessible. Elevated transcription in meiotic cells is required for chromosome recombination. In post-meiotic spermatids there is a second round of histone acetylation and transcription, followed by the sequential replacement of histones by transition proteins then protamines. This altered chromatin architecture leads to arrested transcription and differentiation into elongated spermatids (Adapted from review Kimmins et al., 2004).
1.7 Specific male germ cell transcription

Most genes promoters are in a limited state of activity in male germ cells (Kleene, 2001). In these cells, two types of regulators are detected, the common one and the germ cell specific (Sassone, 1997, Eddy et al., 1998, Hecht, 1998). Spermiogenesis is significant because it involves increasing post meiotic transcriptional activity associated with essential post meiotic gene activity in early haploid germ cells. Specific developmentally-regulated male germ cell related factors regulate gene transcription at this time with optimal efficiency. It has been reported the amounts of TBP (TATA-binding protein), and other controlling proteins such as transcription factor IIB (TFIIB), and RNA polymerase II, are increased in early haploid male germ cells in comparison with somatic cells (Schmidt et al., 1995). It has been demonstrated that the expression level of genes in the testes is different from somatic cells not only for presence of general factors but also because of the incidence of testis specific factors.

In contrast to the autosomes, the X and Y-chromosomes are not transcribed in mammalian spermatocytes, because they are condensed (Handel et al., 1994, Solari., 1974). There is special nuclear structure, named the sex vesicle, which is inaccessible to RNA polymerase II (Ayoub et al., 1997) and, as a result, efficient transcription only takes place from the autosomes of differentiating germ cells.

1.8 Paternal X chromosome inactivation

The common feature of X-inactivation happens in the DNA of female mammalian offspring during development, in order to achieve dosage compensation. However, during spermatogenesis another form of X-inactivation occurs, called meiotic sex chromosome inactivation (MSCI). It is an example of meiotic silencing of unsynapsed chromatin (MSUC), in which silenced chromosomes that fail to pair with their homologous don’t attach to the synaptonemal cord, preventing aneuploidy in offspring (Turner., 2007). Also, Huynh and Lee (2003) and Okamoto et al (2004) separately declared that the inherited paternal X chromosome of female-mouse pre-implantation embryos is silenced. Indeed, they have suggested that the paternal X chromosome is already inactive at the point of fertilization, and it is pre-inactivated during
spermatogenesis (Huynh and Lee, 2003). Whereas Mahadevaiah et al (2001) supported the dogma that MSCI appear just in the meiosis duration, because the X and Y-chromosomes retain a repressed state throughout round spermatid development.

It is believed MSCI is mediated in spermatogenesis in much the same way as somatic X chromosome inactivation. It is thought that X-encoded RNA called Xist recruits an array of chromatin-modifying enzymes to the future inactive X chromosome that induce gene silencing by catalysing methylation, ubiquitylation and deacetylation of defined histone residues (Heard and Disteche, 2006). In males, Xist is expressed exclusively in the testis (McCarrey and Dilworth, 1992, Salido et al., 1992, Richler et al., 1992, Ayoub et al., 1997). Another kind of inactivation may establish mono allelic genes in germ line, transfer to all cells in the offspring, and maintain throughout all somatic cells of an organism. Imprinted genes may cause numerous genetic diseases such as imprinting diseases.

1.8 Imprinting defects in ART children

Methylation is responsible for imprinting defects that causes Wiedemann Syndrome, Silver- Russell syndrome, Angelman Syndrome and Parader Willi syndrome. It has been proposed that just 1% of all human genes are imprinted (Luedi et al., 2007). Recently, the risk of imprinting disorders as a consequence of the detrimental effect of human assisted reproductive technology (ART) on embryonic development has been causing concern. Whether the increase in imprinting defects has expressed in children conceived by in vitro fertilization or intracytoplasmic sperm injection, involves DNA damage in the fertilizer spermatozoon defects in the oocyte and/or defects in the conditions used for embryo culture?

Imprinting may involve genetic or epigenetic mechanisms. Patients attending assisted conception clinics may carry epimutations due to inappropriate patterns of DNA methylation. The reasons for epimutations in such patients is unknown but perhaps factors such as subfertility (Ludwig et al., 2005, Doornbose et al., 2007, Kobayashi et al., 2007), the mechanism of ovulation induction (Sato et al., 2007, Fortier et al., 2008), physical manipulation of the conceptus during IVF/ICSI/embryo transfer process (Rivera et al., 2008) and the in vitro incubation of embryo (Doherty et al., 2000, Fauque
et al., 2007) are effective. There is little or no evidence for cytogenetic disorders such as mutations, or DNA deletions/ duplications occurring as a consequence of ART. There are three human imprinting syndromes which are known to be correlated to ART Beckwith-Wiedemann syndrome (BWS), Prader-Willi syndrome (PWS) and maternal hypomethylation syndrome are three syndromes which show epigenetic defects associate.

In conclusion, two important signs of modification are methylation and histone modification and known as gene silencing processes. DNA methyl transferases and UHRF1 protein are two families of proteins that are employed to methylate DNA and histones. We established this research to better understanding of these mechanisms and apoptosis in human sperm DNA.

1.9 Aims and Hypotheses

In this text the literature review has been subjected on the DNA methylation and histone modification characteristics of two essential interacted proteins; i) DNA methyl transferases and SRA domain of UHRF, ii) definition of apoptosis and its signalling pathways. Then our investigation in human spermatozoa was developed based on this information and developed a research with the aims in following chapters. Level of methylation in different gradient of human spermatozoa has been investigated and compared with the level of apoptosis markers.

1.9.1 Chapter3: Pattern of DNA Methylation in different gradient of human spermatozoa

This research was founded on the comparison the level of methylation in human sperm. We hypothesis that the level of methylation in low density gradient of human sperm, might be higher than the same status in high density gradient. This chapter was designed based on the results of methyl status comparison experiments by HPLC and using an antibody against the methyl cytosine via flowcytometry, immunocytochemistry and an intriguing immunoblotting. Hypermethylation in X chromosom, poor quality of sperm and mid piece of sperm as well could be assessed.
1.9.2 Chapter 4: The relation between the quality of sperm and apoptosis?

Fundamental structure of this investigation is the comparison between the level of apoptotic markers in different gradient of sperm by AnnexinV and Caspases and compared with the level of methylation antibody. It was assumed the level of Annexin-V binding and Caspases is related to level of methylation in the sperm cell.

1.9.3 Chapter 5: Chromatin remodelling in human spermatozoa.

In this chapter the level of CMA3 expression in sperm has assessed and the dogma of donut chromatin compaction in human sperm undergo a trial. The experiment has designed to compare the level of methylation and CMA3 expression.

The results of above experiments might be applied in pre ART diagnostic clinically to detect the best sperm and most successfully procedure and saving time and money of clients.
CHAPTER 2

Materials and Methods

2.1 Introduction

The finding that children who are the product of assisted conception procedures suffer from a significant increase in the incidence of imprinting disorders, notably the Beckwith-Wiedemann and Angelman syndromes (Maher et al., 2003), encouraged us to investigate the methylation status of human spermatozoa. In particular, this study aimed to compare the DNA methylation status of spermatozoa isolated from the high and low-density regions of Percoll gradients. Discontinuous gradient centrifugation is known to separate spermatozoa into subpopulations of differing quality; the spermatozoa recovered from the high-density region of Percoll gradients being significantly more viable, motile and exhibiting better morphology than those recovered from the low-density region of such gradients (Aitken and Clarkson, 1988).

The spermatozoa isolated from the high-density regions of such gradients also appear to have more efficiently compacted chromatin (Sakkas et al., 2000) however there is controversy as to whether this technique also isolates sex spermatozoa containing low levels of DNA damage. Sakkas et al., (2000) claimed that the preparation of spermatozoa on Percoll gradients resulted in a significant decrease in DNA fragmentation while Zini et al (1999) found that DNA damage actually increased following Percoll gradient centrifugation.

The methylation of spermatozoa isolated on Percoll gradients is currently unknown. In this thesis, I set out to recover spermatozoa from the high and low-density regions of Percoll gradients, to isolate the DNA and assess this material for its methylation status. In parallel, whole cells were analyzed by flow cytometry to confirm the methylation status of the sperm DNA and determine how this parameter of semen quality correlated
with other indices of functionality including DNA fragmentation, motility, and markers of apoptosis, including Annexin-V binding and caspase activation. Complete information of list of reagents and antibody suppliers and solutions employed in the explained experiments has inserted in the following Appendices:

Appendix A: Reagents, material and antibodies

Appendix B: Buffers and solution.

Statistics: All results were analyzed statistically by SPSS software and P value <0.05 were considered statistically significant. It is necessary to mention that the first data has labeled on the figures legend of every graph on photos are presented automatically on the Y Axis and the second series of the data on the X axis.

2.2 Human sperm samples preparation

Discontinuous Percoll gradient centrifugation was applied to all samples prior to every experiment. The human spermatozoa were obtained by masturbation after at least 3 days abstention. The qualities that were initially assessed comprised both the macroscopic (liquefaction, consistency, volume) and microscopic (sperm concentration, motility, vitality and morphology) aspects of semen quality.

2.2.1 Percoll Gradient

Semen samples were washed in two steps. Firstly, a Percoll gradient was prepared in a 10 ml Falcon tube in such a way that 3ml of 50% Percoll was laid over 3ml of 100% Percoll in for every ejaculate subjected to analysis. Then 1-2 ml of semen was transferred to the top of gradient without disturbing the latter. All tubes were then centrifuged at 1900rpm (~600g) for 30 min. After centrifugation, spermatozoa from the 50%;100% Percoll interface and pelleting at the base of the 100 % fraction were collected. The collected fractions were placed in separate 10 ml Falcon tubes; 8 ml of medium BWW (Appendix B) was then added and the fractions centrifuged at 1900 rpm (~600g) for a further 15 minutes. Following centrifugation, the remaining pellets were resuspended in 1.0 ml of BWW. These samples were then subjected to microscopic
assessment of sperm concentration, motility, viability, morphology and round cell count.

2.2.2 Macroscopic assessment of semen sample

The samples were liquefied before every experiment; any poorly liquefied samples that initially appeared stringy were incubated for 15 minutes at 37°C in order to ensure their complete liquefaction. Consistency, or viscosity, was estimated visually by aspiration into pipette and then allowed the semen dropped by gravity to observe the thread length. Samples which left the pipette as small discrete drops were assessed as normal while abnormal samples generated elastic threads more than 2cm long. The volume of the ejaculated semen measured in a graduated 10 ml Falcon tube.

2.2.3 Microscopic assessment of semen sample

Some parameters of sperm quality were measured microscopically including concentration, motility and vitality in both the high and low density Percoll fractions.

2.2.3.1 Cell concentration

In reproductive science, sperm concentration is an important parameter of fertility. It is also an important aspect of semen quality to assess in order to determine that sufficient cells are available for analysis. To determine the number of cells, 5 µl of Percoll gradient prepared sample were diluted in 1 ml BWW was added to a 95µl sperm diluting fluid (Appendix B). The diluted sample was then mixed for 10 seconds, and a 10µl aliquot placed under the cover slip of a Neubauer haemocytometer to determine the concentration of spermatozoa: 5 squares (4 corners and centre) in the centre of the chamber were assessed including those cells lying on the upper and left limiting lines. Only sperm heads were counted. Following this assessment of sperm concentration, all round cells/inflammatory cells were counted in the same way. From the sperm concentration measurements, the total number of spermatozoa in each fraction could be computed from knowledge of the dilution used in preparing the spermatozoa for analysis.
2.2.3.2 Motility and Vitality

To detect sperm motility, 6µl of washed semen was diluted in 1ml BWW and placed on a clean slide and covered with a cover slip. The slide then was viewed immediately using the 40 x objective on a phase contrast microscope. 100 cells were then assessed as either motile or non-motile and expressed as percentage motile cells. Vitality was determined by mixing 5 µl of Percoll prepared spermatozoa with 1ml BWW containing 5µl of 0.5% Eosin/0.9% NaCl in PBS (Appendix B) on a slide. A cover slip was then placed over the sample and viewed immediately using the 40 x objective on a phase contrast microscope. 100 cells were assessed as either viable (sperm cells that appear clear) or non-viable (sperm cells that appear pink). The live count cell was expressed as a percentage of the entire sperm population.

2.2.3.3 Morphology

Morphological characteristics of human spermatozoa including head, neck, mid piece and tail were all considered, in determining the normality of the spermatozoa. For this purpose, spermatozoa were diluted and fixed in sperm diluting fluid (Appendix B) and then allowed to settle by sedimentation. A morphology count was done at any time during the day when convenient, as the samples were fixed. A 10 µl aliquot of the fixed sperm suspension was finally put on a slide, and a cover slip placed over the sample. The morphology of 100 cells was then classified as normal or abnormal using a 40 x objective on a phase contrast microscope.

2.3 Computer Aided Sperm Analysis

CASA is a computer-based method for assaying different parameters of sperm motility according to WHO-based classifications. The internal algorithms employed by CASA systems allow spermatozoa to be identified and differentiated from other cells and debris on the basis of their size, luminosity and movement, when visualized under dark-field illumination. Three important characteristics of sperm suspensions, concentration, motility and morphology are measureable with these devices. However their major application is in determining the velocity of movement. For this purpose 2µl of diluted
semen placed on a 20µm deep chamber and visualized using the CASA system’s internal microscope.

2.4 Leukocytes removal

After counting the sperm cells any contaminated samples with more than 5 leukocytes per microscopic field were treated with CD45 Dynabeads (Dynal As, Oslo, Norway). These beads are polystyrene, 4.5µm in size, and contain an IgG2 antibody which is specific for CD45 of leukocytes (the common leukocyte antigen). The beads were initially washed with BWW in order to remove the unwanted storage media. The suspensions were then placed in a Magna sphere Technology Magnetic Separation (Promega, Madison, WI) chamber to collect the beads. This washed bead preparation was then re-suspended in BWW to get the original volume and 50 µl of that bead suspension added to each low density (50%) Percoll preparation while 20 µl of the same bead suspension were applied to every 100% sample fraction – the smaller volume of Dynabeads added to the 100% Percoll fractions reflecting the reduced prevalence of leukocytes in these samples. The samples were then incubated with the beads at room temperature in a rotator for 30 minutes. These samples were placed in the Magna sphere Technology Magnetic Separation chamber for 5 minutes and the purified sperm suspension carefully removed from the beads.

2.5 Human sperm DNA preparation for methylation study

The integrity of sperm DNA is crucial for epigenetic information transmission to the next generation. Robust methods have been used to examine human sperm DNA. Previous studies have demonstrated that human sperm contain a higher incidence of DNA aberrations than somatic cells (Martin et al., 1989). Different techniques have been applied to analyze DNA damage. In this research extracted DNA from human spermatozoa was quantified, purified, digested and run to the HPLC and MSG for studying human sperm DNA methylation pattern.
2.5.1 Standard DNA extraction

To compare the level of methylation in the different fraction of spermatozoa, DNA was extracted from cell pellets collected from Percoll gradients using the following steps. In first step, aliquots containing a known number of spermatozoa (20 x 10^6 cells) were centrifuged at 500 x g for 5 minutes. The supernatants were removed and the pellets vortexed vigorously in around 40 µl of residual fluid. The pellets were then resuspended in 600 µl STE buffer (500 mM NaCl, 100 mM Tris-HCl [pH 8], and 10mM EDTA) mixed with a pipette. 30 µl of 20% SDS (final concentration 1%), 24 µl of 1M DTT (sigma) and 3 µl of 20 mg/ml proteinase K (Promega, Madison, WI, USA) in its buffer (Appendix A) were all added in an 1.5ml Eppendorf tube. The latter was vigorously inverted then incubated at 55 °C overnight to allow lyses of the cells. After cooling to room temperature, 200 µl of protein precipitation medium was added per tube. The Eppendorfs were shaken at this stage and placed on the ice for 5 minutes before being centrifuged at 13000rpm for 10 minutes.

The supernatant containing DNA was then removed to 1.5 ml Eppendorf tubes, containing 300 µl of 100% isoproperanol (Sigma Aldrich, MO) and frozen over night. The tubes were centrifuged again at 13000rpm for 10 minutes, after which the supernatant was carefully removed and the tubes left inverted on absorbent paper to drain. The DNA pellets were washed with 300 µl of ethanol 70% (Sigma Aldrich, MO) by inverting the container several times. Finally the Eppendorfs centrifuged in 13000 rpm for 10 minutes after which the ethanol was removed and any residual alcohol left to evaporate by leaving the tubes on absorbent paper for 15 minutes. The DNA pellets were then rehydrated with 50 µl distilled water and incubation at 65°C for 1 hour to dissolve.

2.5.2 DNA quantification

Several methods are used to quantify the amount of DNA. The standards and controls run with every sample are important in determining the accuracy of the measurement. Most of these techniques not only quantify the amount of DNA but also clarify the purity of this material.
2.5.2.1 UV Spectrophotometry

In this method, DNA is quantified by UV wave absorbance in a spectrophotometer. So samples must be free of contamination from protein, phenol, agarose or RNA to get the best results from this procedure. The peak absorption wavelength following UV radiation of DNA is 260 nm and for proteins is 280 nm. So the purity of sample could be measured by the ratio of two absorbances A260/A280, a ratio of 1.7 to 1.8 predicting high quality clean DNA. The UV spectrophotometer—Ultrospectrum 2000 (Amersham Pharmacia Biotech) was used for DNA measurements, at 260 nm and 280 nm absorbance. In order to zero the spectrophotometer 100 µl dH2O was used as a standard blank in the cuvette, as the first sample. For measuring other samples, 99 µl of dH2O water and 1µl of the original sample was diluted in a cuvette and inserted into the spectrophotometer. For the greatest accuracy, the DNA had to be completely dissolved and homogenous.

2.5.2.2 NanoVue

Functionally, the NanoVue (GE healthcare) is similar to a UV spectrophotometer although with this equipment the sample was delivered directly onto the measurement area. The system is reliable for the measurement of nucleic acid and protein samples. It possesses intuitive software for easy calculation and graphical display. According to the manufacturer, the 0.2 mm path gives the Nano Vue the capacity to measure DNA concentrations up to values 50 x greater than conventional spectrophotometers. We used 2µl of every sample to quantify dsDNA, employing 2 µl d H2O as the negative standard.

2.5.2.3 Quant-IT™ (fluorescent quantitative double strand DNA)

Quant-IT™ technology (Invitrogen, Carlsbad, CA, USA) uses fluorescent dyes specific for DNA, RNA or proteins. This quantification method provides much higher accuracy than UV absorbance methods; the sensitivity of the method being up to 1000 x greater than the spectrophotometric approach. The Kit (Invitrogen, Carlsbad, CA, USA) contains a fluorescent probe which binds to dsDNA and is applied as follows: tubes were set up for two standards and a number of samples. Working solutions were prepared comprising Quant it TM reagent in Quant iT TM buffer in the proportion
1:200. Then 10 µl of standard was added to the working solution for the first run. The other tubes (Axygen PCR-05-C tubes) contained 190 µl of working solution and 1 µl of every sample. Tubes were vortexed and incubated for 2 min at room temperature, then the tubes were run to a spectrofluorimeter and read. The result was multiplied by 200, which represents the dilution factor, in order to calculate the original amount of DNA in the sample.

2.5.2.4 Agarose gel analysis

To estimate, the general quality and quantity of DNA preparations agarose gel electrophoresis is a valuable analytical technique. This technique is based on the molecular mass and negative charge carried by the DNA. For the separation of DNA fragments in the range 0.1–10 kb, normal grade or low melting temperature agarose is adequate at a concentration of 1%. To make the gel the following process was persecuted; 1g of agarose (Archerfield, QLD, and AUS) was weighed out into a flask and 100 ml of 1 x TAE (Appendix B) buffer was added. The solution was heated in a microwave oven until the agarose dissolved. Ethidium bromide was added to the solution to a final concentration of 1 µg/ml. The gel was then allowed to cool for 15-30 min at room temperature before being inserted into a prepared gel casting mold, in which the ends had previously been sealed and a comb had been inserted in order to create the desired number of sample wells.

After removing comb, the gel was placed in the electrophoresis chamber and covered with Tris /Acetate/EDTA buffer TAE (Appendix B). The loading buffer, comprising 5 x 0.25% (w/v) bromophenol blue in 50 % (v/v) glycerol in a ratio of 1:4 with sample buffer, was then added to the DNA samples. 1.5 µl of 10 x loading buffer was added to 25 µl of DNA solution. The DNA sample and standards ladder (Progmega, Madison, Wi, USA) were then loaded onto gel. The electrophoresic system (Portsmouth, NH) supplied current at 100V for 1 h. Finally, the separated DNA was visualized with an ultra violet trans-illuminator (Bio-Rad Gel Doc 1000, Bio-Rad, Hercules, CA) and the images captured with Kodak 1D v3.6 images analysis system software accompanied by a Kodak DC90 Zoom Digital Camera (Kodak, Rochester, NY, USA).
2.5.3 DNA purification

Purification is another step in preparing DNA to analyze by HPLC in order to detect the levels of 5-methyl cytosine. After extracting DNA from sperm cells, it was quantified using a variety of different methods of DNA quantification. Selected samples with purification ratios between 1.7-2 were run through the process of purification as follows.

Firstly, to eliminate RNA, 2.5 µl of an RNAase cocktail containing (RNAase A and RNAaseT1) were added to the sample in a tube and incubated at 37°C for 2 hours. In the next step phenol-chloroform was added to 1.5 x the volume of the original sample and shaken. Centrifugation was performed at 17 x g for 10 minutes to form layers of solution. The top layer, containing DNA, was aspirated carefully to a new Eppendorf tube. Then for DNA precipitation 5µl of acrylamide was added to every sample on ice for 5 minutes or frozen overnight. Tubes were then centrifuged again at 17 x g for 10 minutes. The isolated DNA was then washed with 70% ethanol 3 x and rehydrated in a small amount of dH₂O.

2.5.4 DNA Digestion

After purification, 3 µg of purified double stranded DNA were denatured at 100°C for 5 minutes and then placed on ice. Then 1.5 µl of nuclease p1 (US biological) was added to every eppendorf tube and incubated at 37°C for 16 hours in a gene amp Thermal cycler (Applied Biosystems). 2 µl of calf intestinal alkaline phosphatase was subsequently added to every sample and the Eppendorfs incubated in 37°C for 2 hours.

2.6 Quantitative HPLC to detect nucleotides

High Pressure Liquid Chromatography (HPLC) systems comprise mobile phase, pump, column, and a dual channel chart recorder. The HPLC systems used in this study were Helix DHPLC. As well a UV absorbance detector, the most modern detectors are able to measure different sensitivities and absorbance. This equipment detects the amount of absorbed UV and changes it to a recordable electrical signal. The target for this study
was 5-methylcytosine possessing a UV absorbance maximum at 280 nm. To access and quantify the amount of 5-methylcytosine, DNA was extracted from human spermatozoa, purified, enzymatically digested, amplified, run into the HPLC and analyzed by Star Reviewer software.

In final step of this process, human sperm nucleotides were run into the HPLC to characterize the level of 5-methylcytosine. 3 µg of standard and purified digested DNA sample were measured and carefully located to a special micro pallet space taking care not to create air bubbles. The samples were injected into a Supelcosil LC-18-DB column. Separation of nucleotides occurred at 37°C and absorbance was monitored in 254-280 nm. Elution in mobile phase took place over 30 min with 50 mM ammonium orthophosphate with 4% methanol.

2.6.1 HPLC analysis

It is the relative amount of mdCMP compared with dCMP that is critical in evaluating the degree of DNA methylation. The area under the peaks of mdCMP and dCMP could be converted to the molar equivalents by dividing the areas by the extinction coefficients of the respective nucleotides. In order to calibrate the system, deoxyribonucleotide 5'-monophosphate standards were dissolved in water and the molarities measured according the Beers law (methods 27 2007 156-161). The peak areas were quantified using Star Reviewer software (Varian Inc) and the 5-methylcytosine content was expressed as a percentage of the total cytosine pool after correction for the extinction coefficient as follows:

\[
\text{Methylation percentage} = \left( \frac{\text{mdc}}{\text{mdc} + \text{dc}} \right) \times 100
\]

2.7 Gas chromatography-mass spectrometry

Gas chromatography-mass spectrometry (GC-MS) is a method that combines gas liquid chromatography and mass spectrometry to detect a variety of different molecular entities including nucleotides. The GC-MS is composed of two parts: the gas chromatograph and the mass spectrophotometer. The gas chromatograph possesses a capillary column with different length and characteristics. The different molecules in a
mixture separate as they are heated. They are then carried along the capillary column by an inert gas (e.g., helium) and elute from the gas chromatograph after varying periods of time (called the retention time). As the separated compounds emerge from the column, they are fed into mass spectrometer that then generates critical information of the mass of the compounds as a prelude to their identification. The mass spectrometry processes require a very pure sample.

2.7.1 Sample preparation

Therefore, we used the samples and standards after purification as described in section 2.5.3 to detect the degree of DNA methylation. Purified DNA and standards were prepared as described and run into a GC-MC (DSQ II GC-MS). 100 µg of standard (citidine 99%; Sigma Aldrich) and every sample were placed in glass tubes and 50 µl of 2% w/v of methoxy amine-HCl in pyridine (MOX) was added to the tubes under Dr. Baker and Tony Rothkirch training and incubated at 60°C for 30 minutes with continuous shaking. Then 100 µl of bis (trimethylsilyl) trifluoroacetamide (BSTFA) Sigma-Aldrich was added to the tubes which were then vortexed and incubated at 100°C with vigorous shaking for 1 hour. The samples were then centrifuged at 4000 rcf for 10 minutes and the supernatants removed to GC vials and located ready to inject into the machine.

2.7.2 GC-MS analysis

Samples were analysed on a Trace GC Ultra-DSQ II GC-MS system (Thermo Scientific) fitted with a TriPlus AS autosampler (Thermo Scientific) with Tony Rothkirch cooperation. Samples were injected using a hot-needle technique onto a 30m x 0.25mm ID x 250um FT Factor FOUR VF-5ms capillary column (Varian Inc). Data acquisition and analysis was performed with Xcalibur 1.4 SR1 DSQ II 1.4.2 (Thermo Scientific). Identification of analyze components was performed with the NIST MS Search Program 2.0f (NIST) using the combined Wiley Registry 8th Edition-NIST08 mass spectral library (John Wiley & Sons).
2.8 Flowcytometry

In flow cytometry, a laser beam is directed onto a narrow stream of fluid containing the cells of interest and detectors are located at the point where the fluid passes. The laser light passes through each suspended particle (from 0.2 to 150 µm) and the fluorescent probe associated with each cell is excited into emitting light at a different wavelength than the light source. So flow cytometers are capable to measuring the fluorescent signal emitted by thousands of cells per minute thereby generating information, the nature of which depends upon the specific probe(s) used in the analysis. In this, study a Becton-Dickson FACSCalibur flowcytometer was used to assess various sperm parameters including the level of DNA methylation, serine externalization, and caspase activation.

2.8.1 5-methyl cytosine antibody staining

To study of 5-methylcytosine by flow cytometry, DNA incubated with a mouse monoclonal antibody against 5-methylcytosine (ab73938; Abcam, Sapphire Bioscience, Redfern). Methylation levels were examined in sperm subpopulations isolated on discontinuous Percoll gradients. After semen sample preparation, 20 million spermatozoa were isolated from each Percoll fraction. For every individual experiment, two controls were used one unlabeled (without primary or secondary antibody) and second was secondary antibody alone. The procedure followed was adapted from Benchaib et al., (1995).

In the first stage of this process spermatozoa were fixed with ethanol (70%) (Merck, Germany) at −20°C for 20 min. Cell pellets were then washed twice in phosphate-buffered saline with Tween 0.5% (Sigma, USA) (PBS-T) for 5 min at 500 x g. Then the spermatozoa were incubated at room temperature in 1 mol/l Tris-HCl buffer, pH 9.5 (Merck), containing 25 mM dithiothreitol (DTT; Sigma) for 20 min.

The cells were then washed twice in PBS-T. To ensure that methylated DNA was accessible to antibody, the sperm DNA was further denatured with HCl (6 N) for 10 min. Then it washed with Tris-HCl buffer 2 x and finally with PBS-T, 1 x. At this stage, spermatozoa for the two control incubations were set aside. The main sample was incubated with anti-5-methylcytosine antibody diluted 1/100 in PBS_T for 30 minutes.
After 2 x washes with PBS-T, FITC labelled secondary antibody (anti-mouse IgG) diluted 1/300 in PBS-T was added to the cell pellets and controls containing cells incubated with buffer instead of the primary antibody. The secondary antibody incubations were conducted for 30 min at 37°C. The cells were then washed 2x in PBS-T and run into the flow cytometer.

2.8.2 Annexin- V and PI staining

Phosphatidylserine (PS) externalization is a sign of apoptosis in human spermatozoa. To measure this event we used Annexin V, a protein that has a high binding affinity for PS, in conjunction with propidium iodide (PI), to measure cell viability. The reason for the simultaneous assessment of Annexin V binding and cell viability is that we want to focus exclusively on spermatozoa that are expressing PS on the outer leaflet of the plasma membrane. When cells die the Annexin V can spuriously generate an intense signal by virtue of its ability to access PS located on the inner leaflet of the plasma membrane. PI staining is used to identify and exclude these cells. Thus 4 categories of spermatozoa were identified in these analyses: i) PS⁺/PI⁻, the primary cells of interest that were viable and showed signs PS externalization by virtue of their capacity to bind Annexin V-FITC (green); ii) PS⁺/PI⁺, dead cells that have under apoptosis; iii) PS⁻/PI⁺, dead cells that have no sign of PS binding and must have undergone a necrotic death with substantial loss of the plasma membrane; iv) PS⁻/PI⁻, completely unlabelled cells that are viable but show no signs of PS externalization.

After Percoll discontinuous gradient centrifugation the two separated sperm populations were isolated from the low density (termed the 50% sample) and high density (termed the 100% sample) regions of the gradient respectively and washed 2 x with BWW (Appendix B) at 3000 rpm for 3minutes. 1 million of these washed cells were suspended in 1ml BWW. Then four tubes established and in each tube 100 µl of sample containing 100,000 sperm cells was pipetted. To these tubes were added: (i) 400 µl PBS to generate the control without any staining; (ii) control labelled with 2 µl PI (500 µl /ml) added just prior to running the samples to the FACS machine in order to assess cell viability; (iii) a tube incubated with 2.5 µl Annexin-V-FITC (Invitrogen, Mount Waverly VIC) for 15 minutes and 2.5 µl PI added just prior to run the sample to the machine; (iv) 2.5 µl
human recombinant Annexin-V-FITC per 400 µl PBS in the dark at room temperature for 15 minutes. Cell pellets were washed after incubation with BWW 2x at 3000 rpm for 4 minutes. The cell pellets were finally resuspended in 400 µl PBS (Appendix B) in FACS tubes in the dark. Then the labelled sperm suspension was passed through a FACS Calibur flow cytometer equipped with a 100-mW argon laser (Becton Dickinson, San Jose, CA) with extinction 488 nm and emission 525 nm.

2.8.3 Caspase Staining

One of the features of late apoptosis is the activation of intracellular proteiases known as caspase. As it mentioned in the Introduction, caspase enzymes have critical role in the executioner pathway of apoptotic death cell. Caspases have two domains, the catalytic domain that targets the amino acids in the substrate molecule and binds covalently to active caspase and can be measured by flow cytometry.

To compare the activated caspase levels in different subpopulations of spermatozoa 2 million cells were collected from each fraction of the gradient and centrifuged at 3000 rpm for 5 minutes. Then the pellets were resuspended in 193.3 µl of BWW except the negative control, which was suspended in 200 µl of BWW. FLICA (150 x stock solution) was diluted with BWW to a 15 x working solution. Then 6 µl of this 15 x working solution of FLICA was added to each tube except for the unstained control. These tubes were then incubated in dark at 37°C for 1 hour. Samples were washed with BWW, centrifuged in 3000 rpm for 5 min, 2x. Then the pellet was resuspended in 500 µl BWW and transferred to FACS tubes. Samples were finally analyzed on FL2 channel of the flow cytometer.

2.8.5 Flow cytometry analysis

Analyses were performed using the FACSCalibur flow cytometer (BD Biosciences, San Jose, CA). Fluorochromes were excited with the 488-nm line of the Enterprise laser (Coherent, San Jose, CA). Green and red fluorescence were detected using FL1 and FL2 detectors, respectively, through a bandpass filters of (BP) 530/30 nm and a BP 695/40 nm. All data were analyzed with Cell Quest Pro 3.1 software (BD Biosciences). For 5-methylcytosine, sperm viability, PI, AnnexinV-FITC/PI, and caspase assays 10 000
events were analyzed. FL1, FL2 fluorescence signals were recorded after logarithmic amplification.

2.9 Immunocytochemistry

Antibodies can be used to determine both the presence and the sub cellular localization of an antigen. An important technique that can be used to visualize the location of specific molecules within cells is fluorescence microscopy. With this method, the binding of fluorescent reagents to target molecules can be demonstrated using a compound fluorescence microscope. Cell staining, in some circumstances, may also be used to determine the approximate concentration of an antigen. In this research, fluorescence microscopy will be used as part of the suite of procedures used to demonstrate the differences between two fractions of human spermatozoa in DNA methylation pattern, PS externalization, caspase level and chromatin compaction. To achieve this aim, fixed spermatozoa were labeled with 5-methylcytosine antibody, Annexin-V, red FLICA dye, and CMA3 and analyzed by fluorescence or confocal microscopy. Negative controls incubations were run with each assay.

2.9.1 5-methylcytosine antibody

In this experiment we used a monoclonal antibody to 5- methylcytosine, raised in mice. This antibody was used to capture immunofluorescent images following microscopic examination of spermatozoa recovered from the high and low-density region of Percoll gradients. The method of sperm preparation for this study was adapted from the Benchaib et al., (2005). To ensure adequate antibody access to its antigen, spermatozoa (20 x 10^5) were fixed in 100% ice-cold methanol at -20°C for 15 minutes. Then according to the procedure described in section 2.8.1 adapted from Benchib et al., (1995) DNA was decondensed amd permeabilized with 0.2% Triton X-100 in PBS for 10 minutes at room temperature. The slides were then rinsed with PBS and blocked for 30 minutes with 10% goat serum and 1% BSA-PBS. After rinsing with PBS, 5-methylcytosine antibody was added in humidified chamber. Following incubation for 30 minutes with primary antibody diluted in 1% BSA-PBS at 37°C, the samples were washed 3 x for 5 minutes with PBS. Finally, the cells incubated for 1 hour with
secondary antibody Alexa Fluor goat anti-mouse IgG mouse diluted in 1% BSA-PBS. Finally, cells were washed 3 x for 5 minutes with PBS. Cells were mounted with MOWIOL then covered with a coverslip and sealed. Antibody localization was finally visualized with a fluorescence microscope.

2.9.2 Annexin-V antibody

Recombinant Human Annexin-V (invitrogen) is an FITC-conjugated calcium-dependent phospholipid binding protein with high affinity to phosphatidylserine in the plasma membrane. This molecular probe was used to detect and compare microscopic signs of apoptosis in human spermatozoa recovered from differing regions of the Percoll gradient. After spermatozoa has been washed 2x with PBS, 1 x $10^5$ cells were resuspended in 1ml PBS. Then the surface antigen, PS, was labeled as follows; the samples were fixed and incubated with BSA-PBS (Appendix B) for 5 minutes at 4°C. Then Annexin –V-FITC was added to the cells slowly in PBS, and incubated for 30 min after which the cell pellet was washed 3 x for 5 minutes with PBS. Cells were then transferred to a clean glass slide and mounted with MOWIOL under a coverslip.

2.9.3 Chromatin Compaction Assay (CMA3)

Chromomycin is specific fluorochrome that binds to the minor groove of cytosine – guanine rich DNA sequences, the site normally occupied by sperm chromatin (Monaco et al., 1982). CMA3 is a very helpful probe to detect defective spermatozoa in which chromatin remodeling has not occurred properly during spermiogenesis resulting in a relative deficiency of protamines and an abnormally high histone–protamine ratio. We examined spermatozoa recovered from the high and low-density regions of Percoll gradients to compare the chromatin compaction patterns in them. Cells were collected ($3\times10^5$ ) from each layer, washed and fixed with 100 µl of 4% formaldehyde for 15 minutes. The cell pellets were then washed 3 x with PBS-T. Then an appropriate aliquot of the cell suspension was placed on a poly-L- Lysine coated coverslip and allowed to settle down overnight at 4°C. Then 50 µl of 0.2% Triton X-100 was added to every coverslip, incubated at room temperature for 15 minutes and rinsed with McIlvaine buffer.
For CMA₃ staining according to Manicardi et al (1995) coverslips were incubated with 20 µl of CMA₃ solution (0.25 mg/ml CMA₃= 25 µl of CMA₃ stock/mL McIlvaine buffer, pH 7.0) for 20 min. They were then washed in McIlvaine buffer, air-dried and mounted with MOWIOL, sealed and saved in dark. To view the slides Fluorescence analysis was performed using a Zeiss Filterset 09.® III (Zeiss, Oberkochen, Germany). A total of 100 cells per every fraction of sperm counted randomly and bright cells scored as unprotected cell because of protamine deficiency and expressed by percentage.

2.10 Fluorescence microscopy

A fluorescence microscope (colloquially synonymous with epifluorescence microscope) is a light microscope used to image the content of cells by reflection or absorbance of fluorescent. A common use in biology is specimen staining in order to visualize a protein or other cellular content. We visualized samples prepared as mentioned above using an average of 500,000 cell /ml under a Zeiss Filterset 09.® III (Zeiss, Oberkochen, Germany. Green and red fluorescence were respectively detected using (480–520 nm) filters. Images were captured by a CollSnapfx camera (Roper Scientific, Evry, France) using Meta Imaging 4.6.6. Software (Universal Imaging, Downingtown, PA, USA).

2.11 Protein Extraction and Analysis

2.11.1 Standard Protein Extraction

Since it was of interest to examine the presence of enzymatic proteins such as DNA methyl transferases and non-enzymatic mediator proteins like in different fractions, proteins were extracted from 100% and 50% Percoll fractions using an SDS extraction method. In this method after centrifuging the sperm in 3500 rpm for 5 minutes, the pellets were resuspended in 50 µl SDS extraction Buffer (Appendix-B) and shaken thoroughly. Then the tubes heated in 100°C for 5 minutes and centrifuged in 16000 rpm for 10 minutes to pellet unwanted cell constituents and DNA. The supernatants containing protein were stored at -20°C until required.
2.11.2 Protein Quantification

There are different methods and kits to quantify the extracted proteins. In this research the BCA TM Protein assay kit (Thermofisher scientific, Waltham, MA, USA) was used and manufacturer’s instructions followed. The kit used comprises the detergent compatible bicinchoninic acid (BCA) for colorimetric detection and protein quantification. This kit produces a purple color in the reaction with the protein substrate, measureable by spectrophotometry with an absorbance of 562 nm. Using this absorbance, a standard curve for protein concentration was prepared.

Extracted proteins were measured using the following steps: a standard solution containing 2 mg BSA in 1 ml SDS extraction buffer was used to establish a range of standards with known protein concentrations ranging from 0.2 mg/ml to 2 mg/ml. A 5 µl aliquot of standard plus 25 µl of reagent AS was applied in a 96 micro plate (Sarstedt, Germany) well. Then a final aliquot of 200 µl working reagent B was added to every well. Samples were prepared in a microtitre plate in the same way. 5 µl of every sample and 25 µl of AS reagent were placed in each well and finally, 200 µl of working reagent B was added, mixed gently and incubated in room temperature for 15 minutes. Volume of working reagent AS was determined as follow:

\[
\text{total volume of working reagent} = (\text{number of standards} + \text{number of unknown}) \times (\text{number of replicates}) \times 25 \, \mu l
\]

The microtitre plate was then placed in the imaging system (BioRad, Hercules, CA, USA) and the absorbance measured at 562 nm. The amount of protein was computed from the standard curve and expressed as µg BSA equivalents/ml concentration.

2.12 SDS Polyacrylamide Gel Electrophoresis and Western Blotting

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) is a technique to determine proteins based on their molecular weight. For this approach, proteins first must be treated with a reducing agent such as 2-mercaptoethanol. SDS gives a negative
charge to proteins and allows them to migrate to the positive pole in an electrophoretic chamber at a rate that proportional to their molecular size. Western blotting is specific method for determining the presence of proteins of interest in complex mixtures. For this procedure proteins are transferred onto a membrane which is then probed for the presence of a specific protein using primary and secondary antibodies.

In this study, extracted proteins from different fractions of spermatozoa were separated on an 8% polyacrylamide gel with 5% polyacrylamid stacking gel as describe in the Appendix A-B (Bio-Rad). Following this the gels were assembled in an electrophoresis system (Bio-Rad, Mini-Protean 3 cell system) along with SDS running buffer (Appendix B). The same amount (8 μg) of each protein was mixed with an equal volume of the SDS-PAGE sample buffer (Appendix B) and incubated at 100° C for 5 minutes. Samples were loaded into the wells of the stacking gel. Equal volumes of molecular weight standard were run in the first lane while the second lane was left empty. Two electrodes from the electrophoresis tank were connected and the proteins subject to a constant 120 volt current until marker dye reached the bottom of the gel. The gel was then removed separated and used for Western blotting.

2.12.1 Western blot

SRA domain of UHRF1 was used as the primary antibody while the secondary antibody was a labeled goat anti rabbit antibody. Hybond extra nitrocellulose membrane (Amersham Bioscience Australia) was used for the transfer of proteins from the polyacrylamide gel. The SDS gel was placed on the membrane between three filter papers and sandwiched with two fiber pads. The preparation was soaked in Western blot transfer buffer for 5 minutes. The whole cassette was then closed and located in the Western Blotting module. The power supply was activated and, run at 350 mA for 1 hour. The membrane was subsequently used for immunoblotting.

2.12.2 Immunoblotting

The nitrocellulose membrane was blocked overnight at 4°C with blocking solution (3% w/v BSA in Tris buffered saline) on an orbital rocker. The blocking solution was then removed and rinse briefly with dH2O. Then primary antibody diluted in 10 ml antibody
buffer (Appendix B) was added and incubated with membrane at room temperature for an hour on an orbital shaker. Then the blot was washed 3 x with wash buffer for 10 minutes each on the orbital shaker. After subsequent incubation with 10 ml of diluted secondary antibody in buffer and incubation at room temperature for an hour on an orbital shaker, the membrane was washed 3 x with wash buffer.

Then the membrane was developed for chemiluminescence detection with ECL or ECL Plus kits (Amersham, Buckinghamshire, England) by following the manufacturer’s instructions. The protein side of membrane was covered in a 1:1 mix (usually 6 ml) of solution and left for 1 one minute. The liquid was then poured off and the membrane, protein side up, was put in Luminiscent image Analyzer LAS-4000 visualized with Image reader LAS-4000 software and photographed.
Chapter 3

Pattern of DNA Methylation In Different Gradient of Human Spermatozoa

3.1 Introduction

Throughout the introduction, it has been mentioned that the pattern of DNA methylation could be a potential biomarker for ART, imprinted diseases and early cancer detection (Ohki et al., 2001). DNA methylation plays a crucial role in epigenetic modification and regulation of many homeostatic mechanisms during cell proliferation and cellular development. In a reproductive context, one of the key questions to arise from this work is whether relationships exist between sperm quality and the patterns of DNA methylation recorded in these cells and how is this epigenetic information maintained. To answer this question, research has been directed at two major epigenetic modification factors in DNA; methylation of cytosine at the C5 position on CpG islands (Bird, 2002) and post-transcriptional histone modification during spermatogenesis.

However, relatively little is known about how DNA could be the target of methylation and how the pattern of methylation in human sperm is established or maintained. Two components play a key role during DNA methylation in eukaryotes; the SRA domain of UHRF1 protein (called ICBP90 in humans) orchestrates the establishment of the \textit{de novo} 5-methyl cytosine pattern of genomic DNA, and the DNA methyltransferases (Dnmts) act as enzymatic agents for maintaining methylation on cytosine. In this chapter we measured the level of global methylation by HPLC and investigate the pattern of methylation in sperm sub-populations of varying quality using flow cytometry and an anti-5-methyl cytosine antibody.

The global status of methylation in different somatic cells has been well established. In 1982, Ehrlich reported 70% to 80% of all CpG dinucleotides in the genome are methylated on carbon 5 of cytosine, which it accounts for nearly 1% of all DNA bases. The overall pattern of methylation exhibits some temporal and spatial variation. For instance, during a discrete phase of early mouse development, methylation levels
decline sharply to nearly 30% of the typical somatic level (Monk et al. 1987). In addition, a significant fraction of all human CpG islands are prone to progressive methylation in certain tissues as aging occurs (for review, see Issa 2000). In this chapter, the level of global methylation in human sperm DNA was measured by HPLC in a collaborative research project involving the Aitken and Scott laboratories at the University of Newcastle. This result was then confirmed in a series of flowcytometry experiments in which sperm DNA was labeled with a 5-methylcytosine antibody.

The protocol for labeling sperm cells with the 5-methyl cytosine antibody has been adapted from (Benchib et al., 2005) study of the level of methylation in human spermatozoa. The same antibody has also been used to define the localization of 5-methylcytosine residues using an immunocytochemistry approach. The antibody used in this study is a mouse monoclonal generated against bovine serum albumin conjugated with methylated DNA. According to Ball et al., (1983) a highly mobile group (HMG) of proteins are associated with the methyl group in DNA and contribute to the production of antibodies against m5C. Recently, it was revealed that the protein UHRF1 plays a central role in transferring DNA methylation status from mother cells to daughter cells. The SET associated RING finger-associated (SRA) domain recognizes hemi-methylated DNA that appears in newly synthesized daughter DNA strands during DNA duplication through S phase (Arita et al, 2008, Avvakumov, 2008, Hashimoto et al, 2008).

Therefore it is conceivable (but not demonstrated) that the methyl cytosine epitope targeted by this antibody includes the SRA domain of UHRF1, that according to Hashimoto et al, 2008 naturally surrounds the flipped-out methyl cytosine residue within the DNA helix. In future, it could be interesting to determine whether the epitope targeted by the 5-methyl cytosine antibody involves one domain of UHRF1 such that the 5-methyl cytosine antibody recognizes this domain or not. UHRF1 is a protein involved in cell cycle regulation (Fujimori et al 1998, Hopfner et al., 2000, Muto et al., 2002, Mori et al., 2002, Bonapace et al., 2002, Mousli et al., 2003). It is a regulator protein for cell proliferation especially for transition from G1 to S-phase (Bonapace et al., 2002, Arima et al., 2004).
Interestingly among all tested domains of this protein, only SRA domain showed similar kinetics as full-length Np95 (Rottach et al., 2009). - The aim of this chapter was to study methylation as an important indication of the epigenetic modifications exhibited by human sperm populations of differing quality. The results emphasize that epigenetic differences do indeed exist between good and populations of spermatozoa as confirmed by a variety of different independent, methodologies.

3.2 Results

3.2.1 HPLC

To quantify 5-methyl-2-deoxycytidine, the most important sign of gene silencing, as well as other components in human sperm DNA, high-pressure liquid chromatography (HPLC) was performed. The two different fractions of human sperm (high and low-density Percoll fractions) were isolated and washed as previously described (section 2.2) DNA was extracted, purified, quantified and digested in a sequential enzymatic process and incubated in a gene amplifier over night (section 2.5.1-2). Then the nucleotides generated following DNA hydrolysis were injected into the Helix analysis column under a high pressure mobile phase. The four essential bases and 5- methyl- 2’-deoxycytidine were eluted under pressure according their molecular size. 2’-deoxycytidine with the smallest size was eluted first. These 5 bases produced different peaks in the chromatogram because of specific variation in UV absorbance’s (Fig 3.1, 2). The results were calculated by Star LC Workstation version 5 (Table 3.1).

The Figures (3.1, 2) show the typical chromatogram of five bases in different sub-populations of human spermatozoa from 2 µg digested DNA. In addition, the pattern of these bases has been compared in good and poor quality spermatozoa obtained from different donors. The eluted peak at nearly 7 min indicates 2’-deoxycytidine with molecular weight 227.1, while the peak at nearly 14 min shows the 5-methyl-2’-deoxycytidine a molecular weight 241.2. The results obtained with human sperm DNA extracted from 16 donors between ages 20 and 23 showed a variation in the methylation status from a minimum 0.2% in the 100% fraction of donor number 345 to a maximum 7.58% in a 50% pooled fraction. Overall the results shows that the average percentage
of 5-methyl-cytidine in high density, high quality spermatozoa is 2.4% while in low density poor quality sperm samples the equivalent value was 4.06%.

The calculated data from the area under these two peaks is presented in the Table 3.1. Percentage methylation was calculated according to the following equation:

\[
\text{Percentage methylation} = \frac{\text{5-methyl-2' deoxycytidine}}{\text{(5-methyl-2'-deoxycytidine+2'-deoxycytidine)}} \times 100
\]

Quantitative and qualitative results of the HPLC analysis for hydrolysed sperm DNA for the 2’-deoxycytidine and 5-methyl- 2’-deoxycytidine (Table 3.2) has demonstrated different levels of methylation in spermatozoa recovered from different regions of the Percoll gradients. Although the ratio of 2’-deoxycytidine and 5-methyl-2’-deoxycytidine revealed clear differences between the 100% and 50% Percoll fractions, the absolute amount of DNA loaded onto the columns from these sources, as reflected by the area-under-the-curve analysis, suggested clear differences. The reason behind these differences is not certain at this stage; however, such inconsistencies do not detract from the overall conclusion that the relative level of DNA methylation (proportion of methylated 2’-deoxycytidine) is elevated in populations of defective spermatozoa. In order to consolidate this finding, further experiments were performed, using flow cytometry in conjunction with an anti-5 methylcytosine antibody, to confirm that poor quality spermatozoa are hypermethylated compared with high quality cells.
Table 3.1 Statistic analysis of the HPLC result of hydrolysed sperm DNA for the 5-methyl-2’-deoxycytidine.

<table>
<thead>
<tr>
<th>Sperm quality</th>
<th>number</th>
<th>Mean±SEM</th>
<th>Max</th>
<th>Min</th>
<th>Median</th>
<th>Std deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>100%</td>
<td>11</td>
<td>3.04±0.34</td>
<td>7.470</td>
<td>0.20</td>
<td>3.520</td>
<td>1.615</td>
</tr>
<tr>
<td>50%</td>
<td>5</td>
<td>4.6±0.56</td>
<td>8.630</td>
<td>2.57</td>
<td>3.880</td>
<td>1.778</td>
</tr>
</tbody>
</table>

Table 3.2 Quantitative and qualitative results of the HPLC analysis of hydrolysed sperm DNA for the 2’-deoxycytidine 5-methyl-2’-deoxycytidine.

<table>
<thead>
<tr>
<th>Sperm quality</th>
<th>Mean area under peak</th>
<th>Extinction coeffs</th>
<th>Mean Molar equivalent</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Poor&amp;good</td>
<td>5mdc (5-methyl-2’-deoxycytidine)</td>
<td>dc (2’-deoxycytidine)</td>
<td>5mdc</td>
<td>Dc</td>
</tr>
<tr>
<td>100% (good)</td>
<td>77128.27273</td>
<td>2937459</td>
<td>8.55</td>
<td>7.95</td>
</tr>
<tr>
<td>50% (poor)</td>
<td>144659.2</td>
<td>3177130</td>
<td>8.55</td>
<td>7.95</td>
</tr>
</tbody>
</table>
Figure 3.1. Typical HPLC chromatogram profile of hydrolysed human sperm DNA from high-density gradient Percoll fraction (100%).

**HPLC analysis of hydrolysed sperm DNA from good quality spermatozoa after complete digestion and hydrolysis by enzymatic agents.** The DNA eluted peak at ~7 minute corresponds to 2’-deoxycytidine (227.1KD) dc and the peak eluted at ~15 min represents 5-methyl-2’-deoxycytidine (241.2 KD) mdc.
Figure 3.2 Typical HPLC chromatogram profiles of hydrolysed human sperm DNA from low-density gradient Percoll fraction (50%).

*HPLC analysis of hydrolysed DNA from poor qualityperm populations after complete digestion and hydrolysis by enzymatic agents. The DNA eluted peak at ~7 minutes corresponds to 2’-deoxycytidine (227.1 KD) dc and the peak eluted at ~15 min represents 5-methyl-2’-deoxycytidine (241.2 KD) mdc.*
DNA extracted from 11 good quality and 5 poor quality human spermatozoa were hydrolysed by enzymatic processes in order to detect and compare the percentage of 5-methyl-2′-deoxycytidine in different subpopulations of human spermatozoa. Analysis of the extracted DNA from donors aged between 20 to 23 by HPLC showed an average 2.4% 5-methylcytidine in good quality spermatozoa while in poor quality samples the value was 4.06%. (P < 0.001)

3.2.2 Identification the pattern of 5-methyl cytosine in human spermatozoa by Gas chromatography Mass spectrophotometry

Gas chromatography-mass spectrometry (GC-MS) is a method that combines gas liquid chromatography and mass spectrophotometry to detect a variety of different molecular entities including nucleotides. The mass spectrometry process requires a very pure sample. Therefore, we used the samples and standards after purification as described in section 2.5.3 to detect the degree of DNA methylation. Purified DNA and standards were prepared as described (Section 2.5.3) and run into a GC-MC (DSQ II GC-MS). 100 µg of standard (cytidine 99%; Sigma Aldrich) and every sample were placed in
glass tubes and 50 µl of 2% w/v of methoxy amine-HCl in pyridine (MOX) was added to the tubes and incubated at 60°C for 30 minutes with continuous shaking. Then 100 µl of bis (trimethylsilyl) trifluoroacetamide (BSTFA) Sigma-Aldrich was added to the tubes, which were then vortexed and incubated at 100°C with vigorous shaking for 1 hour. The standard samples were then centrifuged at 4000 rcf for 10 minutes and the supernatants removed to GC vials and injected into a Trace GC Ultra-DSQ II GC-MS system and component graphs were analyzed by the NIST MS Search Program2 combined Wiley Registry 8th Edition-NIST08 mass spectral library (Fig 3.5 A) and compared with the model (Fig3.4) adapted from (Tardy-Planechaud et al 1997) and also our obtained result from HPLC analysis (Fig 3.5 B).

![Figure3.4 Sum of the selected ion chromatograms oligodeoxynucleotides.](image)

Oligodeoxynucleotide hydrosylates (top) all normal bases, (center) an oligonucleotide in which one cytosine has been replaced by 5mC, and (lower) an oligonucleotide in which one cytosine has been replaced by hmC. The base sequence corresponds to the upper strand (Materials and Methods) (Tardy-Planechaud et al 1997)
Figure 3.5 Typical gas chromatography mass spectrophotometry profile of standard cytidine compared with the HPLC chromatogram of hydrolyzed human sperm DNA.

100 µg of standard (cytidine 99%; Sigma Aldrich) were derivatized; every sample was placed in a glass tube and 50 µl of 2% w/v of methoxy amine-HCl in pyridine (MOX) was added to the tubes and incubated at 60°C for 30 minutes with continuous shaking. Then 100 µl of bis (trimethylsilyl) trifluoroacetamide (BSTFA) was added to the tubes, which were then vortexed and incubated at 100°C with vigorous shaking for 1 hour. The samples were then centrifuged at 4000 ruff for 10 minutes and the supernatants removed to GC vials and located
ready to inject into the GCMS (DSQ II GC-MS) machine. The Graphs were analyzed by the Program2 combined Wiley registry 8th Edition-NIST08 mass spectral library Software top (Figure 3.5) which it could be compared with eluted picks of HPLC of analysis sperm DNA at ~7 and ~15 min corresponding to 2′-deoxycytidine (227.1 KD) and the peak eluted at double time 5-methyl-2′-deoxycytidine (241.2 KD).

3.2.2 Identification of the pattern of 5-methyl cytosine expression in human spermatozoa by flowcytometry

The HPLC analysis of global methylation patterns in human spermatozoa was characterized by a great deal of variation and a large standard error. Therefore, we decided to examine the level of 5-methylcytosine in different populations of human spermatozoa, applying a monoclonal antibody against 5-methylcytosine raised in mouse. For this purpose, DNA was prepared from human sperm populations recovered from low and high-density Percoll fractions (section 2.8), incubated with antibody then analyzed with a FACSCalibur flow cytometer (Becton Dickinson) with green fluorescence 580nm emission using the FL2 filter set. A typical frequency histogram of the samples is presented in Fig 3.6 including a comparison of the pattern of methylation in different quality sperm populations from the same donor.

The results of flow cytometry using the 5-methylcytosine antibody in 100% and 50% gradient Percoll fractions of human spermatozoa indicate that the level of 5-methylcytosine expression in human sperm DNA varies between individuals as well as between different Percoll fractions (Fig 3.7). In particular, the data demonstrated a nearly two-fold increase in 5-methylcytosine expression in the poor, low-density sperm fraction, in comparison with the high-density cells (Table3.3).
Figure 3.6 Typical histogram profiles of good (100% Percoll) and bad quality (50% Percoll) human sperm DNA.

Flow cytometry analysis of spermatozoa labelled with 5-methylcytosine antibody staining with the method adapted from Benchaib et al. (2005). Sperm DNA from the high density region of the gradient in top panel and poor quality cells in the lower panel after treating with HCl and DTT and incubating with a 5-methylcytosine antibody. The cells were then analysed by FACSCalibur flow cytometer (Becton Dickinson) with green fluorescence 580nm emission (FL2).
Figure 3.7 The individual pattern of methylation in low and high-density percoll gradient fraction of human spermatozoa by flowcytometry.

Sperm samples from 17 semen donors were prepared and labeled with 5-methylcytosine antibody (2.8.1) using the method adapted from Benchaib et al. (2005). After treating the cells with HCl and DTT they were incubated with 5-methylcytosine antibody then analyzed by FACSCalibur flowcytometer (Becton Dickinson) with green fluorescence 580nm emission (FL2). The results are presented as the percentage of cells methylated in every fraction counted. The graph illustrates the percentage level of methylation in the Percoll fractions recovered from individual donors. The level of methylation is not only different in the high and low-density fractions of the Percoll gradients but also differed markedly from individual to individual.
Table 3.3 Results of the flow cytometry analysis of sperm DNA for 5-methyl cytosine.

<table>
<thead>
<tr>
<th>Sperm quality</th>
<th>number</th>
<th>Mean±SEM</th>
<th>Max</th>
<th>Min</th>
<th>Median</th>
<th>Std deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>100%</td>
<td>17</td>
<td>14.37±2.719</td>
<td>56.80</td>
<td>14.37</td>
<td>33.30</td>
<td>11.21</td>
</tr>
<tr>
<td>50%</td>
<td>17</td>
<td>37.00±3.893</td>
<td>92.66</td>
<td>37.00</td>
<td>64.02</td>
<td>16.05</td>
</tr>
</tbody>
</table>

Figure 3.8. The pattern of methylation in different fractions of human spermatozoa.

Samples from 17 semen donors were prepared on discontinuous Percoll gradients (see Section 2.8.1) and labeled with 5-methylcytosine antibody using a method adapted from Benchaib et al. (2005). After treating with HCl and DTT, the cells were incubated with 5-methylcytosine antibody then analyzed by FACSCalibur flow cytometer (Becton Dickinson) with green fluorescence 580nm emission (FL2). The above graph demonstrates that sperm from the low-density Percoll fraction possess nearly double the level of 5-methyl cytosine compared with the high density fraction. It suggested that hypermethylation of human sperm DNA is associated with defective sperm function.
3.2.3 Immunocytochemistry of 5methyl cytosine antibody

The results of the HPLC and flow cytometry analyses suggest minimum DNA methylation in high quality human spermatozoa. However, the data also indicate a significant variation in 5-methylcytosine expression in individual semen donors. In order to generate more insights into the underlying cause of this variation in expression immunocytochemical analyses were performed with the 5-methylcytosine antibody in order to reveal the pattern of DNA methylation in individual sperm samples.

For this analysis, sperm populations were recovered from discontinuous Percoll gradients and prepared in two different ways. In first method, which was adapted from Benchaib et al. (2005) the sperm DNA was prepared by treating cells with HCl (6N) and DTT (25 mM). By contrast, in second method, neither acid nor detergent treatment was used in order to focus on mitochondrial sperm DNA. Then samples were fixed and incubated with a 5-methylcytosine antibody raised in the mouse and then sequentially with green fluorescent mouse secondary antibody (Appendix A.2). The appropriate amount of the sample was then spread onto poly-L-lysine coated microscope slides and air-dried. After labelling, the pattern of antibody expression was illustrated by fluorescent and confocal microscopy (Fig 3.9-11) by green fluorescence microscopy using 480nm emission FL2 filter set.

The top row of photographs (3.9 a) illustrated that high density Percoll spermatozoa show the lowest level of antibody localization except for three focal points of staining that were detectable in some samples (3.9g,h) which might, in part, be accounted for by the hypermethylated X-chromosome. The photographs depicted in Fig.3.9 (panels b,c,d,e and f) illustrate the pattern of hypermethylated DNA seen in poor quality spermatozoa from the low density region of Percoll gradients. These cells revealed a different pattern of antibody distribution compared with the high-density fraction (Fig.3.9, panels a,g and h). Immunochemical analysis of spermatozoa with a 5-methylcytosine antibody in the absence of acid and detergent treatment gave an opportunity to study methylation of the mitochondrial DNA. In this case, even after treating cells with acid and detergent, we could demonstrate that the DNA in the sperm mitochondria is hypermethylated prior to fertilization (Fig 3.10 a, b).
Chapter 3: Pattern of Methylation

Figure 3.9 Pattern of methylation in human spermatozoa by immunochemistry of 5-methyl cytosine antibody.

Sperms were collected from different Percoll gradient panels and incubated with antibody after denaturation with HCl and DTT (a,b,c,d,e,f,g,h). The appropriate amount of sample was then spread onto poly-L-lysine coated microscope slide and air-dried. After labelling, the pattern of antibody binding was detected by fluorescence microscopy using 480nm emission filter set (FL2). The results show that the pattern of methylation in high quality spermatozoa featured in the top left (a) possess low levels of cross reactivity with the antibody and were often unstained, except for a few minor areas as demonstrated in panels g and h). In contrast, the right hand panels representing the low density Percoll fraction (b) exhibit significant nuclear staining, as emphasized by the enlarged images depicted in panels c,d,e and f). A comparison of these two groups of photos therefore demonstrates significant levels of methylation in the low density Percoll gradient spermatozoa, in contrast to the high density cells which show minimal staining with the 5-methyl cytosine antibody.
Figure 3.10 Immunocytochemistry of mitochondrial DNA with 5-methylcytosine antibody.

Sperm DNA stained with antibody without treating with acid or detergent (a) and after treating acid and DTT (b). In first experiment, the samples were fixed and incubated with 5-methyl cytosine antibody raised in mouse and then developed with a fluorescent mouse secondary antibody. The sperm were then spread onto poly-L-lysine coated microscope slides and air-dried. After labeling, the pattern of antibody binding was revealed by fluorescent microscopy. As the midpiece of the spermatozoa has stained with the 5-methyl cytosine antibody and this region of the cell only contains mitochondria in a minimum of cytoplasm, it is suggested that mitochondrial DNA has been targeted by this antibody (a). In the second experiment the sample has been treated with HCl and detergent followed by the staining method employed in the first experiment (b). As illustrated, the mitochondrial DNA of human spermatozoa still appears to be labelled by the antibody after acid treatment, although the degree of staining is significantly reduced compared with (a). Also, methylated DNA is visible in the sperm head before and after acid treatment [circled areas in (a) and (b)].
Figure 3.11 Immunocytochemistry of good quality of sperm DNA with 5-methylcytosine antibody location of hypermethylated chromosome.

Sperm DNA was treated to HCl acid and DTT according the method of Benchaib et al (2005). The spermatozoa were then incubated with 5-methylcytosine antibody raised in mouse and developed with a fluorescent mouse secondary antibody. The spermatozoa spread on to the poly–L-lysine coated microscope slides and air-dried. After labeling, the pattern of antibody expression was observed by confocal microscopy. I observed two dumbbell shape packed DNA one located in the rightpart of the sperm head and other placed behind the fluorescent DNA; the identity of this hypermethylated domain is unknown however we might speculate that structure is the inactive-X chromosome in the left upper part of the head of sperm. The shadow of Mitochondrial DNA even after treating with acid and detergent still is detectable like a ladder in mid piece. In the attachment place of the head to neck at right hypermethylated foci could be speculated to hypermethylated precentriols.
3.2.4 Immunoblotting of the 5methyl cytosine

In previous experiments we have found that the DNA of normal functional spermatozoa exhibits a minimal degree of methylation. To further explore potential methylation pathways in human spermatozoa, we have employed a mouse monoclonal antibody raised in the mouse against the 5-methylcytosine site (Ball et al., 1983), according to Hassimotto et al., 2008 and Rottach et al., 2010 this site includes SRA domain of UHRF1 nuclear protein that it may concepted as a major epitope.

Spermatozoa were collected from Percoll gradients and were washed and prepared for protein isolation as described in Section 2.2.11. The proteins were then separated by electrophoresis on 8% polyacrylamide gels, as outlined in section (2.2.12). The results of this electrophoresis (Fig 3.12), interestingly demonstrated the presence of a series of cross-reacting protein bands corresponding to the molecular weight of all known DNA methyltransfrases. The putative DNA methyltransfrase1 exhibited a molecular mass less than somatic Dnmt1 (160-190KD) because, as explained in the introduction, Dnmt1o in the male germ line is smaller than its homolog in other tissues (Herman et al. 2004). Putative Dnmt3a was represented as a band at 90 KD while Dnmt3b may have been represented by a band near to 70 KD. Another enzyme, which was expressed in poor quality sperm as a band with molecular mass of 60 KD, could be Dnamt3L. The last band, which was present in all sperm samples at 50 KD, could have been the omni present Dnmt2.

In presenting interpretations it is fully recognized that the epitope targeted by the antibody is still uncharacterised and that proteins cannot be identified on the basis of molecular mass alone. Independent studies with antibodies targeted against epitopes that are unique to each subtype of Dnmtses will be needed to determine whether these enzymes are indeed present in mature spermatozoa. However as it demonstrated in Fig1.5 all methyl transfrases composes motif V and motif Viii in C- terminal catalytic part of their structure (Herman et al., 2004). Also, according to Klimasaukas et al. 1994 and Snakpal et al., 2002 both of these two motifs interact in detecting the cytosine and make a pocket around the flipped out cytosine residue. Therefore it may concepted the antibody is against these epitops and may detect methyl transfrases who are compulsory associated with 5-methyl cytosine.
Figure 3.12 Detection the pattern of methylation via examination of the level of methyl transferases in human sperm.

The extracted protein from different gradient of semen were prepared (section 2.2.11) run to the 8% SDS gel the nitrocellulose membrane probed with monoclonal 5-methylcytosine antibody. The first columns is control, second and third are prepared protein from 100% fraction and the last couple of columns in the right presented of extracted protein from 50% fraction percol gradient. The identity of these bands is unknown however; the bands correspond to molecular masses of DNA methyl transferases. If this were the case, it might carry implications for the capacity of human spermatozoa to engage in de novo DNA methylation following ejaculation.
3.3 Discussion

The results obtained in this section of the study relate to the mystery of DNA methylation in human spermatozoa. The data from the HPLC analysis of hydrolysed human sperm DNA as well as the flow cytometry of spermatozoa labeled with a 5-methylcytosine antibody, suggested that poor quality cells are hypermethylated. In addition, immunochemistry of sperm with 5-methyl cytosine antibody demonstrated unusual patterns of methylation in the sperm nucleus and clear evidence for the methylation of mitochondrial DNA. In high quality sperm, with the exception of some small regions of chromatin, which are hypermethylated, the DNA is mostly unmethylated.

These particular hypermethylated areas on good quality sperm (Fig3.9 a,g,h) could be specific genomic regions, such as the X chromosome (Fig 3.11)(Turner, 2007). The result of immunoblotting (Fig 3.12) suggested the possible presence of DNA methyltransferases in human spermatozoa. However, it is difficult to imagine how these putative enzymes could be active in the tightly compacted chromatin structure characteristic of mature spermatozoa. However, they may have been very active during the extensive chromatin remodeling that occurs during spermiogenesis. Interestingly results from all the above-mentioned methods support the idea that high quality spermatozoa are hypomethylated.

In 1982, Ehrlich analysed the total base composition of DNA from seven different normal human tissues and eight different types of homogeneous human cell populations. The results demonstrated that the level of methylation in cytosine residues is remarkably tissue and cell specific. It reported the lowest levels of methylation from in vivo sources were placental DNA and sperm DNA. He reported the level of 5-methylcytosine to be 1% of the total base pool in human DNA and to be resent in 70%-80% of all CpG dinucleotides in the genome (Ehrlich 1982). Another report from early mouse development, revealed a dramatic decline in embryonic cells to nearly 30% of the typical somatic cell level (Monk et al. 1987, Kafri et al. 1992).
Despite the controversies, in the level of methylation in human sperm DNA according to the HPLC analysis (Table 3.1) demonstrated a fluctuation in the percentage level of 5-methyl-2′-deoxycytidine to total level of cytidine (5-methyl-2′-deoxycytidine+2′-deoxycytidine). This variation could reflect the precise role that methylation is playing during spermatogenesis in gene silencing. This diversity has been demonstrated (Table 3.1) from a minimum 0.21% to a maximum 7.47% in good quality and a minimum of 2.57% to 8.63% in poor quality sperm fractions. The variation of 5-methylcytosine in different quality sperm populations from the same donor may reflect the precise role of methylation in the control of gene expression during differentiation of the male gamete. Since the ratio of the percentage of 5-methyl-2′-deoxycytidine in low and high density sperm fractions was 1.7, it could be concluded that nearly double (1.7) the level of gene silencing was occurring in the poor quality spermatozoa compared with the high quality cells. The HPLC results therefore promote the idea that high quality sperm shows the lowest amount of DNA methylation. In contrast Monk et al (1987) reported that sperm DNA tends to be hypermethylated. To confirm our hypothesis and reject this idea we have used additional methods to assess the methylation status of human spermatozoa.

While in some references, 5-methylcytosine has been declared the fifth base of human DNA (Widschwendter. 2007), the result of human sperm DNA flowcytometry shows a wide variation in the level of this modified base (Fig 3.1). In some reports, primordial germ cells have been shown to exhibit a sharp reduction in the degree of DNA methylation (Tada et al. 1997, Reik et al. 2001). However, our findings indicate a different level of DNA methylation in every sperm donor, all evidence indicates to lower level of methylation in high density level percol fraction. To examine the factors underpinning these varying levels of DNA methylation in more detail, immunocytochemistry was indicated.

Previously, the 5-methylcytosine antibody has been used to detect DNA methylation in germ cells and spermatocytes (Baumann et al., 2008). The immunochemical images depicting the binding of this antibody to human sperm chromatin has revealed three types of spermatozoa; i) spermatozoa, usually encountered in the high quality sperm populations recovered from the high density region of Percoll gradients that exhibit little, if any, signs of DNA methylation; ii) spermatozoa from the high density Percoll
fraction that exhibit three small foci of antibody reactivity; iii) spermatozoa collected from the low-density region of Percoll gradients that frequently exhibit evidence of hypermethylation (Fig 3.9 b-f).

Moreover, in the immunoblots generated with a monoclonal antibody against methylated DNA, it has shown that the pattern of cross reactive bands differed between different sperm populations. As indicated above it concepted that may the epitope targeted by this antibody is involved in high mobile group of protein (HMG) including a methyl transfrases. As a result, it seemed possible that an antibody directed against the DNA methylation site might target methyltransferases that normally are attached to NP95 (Rottach et al., 2010). The results of the Western blot analysis did indeed indicate the presence of a number of cross reactive protein bands with molecular masses and behavior corresponding to the major forms of Dnmts. Of course, additional studies with the appropriate antibodies would be needed to ratify such speculation and confirm that mature human spermatozoa do indeed possess an array of methyltransferases that might modulate the methylation status of these cells.

It can be concluded from these studies that a major epigenetic modification, DNA methylation, arias in concert with the quality of the spermatozoa. Poor quality spermatozoa from the low-density region of Percoll gradients were found to be hypermethylated using three independent methods. Moreover, these defective cells might also be characterized by the over-expression of DNA methyltransferase. Additional studies will be needed to confirm this point.
Chapter 4

The relation between the quality of spermatozoa, apoptosis and DNA methylation?

4.1 Introduction

Two important related factors in human sperm fertility are DNA damage and apoptosis. The importance of research in these fields is in the diagnosis of male infertility, where the results might contribute to the correction of impaired reproductive function. As reviewed before, checkpoints during the cell cycle could stop the replication of damaged DNA at different stages of cell division. In addition, checkpoints could slow down the replication or increase the transcription of DNA repair genes or trigger cell death via different chemical pathways (Kastan and Bartek, 2004). Apoptotic cells are characterized by a cascade of morphological and chemical events. In this chapter, a brief analysis of apoptosis has been attempted to investigate early and late events in this pathway in human spermatozoa of differing quality through the application flow cytometry. We have detected significant relationships between human sperm characteristics, phosphatidylserine (PS) externalization, and total caspase activity, and demonstrated to what extent these events are correlated with DNA methylation.

Apoptosis is a form of programmed death cell, involving a cascade of well defined biochemical events with the ultimate purpose of eliminating damaged or surplus cells. The morphological and biochemical characteristics of affected cells changes as they progress along the apoptotic pathway to include such processes as: shrinkage, blabbing, nuclear condensation, loss of the integrity of the mitochondrial membrane potential, DNA fragmentation and finally presentation of apoptotic bodies (Wyllie et al., 1980). Translocation of phosphatidylserine (PS) from the interior of the cell to the external surface is another important early chemical sign of apoptosis. This translocation results in changes to phospholipids’ asymmetry within the lipid bi layer (Martin et al., 1995) and results in the cellular expression of high affinity towards AnnexinV, which binds to
exposed phosphatidylserine residues, making this an important landmark in the apoptotic process (Martin et al., 1995, Vermes et al., 1995).

Theoretically, the sperm plasma membrane should be very fluid due to the unusually high proportion of long chain polyunsaturated fatty acids (Flesch and Gadella, 2000). Approximately 50% of the fatty acid in a human spermatozoon is polyunsaturated (largely arachidonic and decosohexaenoic acids) and 70% of the total lipid composition of the mammalian sperm plasma membrane is on the form of phospholipids, while around 3% of the phospholipid is in the form of phosphatidylserine (Jones et al., 1979, Mann and Lutwak., 1981). A significant association between lipid peroxidation and apoptosis (Niggli and Burger., 1987, Mombers et al., 1979, 1980) has been reported. Intracellular oxidations initiate apoptosis and induce an irreversible cascade of cellular damage to proteins, carbohydrates, lipids, and DNA (Bennett, 1985, Niggli and Burger. 1987). Some researchers have reported that intracellular oxidation signaling could be the consequence rather than a cause of apoptosis because the latter is frequently associated with the enhanced generation of reactive oxygen species (ROS), particularly from the mitochondria (Simon et al., 2000). On the other hand, others have suggested that lipid peroxidation is an initiator for apoptosis in human spermatozoa (Kurose et al., 1997).

Either way, the presence of high levels of polyunsaturated fatty acid in human spermatozoa means that these cells are particularly vulnerable to oxidative stress and the initiation of an apoptotic cascade associated with mitochondria ROS generation (Koppers et al., 2008), lipid peroxidation (Aitken et al., 2007) phosphatidylserine exposure on the sperm surface (Barroso et al., 2000) and the activation of a suite of intracellular proteases known as caspases. Two family of specialized cysteine-dependent aspartate proteases known as caspases (Alnemri et.al., 1996) and including initiator and executioner caspases, control the process of apoptosis. Of 14 sequenced caspases that have been reported only 11 are of human origin. Initiator sub-families of caspases, including Caspase -2, -9 and -7 induce apoptosis via protein mediators including the death domain-containing protein, PIDD, and the adaptor protein RIP associated Ich-1/CED homologous protein with death domain ( RAIDD ) (Tinel , Tschopp, 2004). Caspases-9 and its special adaptor Apaf-1 are specific for DNA damage. Caspases are activated via extrinsic and intrinsic stimulators. Executioner
members of the caspase family, include Caspase-1, -4, -5, -11, -12, and -13 all possess a nucleophilic cys residue are cleave their substrate after an Asp residue.

The Execution phase signals the completion of both the intrinsic and extrinsic apoptotic pathways. The executioner caspases fulfill their enzymatic role by cleaving various substrates including cytokeratins, PARP, the plasma membrane and cytoskeletal elements during the active phase of apoptosis (Slee et al., 2001). The most important executioner is Caspase-3, which is activated by any of the initiator caspases (caspase-8, caspase-9, or caspase-10). Caspase-3 induces the separation of endonuclease CAD from the inhibitory complex ICAD. The released CAD then translocates to the nucleus and induces DNA fragmentation, leading ultimately to chromatin condensation, cytoskeletal reorganization and, finally, the formation of apoptotic bodies.

Because apoptosis can occur in human spermatozoa and constitute a major cause of DNA damage, it is of direct relevance to the assisted conception industry. The latter inevitably involves the attainment of conceptions in vitro that would have been impossible in vivo because of defects in the fertilizing potential of the spermatozoa or their ability to initiate and normal embryonic development. If apoptotic cells are used for assisted conception in the form of in vitro fertilization (IVF) or intra-cytoplasmic sperm injection (ICSI) then the DNA damage associated with this process would be anticipated to have major impacts on the development of the embryo and the health and wellbeing of the progeny (Aitken et al., 2009). This could be the reason that strenuous efforts are being made to identify and remove apoptotic cells from sperm suspensions that are being used for assisted conception purposes (Dirican et al., 2008).

While this represents a potential short-term solution to the problem, improving the safety of assisted conception will ultimately depend on understanding the factors that result in the formation of apoptotic spermatozoa in the first instance. In this Chapter, we examine two features of the apoptotic process, including PS externalization and total caspase activation, as markers for early and late apoptosis respectively. The expression of these markers is then compared with the methylation status of the chromatin as measured by 5-methylcytosine expression, in order to determine whether the tendency of spermatozoa to undergo apoptosis is correlated with the quality of chromatin remodeling during spermatogenesis.
Chapter 4: Apoptosis and Quality of Sperms

4.2 Results

4.2.1 Flowcytometry of Annexin V/PI expression in Different Sperm fractions.

In this part of the study, apoptosis was the focus of attention. To achieve this aim, we have used flow cytometry to monitor: (i) Annexin-V binding to phosphatidylserine at the cell surface, as an early marker of apoptosis, and (ii) propidium iodide (PI) staining in order to monitor cell viability. These assessments were conducted on good and poor quality spermatozoa isolated from the high- and low- density regions of Percoll gradients respectively.

The method of double staining with propidium iodide and Annexin-V allowed us to analyze changes in apoptosis and cell viability within 3 hours of ejaculation. One million spermatozoa isolated from the different regions of Percoll gradients were prepared as described in Section 2.2 and incubated with 5µL / ml of a 35 kDa Ca\(^{2+}\)-dependent phospholipid-binding protein that binds to phosphatidylserine (PS), Annexin V01human recombinant FITC. Three types of sample were prepared: unstained sample, stained with PI (2.5 µL of 0.1 mM PI in 500 µL PBS,) and double stained with Annexin-PI. Samples were analyzed after 15 minutes incubation with Annexin-V in a FACS calibur\(^{TM}\) flow cytometer (Becton-Dickinson).

The total number of counted events in every sample was 10,000. Forward-angle light scatter and side-angle light scatter filters were applied to exclude electronic noise and debris. The lower left quadrant contained viable unstained cells, the lower right quadrant presented early apoptotic cells labeled with recombinant human Annexin-V, and the upper right quadrant represented necrotic cells, labeled with Annexin-V plus PI, while the upper left quadrant is demonstrated necrotic sperm stained with PI alone. The Annexin-V signal was measured in the FL1 channel and the PI-stained spermatozoa were analyzed in FL2 channel of the flow cytometer, as illustrated in Figure 4.1. The statistical significance of the results was determined using the SPSS statistical package (Table 4.1) and the graphs (Figure 4.3) were analyzed, using Graph pad software.
Chapter 4: Apoptosis and Quality of Sperms

Figure 4.1 A comparison between the level of Annexin-V and Annexin-PI expression in different fractions of human sperm.

Following incubation of 1 million human sperm with 5μL Annexin-V recombinant conjugated antibody per 500 μL PBS for 15 min after Percoll preparation samples were analyzed using a FACS calibur™ flow cytometer. The result were analyzed by cell Quest software. The typical graph of result exhibited in quadrant that unstained cells appeared in the lower left quadrant; cells that are alive and stained with Annexin-V are in the lower right quadrant and the cell that are dead because of apoptosis are labeled with Annexin-V and PI with both probes, are present in the top right quadrant. The top left panel of Fig (a) demonstrates that 8.39% of the spermatozoa were Annexin-V positive in 100% sperm fractions whereas the top right panel of this Figure (b) reveals a value of 19.44% for poor quality spermatozoa from the same donor recovered in the 50% Percoll fraction. The panel (c) demonstrates that 5.21% of Annexin-V positive cells were dead in the 100% Percoll fraction whereas the corresponding figure for the 50% fraction demonstrated 9.13% dead cell (d).
Chapter 4: Apoptosis and Quality of Sperms

A)

Figure 4.2 Time dependent presentation of phospholipid asymmetry in two different populations of human spermatozoa separated on discontinuous Percoll gradients.

The above graph in panel (A) shows the time-dependent progression of apoptosis in human spermatozoa. Thus beginning at 6 h post incubation, a shift in the population of spermatozoa that are Annexin V positive but still retain their vitality is clearly evident. By 9 h this group of apoptotic cells has become a discrete subpopulation, clearly separated from the main body of unstained cells. After 12 h incubation, a majority of the cells are in the early stages of apoptosis. However, it is also clear that at this time point a significant number of Annexin V positive cells are dead and staining positively with PI. This loss of vitality became even more evident after 16 h incubation and after 24 h, a majority of the cells examined were Annexin V positive but had lost their vitality.
The lower panels (B) compare the greater tendency of spermatozoa from the low density Percoll fraction (50%) to enter the fourth quarter (necrotic death) compared with those from the high density regions of these gradients. In the particular example given, 65% of cells from the high-density region of the Percoll gradient had lost vitality but still remained in third quarter whereas, in the low density sample only 35% of samples were PI and Annexin-V positive, and most of them have left the quarter three to stage four. It demonstrates the low-density gradient have a higher tendency to promote the apoptosis to necrotic death.

Table 4.1 The expression level of Annexin-V and Annexin-PI in different gradients of human sperm.

<table>
<thead>
<tr>
<th>Sperm quality/</th>
<th>Number</th>
<th>Mean ± SEM</th>
<th>Max</th>
<th>Min</th>
<th>Median</th>
<th>Std</th>
</tr>
</thead>
<tbody>
<tr>
<td>100% An</td>
<td>20</td>
<td>5.389 ± 1.152</td>
<td>18.96</td>
<td>0.1700</td>
<td>3.520</td>
<td>5.154</td>
</tr>
<tr>
<td>50% An</td>
<td>17</td>
<td>9.228 ± 1.765</td>
<td>25.35</td>
<td>1.180</td>
<td>6.810</td>
<td>7.3</td>
</tr>
<tr>
<td>100% An-PI</td>
<td>16</td>
<td>5.17 ± 1</td>
<td>14</td>
<td>0.88</td>
<td>3.85</td>
<td>4.1</td>
</tr>
<tr>
<td>50% An-PI</td>
<td>16</td>
<td>7.85 ± 1.5</td>
<td>20.8</td>
<td>0.43</td>
<td>6.8</td>
<td>5.9</td>
</tr>
</tbody>
</table>
Figure 4.3. Comparison between the phospholipid externalizations (Annexin V expression) in different fractions of human sperm.

Histograms illustrate the mean ± SEM for Annexin V (Ann) and Annexin V +PI (An-PI) for sperm populations recovered from the high and low density Percoll gradients. These fractions gave Annexin V expression levels of 5.389 ± 1.152% for the high density Percoll fraction (n=20) and 9.228 ± 1.765 (n=17) for the low density fraction respectively the difference was significant (P < 0.05). Similarly, the mean ± SEM for the Annexin V +PI results were 5.17 ± 1 (n=16) and 7.85 ± 1.5 (n=16)( P<0.0001) from the poor and good quality respectively. All the samples assessed by the two tailed paired t test. The graph shows in both fractions the level of Annexin-V expression is significant and rejects the null hypothesis strongly. The absolute levels of apoptosis experienced by these two populations (AnnexinV+ and AnnexinV-PI+) are higher in spermatozoa from the low-density Percoll region significantly than the high-density fractions.
Figure 4.4. Linear correlation between the phospholipid externalization (Annexin-V binding) in different fractions of human sperm.

Scattergram shows the expression of AnnexinV in different populations of human spermatozoa recovered from the low and high density regions of Percoll gradients using flow cytometry. A total of 17 paired high (G) and low (B) density Percoll fractions were used in this determination. The results of this analysis demonstrated that the levels of Annexin V expression in the high and low regions of Percoll gradients were highly correlated with a correlation coefficient of $r = 0.75; \, P < 0.01$. This result suggests that the tendency of these sperm populations to become apoptotic in varies samples and is a property shared by all of the spermatozoa in an ejaculate irrespective of which fraction they came from.
Figure 4.5. Linear correlation between the levels of (AnnexinV-PI expression) in different gradients of human sperm.

Scattergram shows the expression of AnnexinV-PI in different populations of human spermatozoa recovered from the low and high-density regions of Percoll gradients using flow cytometry. A total of 15 paired (high (G) and low (B) density Percoll fractions) were used in this analysis quality. The results of this analysis demonstrate that the levels of AnnexinV-PI expression in the high and low regions of Percoll gradients are highly correlated at \( r = 0.75; \ P < 0.004 \). This result suggests that the tendency of these sperm populations to become exhibit cells death in association with Annexin V positivity is a property shared by all of the spermatozoa in an ejaculate irrespective of which fraction they came from.
Figure 4.6. Relationship between the expression level of Annexin-V (phospholipid externalization) in high-density fraction of percoll gradient and volume of ejaculated human semen.

The above scattergram illustrates the relationship between AnnexinV expressions with the volume of ejaculated human in 11 high-density gradients of semen samples. The level of AnnexinV expression did not demonstrate any statistical relationship with the volume of ejaculated semen.
Figure 4.7 The correlation between the expression level of Annexin-V (phospholipid externalization) and viability of human sperm.

As revealed in the left hand Fig. 4.7, the results of this analysis indicated an extremely powerful inverse relationship between AnnexinV+ expression and cell vitality in good quality spermatozoa recovered from the high density region of the Percoll gradients (r = 0.81; n= 15; **p< 0.01 (2-tailed) pearson test). An equally strong relationship was encountered in the low-density Percoll fractions as indicated in right hand Figure (r = 0.80; n= 16; P<0.01). The results of these analyses are consistent in demonstrating that apoptosis is the major mechanism by which spermatozoa lose their vitality. It may not be the only mechanism however because the proportion of non–viable cells at any point in time always exceeded the percentage that were Annexin V positive for both the high and low density Percoll fractions (P < 0.0001: Wilcoxon signed rank test).
Figure 4.8. The correlation between the percentage level of Annexin-V in human sperm and motility.

After Percoll preparation two different fractions of spermatozoa of differing quality were collected from the high and low-density regions of Percoll gradients and incubated with Annexin-V and PI. The samples were then analysed by flowcytometry. The linear regression analysis reveals a significant negative correlation between sperm motility and Annexin-V expression in good quality spermatozoa recovered from the high-density region of Percoll gradients ($r = 0.76; n = 11; P<0.01$). Thus, within this population, apoptosis appears to be a major reason for motility loss.
4.2.2 Flowcytometry of Annexin-V and 5-methylcytosine labelled sperm cell.

In this section of the study we examined the expression of Annexin- V, as an early marker for apoptosis, and the level of 5-methylcytosine to examine the hypothesis that the tendency of spermatozoa to undergo apoptosis and externalize phosphatidylserine would be correlated with the methylation status of these cells. For this purpose, after spermatozoa had been recovered from the high- and low- density regions of discontinuous Percoll gradients they were divided into 2 aliquots: one million spermatozoa were incubated with recombinant human Annexin-V0 while 10 million cells were assessed for 5-methylcytosine content using the flow cytometry protocols (Section 2.8).

Flow cytometry was used in these experiments and the results expressed as a percentage of the cell population after analyzing 10000 events. In these experiments, the time point at which the cells were analyzed for Annexin-V binding is important to define precisely because this is a dynamic process, which changes as spermatozoa capacitate and then become senescent as illustrated in (Fig 4. 1,2). We used the cells without fixation at the 3 to 4 hour point time after incubation. The results obtained from the two human sperm fractions using flow cytometry were analyzed employing the SPSS the software package and the results are presented in Figure (4.4-8).
Figure 4.9. Presentation of linear correlation of Annexin-V and DNA methylation in human sperm.

After Percoll separation the two different fractions of spermatozoa were collected; one group was incubated with Annexin-V and PI while the second was labelled with a 5-methylcytosine antibody. The 100% and 50% Percol fractions were analysed by flow cytometry and the results integrated in a single graph. In order to test for correlations linear regression analysis was used while the Wilcoxon signed-rank test was employed to determine whether there were significant differences between groups. This scattergram presents a highly significant relationship between AnnexinV binding and 5-methylcytosine expression in human spermatozoa (r = 0.79; n = 17; P < 0.01). Thus, the higher percentage of cells expressing 5-methylcytosine in the higher percentage binding Annexin-V. These results suggest that hypermethylation is a feature of defective sperm populations that have a high tendency to undergo apoptosis. This relationship is unlikely to be direct because the proportion of cells expressing high levels of DNA methylation far exceeded the percentage that was Annexin-V positive (P < 0.0001; Wicoxon sign rank test). It is more likely that hypermethylation and a tendency to undergo apoptosis are both reflections of some fundamental defect in the underlying process of spermiogenesis.
4.2.3 Flowcytometry of caspases

In order to further explore the capacity of human spermatozoa to undergo apoptosis we have used flow cytometry to monitor the presence of activated caspases using the FLICA stain as a pan-caspase marker. For this purpose, samples were prepared on discontinuous Percoll gradients and then stained with a red FLICA kit as described in the Materials and Methods (Section 2.9). The kit contained either fluorescent-labeled VAD, a sequence of amino acids targeted by all caspases, or DEVD, targeted by caspase-3. These probes enter the spermatozoa and covalently bind with the heterodimer of reactive caspase at cysteine resides and in the process, suppress any further activity of the enzyme.

After one-hour incubation with the FLICA probe the samples (and a heat-shocked positive control) were, pelleted, washed to remove excess fluorescent probe and analysed on a FACS calibur™ (Becton Dickinson) flow cytometer. The Forward Scatter (FSC) was set such that caspase activation would only be measured in spermatozoa and not any other contaminating cell type. In our experiments, higher levels of caspase expression was detected in poor quality spermatozoa recovered from the low density region of Percoll gradients as long as the samples were analysed with 3 hours of incubation and not later.

Typical histograms of the experiment are illustrated in Figure 4.10 in which the top row describes spermatozoa recovered from the high density region of Percoll gradients, comprising an unlabeled negative control, caspase labeled spermatozoa and a heat shocked positive control. The second row presents the same data for spermatozoa recovered from the low-density region of Percoll gradients. The FLICA stained samples in 100% and 50% fractions were visually compared and the results statistically presented in the Table 4.2. The latter shows that the mean ± SEM level of caspase activation in the good quality, high-density Percoll fractions (29.95± 5.770%; n = 9) was significantly lower ($P < 0.001$) from the values recorded for the poorer quality, low-density Percoll fractions (52.99± 6.628%; n = 10).
Spermatozoa were fractionated into high and low density Percoll fractions (Section 2.2.) and incubated with the red FLICA kit for one hour. This probe is an inhibitor that covalently binds to reactive cysteine residues in activated caspases and further inhibits their activity. After two washes to remove excess probe the samples were analysed by FACS (Becton Dickinson) after 3 hours of incubation with argon laser excitation (Enterprise II, Coherent, Santa Clara, CA) emitting 60 mW at 488 nm; the emission was recorded through specific band pass filters: 530-630 nm for FITC (FL2). The frequency distribution of caspase labeled cells is presented in high density Percoll fraction in the top row, low density Percoll fraction in bottom row. The first histogram in top and low (a & c) demonstrated unlabelled negative controls, FLICA stained cells in the middle showed the lower level of caspases in high density percoll fraction (b) than low density (d) and the last photos in both rows are heat shocked positive controls.
Table 4.2 The statistical characteristic of Caspase expression in two different gradients of human sperm

<table>
<thead>
<tr>
<th>Sperm quality/number</th>
<th>number</th>
<th>Mean ± SEM</th>
<th>Max</th>
<th>Min</th>
<th>Median</th>
<th>Std</th>
</tr>
</thead>
<tbody>
<tr>
<td>100%</td>
<td>9</td>
<td>29.95 ± 5.8</td>
<td>67.54</td>
<td>3.750</td>
<td>26.96</td>
<td>17.31</td>
</tr>
<tr>
<td>50%</td>
<td>10</td>
<td>52.99 ± 6.6</td>
<td>97.00</td>
<td>21.24</td>
<td>55.50</td>
<td>20.96</td>
</tr>
</tbody>
</table>

Figure 4.11. The comparison of mean and median of total caspases expression in different fractions of human sperm.

*Box plots show the level of caspase expression in high density Percoll fractions (blue) compared with low density fractions (red). In this graph the maximum, minimum and median of the two groups of samples are illustrated. A significant difference between these two fractions has been shown with $P<0.0001$ calculated by one sample two tailed $t$ test and $P<0.05$ by one sample Kolmogrov Smirnov test. As expected, the mean level of caspase activation in poor quality spermatozoa is higher than that seen in high density cells.*
4.2.4 Flowcytometry of Annexin-V, FLICA Caspases and 5-Methyl cytosine labelled sperm cell.

In this section, we compare the markers of apoptosis employed in this study, Annexin-V expression, and caspase activation. For this purpose after preparing sperm cells on Percoll gradients the individual fractions were divided to three parts, one million cells for incubation with recombinant human Annexin V FITC conjugate another one million cells for incubation with the red FLICA caspase probe (Section 2.8.3) and 10 million for incubation with the 5-methylcytosine antibody as previously discussed.

Flow cytometry was applied for the three assays and expressed as the percentage of spermatozoa after analyzing 10000 events. In this experiment the time selected for analyzing Annexin-V binding and caspase activation by flowcytometry is very important because, as mentioned before, Annexin-V and caspases are both dynamic criteria that are continuously changing as the spermatozoa are incubated in vitro. We used the fresh cells without fixing them and analyzed results from the two Percoll fractions by flow cytometry before running the 5-methylcytosine labeled samples. The time point selected for these analyses was after 3 hours incubation; samples incubated with Annexin-V were run first and the 5-methyl experiments were run last. The results were analyzed using the SPSS software program and are presented graphically in Figure (4.11-12).
Figure 4.12 Annexin-V and caspase activation in different fractions of human sperm.

Scattergram shows the results of the analysis the relationship between the levels of caspases activation and the levels of Annexin V binding observed in sperm from both populations, high and low density gradients percol fractions ($r = 0.59$; $n = 9$; $P < 0.09$). Although $p$ value is not significant further examination may show a direct tendency of both caspases and Annexin-V.
Figure 4.13. Relationship between the caspases expression and 5-methylcytosine level in human sperm.

Only a limited number of samples were analysed for caspase activation and DNA methylation. What data there is suggests the same linear correlation between DNA methylation and caspase activation as we saw for Annexin V binding ($r = 0.64; n = 6; P$ value was not significant). That is, although poor quality cells with a high tendency to undergo apoptosis tended to exhibit high levels of methylation this relationship was not significant. Moreover, there was no significant difference between the mean percentage of caspase positive cells and the percentage exhibiting high levels of methylation.
4.5 Discussion

During the process of spermatogenesis developmental defects may occur in both the cytoplasmic or nuclear compartments of the germ line that result in the release of immature spermatozoa from the germinal epithelium (de Kretser et al., 1998; Huszar et al., 2000). One of the mechanisms by which germ cell development might become disrupted is through the induction of apoptosis. Proteins, including enzymes such as caspases, regulate the process of apoptosis. Immature, ejaculated spermatozoa show a proportionally higher level of apoptotic signs, including caspases expression (Sevil et al., 2004), suggesting that these cells are particularly unstable. Apoptosis can occur at any stage of spermatogenesis however, after meiosis the germ cells progressively lose their capacity to become eliminated via this mechanism. In this section of our research program we hypothesized that the level of phosphatidylserine asymmetry in low density sperm suspensions would be higher than observed in normal cells recovered from the high density region of Percoll gradients. Furthermore, we postulated a relationship between apoptosis as reflected in high rates of phosphatidylserine exposure and caspase activation and abnormally high levels of DNA methylation.

As expected, our results revealed high levels of phosphatidylserine exposure (AnnexinV–positive cells) in defective cells, reflecting the spontaneous tendency of these cells to undergo apoptosis on incubation. In addition, within individual samples, we observed a positive correlation between apoptosis in the low and high density Percoll fractions \((r = 0.75; P< 0.01)\), suggesting that the level of apoptosis is not just by the fractionation of sperm subpopulations on Percoll gradients but also by the overall quality of the semen sample (Fig 4.5). Thus, defects in the processes of sperm production or maturation will set the relative level of apoptosis observed in a sperm population, irrespective of which Percoll fraction is analyzed.

Whatever the factors are, that set the overall level of apoptosis observed in human sperm populations they are not reflected in the original volume of the semen sample (Fig 4.6). However we did find an extremely powerful inverse correlation between Annexin-V binding and viability of sperm in both high density left Figure 4.7 \((r = 0.81; n= 15; **p< 0.01)\) and low density Percoll fractions right Figure 4.7 \((r = 0.80; n= 16; p<0.01)\). Similarly an inverse correlation was observed between sperm motility in the
100% fraction (Fig 4.8: \( r = 0.76; n = 11; p<0.01 \)) as also found by Barrosso et al (2000), Shen et al (2002). In contrast, we could not find a significant correlation between motility and apoptosis in the 50% fractions; possibly because in these suspensions many spermatozoa are immotile for reasons that are independent of apoptosis but reflect other structural/biochemical abnormalities in these highly abnormal cells.

If the high level of Annexin-V binding seen in defective sperm preparations was indicative of abnormalities occurring during sperm differentiation in the testes then we might see a correlation between this apoptotic marker and DNA methylation – which is dynamically regulated during spermatogenesis. In focusing upon the relationship between the levels of 5-methylcytosine and Annexin-V binding in human spermatozoa, we examined both high and low density Percoll fractions for this purpose. The results (Fig 4.9) show a highly significant relationship between Annexin-V binding and 5-methylcytosine expression in human spermatozoa \( (r =0.79; n =17; *p<0.01) \). The scattergram demonstrates just how powerful a correlation was observed between AnnexinV and 5-methylcytosine in human spermatozoa.

This report indicates that membrane changes associated with apoptosis and occurring in the first hours after ejaculation are correlated with the level of DNA methylation occurring during the differentiation of the germ line. These findings contradict a recent analysis by Tunc and Tremellen (2009) who found a significant negative correlation between sperm DNA methylation and sperm DNA fragmentation. However in our case immunocytochemistry with a 5-methylcytosine antibody also indicated high levels of DNA methylation in poor quality spermatozoa isolated from the low density regions of Percoll gradients (Fig. 3.10). We hope that the combined force of these assays will allow a more profound understanding of the relation between DNA methylation and apoptosis induced during the human sperm development.

To populations distinguish the process of late apoptosis we have compared the evidence of caspase catalytic activity in high and low density sperm. The pan-caspase substrate is a sensitive marker for caspase activation (Parvathenani et al., 1998, Morris et al., 2002). The results presented in Fig 4.10 shows variable caspases expression amongst the different populations of human spermatozoa with higher expression of active caspases in poor quality spermatozoa. As the data in Table 4.2 shows, the mean± SEM (9.96±
5.55%) level of caspase expression in the high density sperm populations is significantly lower than the value recorded (52.99 ± 6.63%) for poor quality spermatozoa recovered from the low density Percoll fraction (P<0.0001). The previous report from Weng et al., (2002) is supportive of these data.

Given the above results, we would naturally have anticipated positive correlations between Annexin-V binding to human spermatozoa and caspase activation as well as caspase activation and methylcytosine expression. The results of these analyses are illustrated in Figs 4.12 and 4.13. Both scattergrams are suggestive of the positive correlations anticipated, however the regression analyses were not statistically significant because of the small value for ‘n’. If time had permitted these analyses would have been replicated several more times in order to firmly establish their statistical significance.

We conclude that during the process of spermatogenesis defective spermatozoa may be produced that have an enhanced capacity to undergo the process of apoptosis. The entry of these defective cells is indicated by the high levels of Annexin-V binding and caspase activation seen in poor sperm populations. The suggestion that these cells are defective because of developmental errors is clearly suggested by the high levels of methylcytosine expression seen in these cells. Clearly, more samples need to be analyzed to confirm some part of our findings. However the high level of correlation seen between defective DNA methylation and apoptosis in this study is strongly suggestive of a developmental origin to defective sperm quality. What perturbs the pattern of DNA methylation is now the key question that needs to be addressed.
Chapter 5

Chromatin remodelling in human spermatozoa.

5.1 Introduction

The previous sections of this study investigated the most important signs of apoptosis and demonstrated that this phenomenon is correlated with evidence of hypermethylation of the sperm chromatin. In this part, we have examined the efficacy of chromatin packaging in human spermatozoa using the chromomycin A3 (CMA3) fluorochrome in order to generate further evidence that the tendency if spermatozoa to revert to an apoptotic state is associated with errors of spermiogenesis resulting in defective chromatin remodelling.

As it reviewed in the Introduction, during the process of spermiogenesis, mammalian chromatin experiences remodelling, in that nuclear histones are replaced by protamines (Bouvier., 1977, Goldberg et al., 1977, Courtens and Loir 1981, Balhorn., 1982). This process results in the DNA in mature human spermatozoa being sixfold more condensed than in somatic cell chromosomes (Pogany 1981, Pienta et al., 1984, Ward and Coffey 1991). In mammals, the molecular mass of protamine is nearly half that of histones. Protamines are rich in arginine residues, that give these proteins a net positive charge allowing then to integrate easily into the negatively changed DNA, and cysteine residues that take part in inter- and intra- molecular disulphide bridge formation further stabilizing the chromatin of mature spermatozoa (Calvin et al. 1971, Bedford et al., 1973, Marushige et al., 1974).

Two kinds of protamine have been recognised in human sperm nuclei, known as P1 and P2 (P1/P2), which are very similar to each other and exist in a ratio of approximately 1:1 (Balhorn et al., 1988, de Yebra et al.,1993, Bench et al., 1996, Corzett et al., 2002,
Mengual et al., 2003a, Aoki et al., 2005a). The function of the two different protamines may be different. All vertebrates possess the protamine P1 (McKay et al., 1985, 1986, Gusse et al., 1986, Balhorn et al., 1987, Bellvé et al., 1988, Oliva and Dixon, 1991a, Chauvière et al., 1992, Yoshii et al., 2005). However, protamine P2 has been recognised only in a limited number of mammals including human (Balhorn et al., 1977, 1987, McKay et al., 1985, 1986, Gusse et al., 1986, Belaiche et al., 1987, Bower et al., 1987, Bellvé et al., 1988, Oliva and Dixon, 1991a, Yoshii et al., 2005).

As indicated above, the protamines are rich in arginine residues (48% in human) that give the molecule a positive charge. These positively charged residues form a highly compacted structure with the negatively charged DNA (Oliva and Dixon, 1990, 1991a, Retief et al., 1993, Oliva, 1995, Queralt et al., 1995, Lewis et al., 2003a). Furthermore, protamines of Eutherian, but not most Metatherian, mammals include cysteines (Cys) amino acid in their structures that generate strong disulphide bonds between protamines molecules to produce a highly condensed material (Saowaros and Panyim, 1979, Balhorn et al., 1992, Lewis et al., 2003a, Vilfan et al., 2004).

During the remodelling of the sperm chromatin spermiogenesis there is a transitional phase in final stages of spermatogenesis, when histones are replaced by transition proteins (TNPs) that are, in turn, replaced by protamines, in a carefully orchestrated sequence (Mezquita, 1985, Poccia, 1986, Oliva and Dixon, 1991a, Hecht, 1993, Green et al., 1994, Dadoune, 1995, Grootegoed et al., 2000, Meistrich et al., 2003, Kierszenbaum and Tres, 2004, Rousseaux et al., 2005). Protamination occurs to 85% of the sperm DNA with the remaining 15% retaining its nucleosomal structure with associated histones and other proteins (Tanphaichitr et al., 1978, Ammer et al., 1986, Gusse et al., 1986, Gatewood et al., 1987, 1990, de Yebraet al., 1993, Zalensky et al., 2002). Protamines attach to the minor grooves of DNA and in this way block the binding of other reagents, such as the fluorochrome, chromomycin A(CMA3), that also possess an affinity for the minor groove of sperm DNA (Bianchi et al., 1993).

Thus according to the literature, CMA3 and protamine may both compete for the same binding site on DNA (Suau et al., 1977, Berman et al., 1985, Gao et al., 1989). As a consequence, if spermatozoa are exposed to CMA3 them the latter will bind to the
sperm nucleus in a manner that is inversely correlated with the level of protamination (Bianchi et al., 1993).

CMA3 is an anticancer antibiotic glycoside isolated from the bacterium Streptomyces griseous that reversibly binds to guanine-cytosine (G-C) base pairs of DNA. CMA3 inhibits cellular processes like transcription and replication, by binding reversibly with Cytosine –Guanine through the minor groove of double-stranded DNA, in the presence of metal ions like Mg2+. CMA3 and protamine are both in competition to bind to C-G rich regions with the result CMA3 fluorescence is enhanced when the protamination of sperm DNA is defective (Bizzaro et al., 1998). Using this experimental approach, increased protamine deficiency has been detected in the spermatozoa of infertile men (Lolis et al., 1996, Franken et al., 1999, Razavi et al., 2003, Nasr-Esfahani et al., 2004a,b, 2005). Also Nasr-Esfahani et al., 2004a, 2005 have found the correlations between CMA3 staining in spermatozoa and the success of ART.

In this study, we have attempted to assess the level of CMA3 fluorescence in sperm populations of differing quality, taken from different regions of discontinuous Percoll gradients and then looked for correlations with DNA methylation and other apoptotic parameters. In this way, we hoped to examine the hypothesis that defective chromatin remodelling creates a state of vulnerability in the spermatozoa that drives the latter into an apoptotic state.

5.2 Result

5.2.1 Fluorescent microscopy of Chromomycin labelled sperm cells.

In this section, the level of chromatin compaction in immature sperms has been compared in good and bad quality of human spermatozoa recovered from the high and low-density regions of discontinuous Percoll gradients respectively. After Percoll preparation two different fractions were collected (2.2). Specimens stained with CMA3 (2.9.3) and were visualized under a Zeiss Filterset 09. III (Zeiss, Oberkochen, Germany. Green fluorescences was detected using (480 nm) filters. Figure 5.1 presents some typical examples of the microscopic images obtained while the results of 15
paired comparisons were analyzed by SPSS (Table 5.1) after calculation of the percentage of CMA3 stained spermatozoa.

We compared the mean expression of CMA3 fluorescence in spermatozoa recovered from the high and low-density regions of Percoll gradients and the results are illustrated in figure 5.1. At a glance, the histogram shows a higher mean ± SEM 19.20±2.341 expression of CMA3 binding in poor quality spermatozoa recovered from the low density region of Percoll gradients compared with the high density sperm populations from the same donors (mean ± SEM 4.467± 0.9752) as presented in Table 5.1. The data were analysed with the Shapiro-Wilk test, which revealed that the data were not normally distributed; therefore, a non-parametric test (the two-tailed Wilcoxon Signed Rank test) was applied to compare the two sperm populations. The obtained P value (two tailed) of $P < 0.0001$ allowed the null hypothesis concerning the levels of CMA3 fluorescence in the two populations of spermatozoa to be rejected. This means that the efficiency of chromatin protamination was significantly impaired in populations of poor quality spermatozoa recovered from the low-density region of Percoll gradients compared with their high-density counterparts.
Figure 5.1. Chromomycin CMA3 staining in two different population of human sperm.

Two different populations of spermatozoa were prepared on Percoll gradients (section 2.2.) and slides stained with CMA3 fluorochrome (section 2.9.3). They were then analyzed by fluorescent microscopy; green fluorescent emitting 60 mW at 488 nm. The number of bright cells (CMA3 stained) has been expressed as a percentage and compared in the two populations. Three samples in pairs gradients, high-density factions (100%) are on the left side while poor quality, low-density (50%) cells are presented on the right hand side. Poor fraction of samples has demonstrated higher percentage of CMA positive sperms.
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Table 5.1 The expression level of CMA3 in different gradients of human sperm.

<table>
<thead>
<tr>
<th>Sperm quality</th>
<th>number</th>
<th>Mean± SEM</th>
<th>Max</th>
<th>Min</th>
<th>Median</th>
<th>Std deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>100%</td>
<td>15</td>
<td>4.467± 0.975</td>
<td>15</td>
<td>1</td>
<td>3.520</td>
<td>3.777</td>
</tr>
<tr>
<td>50%</td>
<td>15</td>
<td>19.20± 2.341</td>
<td>40</td>
<td>4</td>
<td>20</td>
<td>9.065</td>
</tr>
</tbody>
</table>

Figure 5.2. Chromatin, stained with ChromomycinA3 (CMA3 in human sperm.

Two different populations of human spermatozoa were prepared (section 2.2.), stained with the CMA3 fluorochrome (section 2.3.9) and analyzed by green fluorescent confocal microscopy. As it demonstrated in photos the chromatids are established a regular special shape through the head of sperm. These photos of the chromatids alignment and other unpublished photos have induced the idea that similarity of human sperm DNA to the idea in somatic cells in Zebra fish previously published. As well does not support the idea of toroid chromatin in human spermatozoa. Bad quality fractions are the cells with more corporate dye in bright presentation. We could find any contracted chromatin neither in poor nor in good quality of sperm.
Figure 5.3. The mean comparison between the CMA3 presentations in different gradients of human sperm.

Box plots showing the mean ± SEM level of CMA expression in populations of spermatozoa recovered from the high and low density regions of Percoll gradients respectively. Data were not normally distributed according to the Shapiro-Wilk normality test therefore the two-tailed Wilcoxon Signed Rank Test was applied to compare two related parameters. The P value (two tailed) of $P < 0.0001$ clearly indicates that there is a significant difference in the levels of CMA3 fluorescence detected in these two populations.
5.2.2 A comparison between the percentage of ChromomicinA3 and 5-methylcytosine antibody labelled sperm cell in human.

We tried to compare the level of CMA3 staining and level of methylation in human sperm DNA of the same donors in independent ejaculates. After Percoll preparation two different fractions were collected (2.2.). Every individual sample was divided into two aliquots, specimens for detection of chromatin compaction, stained with CMA3 (2.9.3) and processed as explained in 5.2.1 and samples for 5-methylcytosine assessment were incubated with the relevant antibody (2.8.1) were measured by flowcytometry.

The results of two experiments (CMA3 staining & 5-methyl cytosine labelling) from the same donors were analysed and illustrated (Figure 5.4, 5). The Scattergram shows the relationship between of protamination efficiency (CMA3) and level of DNA methylation in 9 fractions of 5 semen donors. To compare the overall levels of protamination and methylation in the same donors we analysed the data by SPSS (r = 0.81; n= 9; *p< 0.05) it has demonstrated the strong direct relation between the level of methylation and stained cells with ChromomycinA3.
Figure 5.4. Correlation between the level of protamination deficiency (CMA3) and DNA methylation (5methylcytosine antibody) in human sperm DNA.

Scattergram showing the relationship between protamination efficiency and level of DNA methylation in 4 semen donors. The level of methylcytosine expression is indicated on the y-axis while chromomycin staining is represented on the x-axis. The samples were collected after Percoll wash, every gradient divided to two aliquots and stained separately with CMA3 and 5-methylcytosine antibody and sequencely were analyzed by fluorescent microscope and flowcytometer. To compare these two characteristics in the same donor we used the Related Wilcoxon signed-rank test. Our obtained p value is significant at the level of 0.008 and linear integrity as it demonstrated r=0.81 which means that both parameters show related direct increase in the same human sperm donors. SPSS analysis of data (r = 0.81; n= 9; *p < 0.05)
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Figure 5.5  Comparison between the stained 5-methylcytosine and labeled ChromomycinA3 (CMA3) in different fractions of human sperm.

The four left samples demonstrate the percentage level of 5- methyl cytosine, and CMA3 stained cell in good quality and the right side four samples with the same number presented the same values in the poor quality of sperm. The percentage of samples in these two graphs were analyzed by flowcytometer except CMA3 stained cells which analyzed by fluorescent microscopy.

5.2.3 The correlation between the level of protamin deficiency (CMA3+) and the signs of apoptosis in human sperm DNA.

In this section we have attempted to demonstrate the relationship between the efficiency of chromatin protamination and signs of apoptosis in human sperm. The data gathered by flowcytometry focused on annexing-V expression and caspase activation and the results were compared with the outcome of of CMA3 staining analysed by fluorescent microscopy. The scattergram shows the relationship between the efficiency of chromatin protamination and the level of AnnexinV expression in the same semen donors the analysed data by SPSS ($r = 0.66; n= 13; p <0.05$) has demonstrated a strong direct relation between the level of AnnexinV expression and CMA3 positivity.
We also tried the level of CMA3 staining and caspases expression in human sperm cells (Fig 5.7) the collected data were analysed by SPSS \((r = 0.56; n= 11; p \text{ not significant})\). An increase in the size of the sample population may be needed to generate definitive conclusions in this respect.

**Figure 5.6.** Correlation between the level of protamine deficiency (CMA3) and apoptosis (Annexin-V expression) in human sperm DNA.

Scattergram show the relationship between protamination efficiency and level of AnnexinV expression in the same semen donors. Semen samples were prepared on Percoll and incubated with AnnexinV and CMA3 in separate aliquots section (2.2.) and were incubated with AnnexinV (2.9.2) and Chromomycin A (2.9.3) analysed by flowcytometry and fluorescent microscopy respectively the data analysed by SPSS \((r = 0.66; n= 13; p <0.05)\) has demonstrated a strong direct relation between the level of AnnexinV expression and CMA3 positivity.
Figure 5.7. Correlation between the level of protamination (CMA3) and caspases activation in human sperm DNA.

Scattergram shows the relationship between protamination efficiency and level of caspase activation in the same donors after the samples were prepared on Percoll (except one sample that has just 50% fraction all have been from low and high density percoll fractions) and incubated with CMA3 and red FLICA dye in separate aliquots, were subsequently analysed by fluorescent microscopy and flowcytometry. The data were analysed by SPSS software ($r = 0.56; n = 11; p$ not significant).
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Figure 5.8. Comparison between the percentage level of Annexin-V binding, Caspases activation, 5-methylcytosine and Chromomycin A3 (CMA3) in high and low level density percoll fractions from the same donors.

The three left demonstrated bar charts indicate to the percentage level of Annexin-V, 5-methyl cytosine, Caspase and CMA3 expression in high density percoll fraction while the three right hand groups of bar charts in right side has presented the same values in the low density percoll fraction of human sperm (missing CMA3 data for low density percoll fraction of sample 470).

5.3 Discussion

One of the most important epigenetic events during spermatogenesis is the chromatin-remodelling that occurs during spermiogenesis and leads to extensive protamination and stabilization of the sperm chromatin. The relationship between the success in ART and the ratio or level of protamine in the sperm head is well established (Nasr-Esfahani et al., 2005). As reviewed in the first chapter chromatin compaction protects the genetic information contained within the haploid sperm nucleus from physical and chemical
damage. The efficiency of protamination can be indirectly assessed using CMA3 staining. Therefore, this method could be applied to measure protamine deficiencies in sperm populations prior to ART. A particular subject of interest in this section has been the relationship between two criteria of chromatin quality, the degree of DNA methylation and the efficiency of chromatin remodelling using the CMA3 fluorochrome.

The most popular hypotheses for the function of protamines is protection of the genetic information in spermatozoa (Oliva and Dixon, 1991a; Mengual et al., 2003a). It is believed that incomplete protamination may affect sperm DNA causing more vulnerability to environmental damage or endogenous agents such as nucleases (Szczygiel and Ward, 2002, Sotolongo et al., 2003) or free radicals and oxidative stress (Aitken et al. 2007, Irvine et al., 2000; Alvarez et al., 2002). Our results support this general concept in that poor quality spermatozoa from the low density region of Percoll gradients where shown to exhibit the greatest CMA3 fluorescence and thus the lowest levels of protamination.

Interestingly, we have found a direct relationship between the amount of sperm cell DNA methylation and percentage of CMA3 positivity (Fig 5.4). Scattergram show the relation of protamine deficiency (Chromomycin staining) and level of DNA methylation in 5 semen donors($r = 0.81; n= 9; *p< 0.05$). Thus it appears to be a good relationship between the level of sperm DNA methylation and the level of protamine deficiency in human sperm DNA. It is possible that the methylation level of sperm DNA could affect on the packaging, shape and organization of human DNA in the space of sperm head (Fig 5.2). Our research indicates that both of the criteria of epigenetic modification examined in this study are elevated in poor quality spermatozoa.

However, our initial microscopic findings (Figure 5.2) do not support the current model, which posits that DNA in mammalian spermatozoa is tightly compacted into linear arrays organized as loop domains (Ward and Coffey, 1991). The detected module for chromatin in human sperm despite bad or good quality of that demonstrated in figure 5.2. We have previously shown that there is a direct link between the 5-methyl cytosine percentage of human sperm and to the guanine-cytosine-specific Chromomycin A3 (CMA3) fluorochrome positivity.
Then we tried to compare the degree of protamine protamination as determined by CMA3 fluorescence with the level of Annexin-V binding and caspase activation in human spermatozoa by flowcytometry. The presented scattergram (Figure 5.6) shows the relationship between protamine deficiency and level of Annexin-V expression in the same semen donor, data analyzed by SPSS ($r = 0.66; n= 13$ (low & high density percoll fraction); $p < 0.05$). Despite of low size of the sample population result fairly establish the relation between protamination and early signs of apoptosis. However the previous results clearly suggest that hypermethylated spermatozoa may be earmarked for early apoptosis and expression of Annexin-V.

Similarly, comparison of CMA3 staining and red FLICA labelling of human spermatozoa (Figure 5.7) reveaed a tendency to positive correlation between these parameters ($r = 0.56; n= 11$; $p$ not significant). This result reinforces the idea that spermatozoa exhibiting signs of poor chromatin protamination are destined for apoptosis as indicated by a higher level of caspase activation.

We hypothesize during the transition from an immature germ cell to a mature spermatozoon; different mechanisms control the quality and progress of the differentiation process. The availability of CMA3 sensitive sites in the C-G rich regions of sperm chromatin and the level of DNA methylation in the same sperm donors could mean that during remodeling and protamination, hypermethylation might affect the ability of super coiled DNA to resemble effdectively. In addition, we demonstrated that such poorly protaminated hypermethylated cells have a tendency to default to an apoptotic pathway associated with phosphatidylserine externalization and caspase activation.
Chapter 6

Final Discussion and Future Direction

6.1 Introduction

Methylation in human male germ line is a major epigenetic signal involved in the silencing of genes and in propagating chromatin status during cell division. However, the gene silencing procedure may happen via genetic or epigenetic events. In present thesis the most important signs of epigenetic modification, DNA methylation and histone modification, have been investigated in human sperm sub-populations of differing quality. The results obtained illustrate that the low-density gradient Percoll fraction comprising largely defective human spermatzoa express a higher level of methylation than the high-density fraction. In addition, to better understanding the pattern of methylation, the role of methyltransfrases and UHRF1, the most important players during sperm DNA methylation has been reviewed. Then the level of methylation has been measured and its link to signs of apoptosis has been examined. Moreover, the higher level of methylation in low-density defective sperm populations has been confirmed by immunocytochemistry, and related to CMA3 - mediated assessments of the histone to protamine transition. In following text I discuss systematically the major results secured in this investigation and review their implications for the direction of future studies.

6.2 The pattern and pathway of DNA methylation in different subpopulations of human spermatozoa.

The aim of this thesis has been to study the methylation of human spermatozoa. It is believed that DNA methylation could be a marker for sperm quality in the context of ART as well as relevant to imprinting syndromes and early childhood cancers (Feinberg and Tycko, 2004) or psychiatric disorders (Jiang et al., 2008). Some references portray 5-methylcytosine (5mC), as the most important regulator of DNA modification and as
the fifth base of human DNA (Widschwendter, 2007). The results generated in this study on the expression of 5mC in different sperm populations using a variety of different techniques has demonstrated a higher level of DNA methylation in poor quality of sperm.

In somatic cells, 5mC accounts nearly 1% of total bases in human DNA and it contains 70%-80% of all CpG dinucleotides in the genome (Ehrlich 1982). Tada et al. (1997), Reik et al. (2001) has reported a sharp reduction of methylation in germ cells during spermatogenesis. Monak et al (1987) and Kafri et al (1992) have also reported a dramatic decline in methylation level to nearly 30% of the typical somatic cell level during embryonic demethylation in mice. Different references from different tissues have reported different degrees of methylation. Our HPLC result of hydrolysed, extracted, DNA from 11 good quality and 5 poor quality populations of human spermatozoa detected levels of 2.4% and 4.06% respectively of 5-methyl-2´-deoxycytidine relative to the total level of 5-methyl-2´-deoxycytidine+2´-deoxycytidine).

Considerable variation in the levels of methylation in human spermatozoa isolated from different regions of the gradient has been demonstrated by HPLC from a minimum of 0.2% in the 100% Percoll sperm fraction to a maximum of 7.58% in the defective .50% fraction (Table3.1). Using HPLC, the 5-methylcytosine levels in the poor quality sperm fractions were found to be 1.5 fold higher than those observed in the high quality fractions, giving mean±SEM values of 4.60±0.56% and 3.04±0.34% respectively. When flow cytometry was used to assess the percentage of cells exhibiting a high level of DNA methylation, a 2.6 fold difference was observed, 14.37±2.72% of the 100% Percoll fraction being positive compared with 37.00±3.89 % in the low-density sperm fraction.

Thus, poor quality samples are characterized by both a higher methylated DNA content and a higher percentage of cells exhibiting high levels of 5mC. This finding may have important implications for the normality of embryonic development, should an oocyte be fertilized by a hypermethylated defective spermatozoon, as is likely when ICSI is being used as insemination procedure. Prior to this study, global DNA demethylation has been reported in the one-cell stage through to the morula stage, using
immunofluorescence staining with an antibody against 5-methylcytosine (anti-5mC) (Mayer et al., 2000; Santos et al., 2002). It has been shown that the process of demethylation is active in the male pronucleus in the 1-cell stage and passive in each replicated cell at the 2-cell stage. Chromosomes in the male pronucleus are actively demethylated within 4–6 h after fertilization (Mayer et al., 2000; Santos et al., 2002). However, Ehrlich and Wang (1981) observed a lower level of 5mC in human sperm in comparison with other tissues.

Furthermore, Xu et al (2005) concluded their article by stating that active demethylation of the parental pronucleus occurs immediately after fertilization in both mouse and man, but the reasons for this conclusion were not fully explained. Our data obtained using three separate analytical methods (HPLC, flowcytometry and immunocytochemistry) have demonstrated that the high-density functional Percoll sperm fraction is hypomethylated. Therefore, might suggest that only hypomethylated sperm have the capacity to fertilize the oocyte while hypermethylated defective spermatozoa are destined to undergo apoptosis. The result secured through an immunochemical analysis of human spermatozoa with a 5-mC antibody is clearly supportive of this hypothesis.

Immunocytochemistry with a 5-mC antibody has provided information on two different processes. The results obtained following staining poor quality Percoll fractions with this antibody demonstrated high levels of methylation giving multiple high intensity signals within the chromatin. By contrast, high-density Percoll gradient fractions demonstrated different pattern of chromatin packaging (Fig 3.9 a,gh,h&Fig 3.10) after exposing the sperm DNA to acid and detergent. In some of these cells a small discrete hypermethylated region was observed, the identity of which is unknown. A possible hypothesis is that such a highly localized area of methylated chromatin might represent an inactive X chromosome in the subpopulation of X-bearing spermatozoa. Turner (2006) has reported paternal X chromosome inactivation in XX mouse embryos prior to implantation, the paternal X being transcriptionally repressed. However, prior to this study Huynh and Lee (2003), and Okamoto et al., (2004, 2005) have suggested that an inactive X may be provided by the spermatozoa at fertilization or after fertilisation at the four to eight stage cells. Huynh and Lee (2005) have linked X-inactivation to chromosome inactivation during the pachytene stage of male meiosis and Turner et al
(2006) have implied that some degree of X repression remains from the pachytene spermatocyte stage through to the mature sperm.

Furthermore, in this research we have stained the sperm mitochondria with 5mC antibody without treating sperm with acid or detergent to demonstrate the mitochondrial DNA methylation pattern. The DNA of all mitochondria, whether the spermatozoa originate from the low or high-density gradient fraction were found to be hypermethylated (Fig 3.10-11). This overall methylation means that this DNA is not heavily complexed with proteins such as the mitochondrial transcription termination factor 1 or light and heavy strand promoters (Rebelo et al., 2009). Such heavily methylated mitochondrial DNA is typical of replicating cells and may be a legacy from the germ cell’s replicative past. Methylation of the mitochondrial DNA would also ensure transcriptional repression of the paternal mitochondrial genome, which is likely to have experienced a great deal of oxidative DNA damage, during the free-swimming stage of the spermatozoa’s life history.

Thus the sperm mid piece houses not only the mitochondria with their hypermethylated DNA but also orchestrates the senescence of these cells as the result of an intrinsic apoptotic cascade that features PS externalization (Kotwicka et al in 2008) caspase activation, mitochondrial generation of reactive oxygen species (Kopper et al., 2008) and lipid peroxidation (Ferrusola et al., 2009). Since, all these apoptotic signals are associated with poor quality spermatozoa, the mitochondria are mediators of oxidative stress in spermatozoa, as they are in other cell types, and their DNA is likely to be heavily damaged, and therefore, not welcomed by the embryo.

Given that the mitochondrial genome is inherited exclusively through the female germ line, the transcriptional repression of the paternal mitochondrial genome in the zygote is critical. The role of the mitochondria in human spermatozoa is not well characterized except as a source of ATP for the optimal functioning of germ cells including the movement of spermatozoa, although the latter are largely dependent on glycolysis for motility. A role for mitochondrial dysfunction in partial or complete spermatogenesis arrest and male infertility has been suggested (Taylor et al 2004, Cummins et al., 1994, St.John et al., 1997).
Clearly, the embryo does not require the sperm mitochondria and their badly damaged genomes and hypermethylation of this DNA as well as ubiquinination of the mitochondrial proteins are typical of the strategies that are used to silence contributions from the paternal mitochondrial lineage to the embryo. Mutations of mtDNA are responsible for many types of mitochondrial diseases in humans, including myopathy and neurological disorders. Females donate a mixture of mutant and wild-type mtDNA variants to each offspring. The proportion of mutated mtDNA inherited from the mother determines the onset and severity of diseases. Studies have suggested that the mtDNA genome is transmitted through a bottleneck, although the mechanism remains controversial.

We have compared the pattern of methylation in human sperm populations of differing quality, via immunoblotting with the 5mC antibody. The major bands appeared, at least in terms of their molecular masses to correspond to a series of 5-methyl transferase isoforms. Since the antibody has been raised against a methylated DNA complex comprising UHRf1 (Hassimoto et al., in 2008) as well as methyltransferases, such an interpretation is feasible. In addition, according to Rottach et al. (2009) the SRA domain of UHRf1 is necessary and sufficient for DNA binding. This domain penetrates its tips to the DNA from both sides and, like a belt, provides covalent contacts that help flip out the miss-matched cytosine and make it available to the UHRF1 complex (Hassimoto et al., 2008). Therefore, it has been our expectation that the antibody against 5mC could be monoclonal in nature and still target proteins associated with the DNA methylation complex including the methyltransferases.

Following this line of thought, the bands that appeared on the SDS gel (Fig 3.12) might correspond to cross-reacting Dnmts. At present, we have no evidence to support this conjecture other than an alignment of the bands with the anticipated molecular masses of major Dnmt isoforms. If time had permitted, we would have absorbed the 5mC antibody with 5mC to see if the cross-reactivity disappeared. We would also have used bone fide antibodies against the Dnmtses isoforms to confirm the identity of these bands.

The presentation of different isoforms is especially marked in the case of bands corresponding to Dnmt3a and Dnmt3b in the low density fractions of human spermatozoa. Such a result may suggest the importance of de novo methylation of
hypermethylated, poor quality human sperm, rather than a suppression of demethylation activity. Dnmt3L was not detected in the high-density Percoll fraction and was only weakly present in the low density Percoll fraction.

Although Dnmt3L involvement in the methylation of imprinted genes during oogenesis and spermatogenesis has been detected (Bourc'his and Bestor 2004, Bourc'his et al., 2001, Hata et al., 2006), we have found the band corresponding to this molecule to be poorly expressed in spermatozoa. Another interesting point to emerge from this analysis was the remarkable expression of a band corresponding to the omnipresent Dnmt2, which may suggest the importance of this member of the methyl transferase family in human spermatozoa, despite receiving little attention from other tissues. Finally, the over expression of putative Dnmt1o in all samples irrespective of quality suggests a critical role in the maintenance DNA methylation of both fractions of sperm.

Based on the regulatory activity of Dnmt3a and Dnmt3b in the expression of Dnmt3L in mouse ES cells and embryos, Hu et al. (2008) have suggested an auto-repression mechanism for the control of the cellular methylation activity (Fig. 6.1). They have reported that Dnmt3L is a regulator of de novo methyltransferases and would be suppressed after de novo methylation has been completed in genome. The expression of Dnmt3L would therefore be suppressed in the presence of heavy methylation (Hu et al., 2008). In light of these findings, we conclude that the hypermethylation observed in the defective sperm populations results from a breakdown in this negative feedback loop also, assume that according this could be the reason of absence of Dnmt3L in human spermatozoa (Fig3.12).
Figure 6.1 Recruitment of Dnmt3b to the Dnmt3L promoter in ES cells upon differentiation.

Model of methylation-mediated auto-suppression of Dnmt3L expression. Mechanism controlling the access of Dnmt3 proteins to the promoter is unknown. The negative feed-back system may allow silencing of Dnmt3L, thus resulting in a compensation of the methylation activity (Hu et al., 2008).

Overall, we have examined DNA methylation patterns in two different populations of spermatozoa isolated from the low- and high- density regions of Percoll gradients respectively. The results demonstrate the presence of low levels of 5mC expression in the nuclear DNA of good quality spermatozoa. The only exceptions to this rule were discrete areas of hypermethylated chromatin in certain cells, which may represent the presence of an inactive X chromosome. By contrast, poor quality spermatozoa revealed evidence of global hypermethylation, which may have implications for embryonic and childhood development when such defective cells are used for assisted conception purposes. In addition, mitochondrial DNA in both fractions of spermatozoa exhibited high levels of methylation, possibly as a means of suppressing transcription of the paternal mitochondrial genome in the zygote.
6-3 The relation of apoptosis with individual characteristic, and level of DNA methylation in different density gradients of human spermatozoa.

The aims of this series of experiments were to determine the importance of apoptosis in regulating the behavior of human spermatozoa. This study has been persecuted by examination of phosphatidylserine externalization, total caspase activation and sperm vitality, and then comparing these attributes of sperm function with the methylation status of these cells. We have found some interesting biological relationships that may be related to, either capacitation (Gadella and Harrison 2002) and/or the ability of these cells to undergo apoptosis (Aitken., et al 1994).

According to Vermes et al (1995) externalisation of phosphatidylserine is one of earliest signs of apoptosis latter Sakkas et al (1999, 2004) have declared that phosphatidylserine externalization in human spermatozoa may represent that spermatozoa has escaped from elimination during spermatogenesis. If this were the case, then these cells should detect a higher level of phosphatidylserine externalisation in the semen of infertile men compared with normal donors. However, some authorities (Oosterhuis et al., 2000, Duru et al., 2000, Ricci et al., 2002) have questioned the correlation between sperm quality and phosphatidylserine externalisation in human spermatozoa although spontaneous apoptosis has been detected in rat germ line (Lachaud et al., 2004).

Tesarik be Annexin V positive from the moment of ejaculation. Some studies have shown an increase in molecular apoptotic markers associated with abnormal sperm parameters (Sakkas et al, 1999) while Weng et al (2002) has reported higher levels of AnnexinV binding in sub-populationms of spermatozoa exhibiting poor motility.

Our time-dependent analysis of phosphatidylserine externalisation (Fig 4.1-2) revealed the profile of AnnexinV binding and the progressive increase in surface phosphatidylserine expression until these cells lost viability over a 24-hour period. However, it should be noted that the time taken to achieve an apoptotic death varied from cell-to-cell. There were clear differences in the apoptotic status of spermatozoa.
recovered from different regions of the Percoll gradients and between different semen donors.

Intriguingly, our data indicate a direct correlation between the percentage apoptotic cells in the high- and low-density Percoll fractions for individual samples ($r = 0.75; P<0.01$). Thus, while the spermatozoa recovered from the low density Percoll fractions invariably expressed more apoptosis than the high density fractions, the overall level of apoptosis observed was characteristic of an individual semen specimen, regardless of which particular fraction was being examined.

Our results also show an inverse correlation between Annexin-V binding and the viability of spermatozoa in the high and low-density Percoll fractions respectively ($r = 0.81; n= 15; P<0.01$) and ($r = 0.80; n= 16; P<0.01$). In addition, a similar correlation was observed between sperm motility and apoptosis at least in the high-density fractions ($r = 0.76; n = 11; P<0.01$). In contrast, no significant correlations were observed between semen volume and apoptosis signs. In addition, total caspases demonstrated high levels of expression in poor quality of spermatozoa in comparison with high quality cells ($P<0.001$) although the strength of this difference was very sample dependent.

We also examined the level of externalised phosphatidylserine in relation to the methylation status spermatozoa isolated in different regions of the Percoll gradient. The individuals whose spermatozoa demonstrated high rates of externalised phosphatidylserine in the early hours of incubation also exhibited high levels of DNA methylation ($r =0.79; n =17; P<0.01$). Our data confirm the presence of de novo apoptosis in mature human spermatozoa because the capacity for Annexin-V binding increased progressively during incubation in vitro. It might be that hypermethylation of human sperm DNA is indicative of a propensity of these cells undergo apoptosis. It remains to be established whether apoptosis is a cause or a consequence of DNA damage.

It has previously been proposed that DNA damage in mature spermatozoa could be linked to aberrant apoptosis following the targeting of defective cells at critical check points during spermatogenesis. Apoptosis eliminates harmful, irreparably damaged cells
that are not able to pass checkpoint-monitored transitions (Rodriguez 2006). DNA damage and mitotic spindle defects as well as a large number of other environmental stresses trigger apoptotic pathways when the anomaly cannot be repaired in time (review Rodriguez 2006). In the latter review it mentions that apoptosis in germ cells has a major role to safeguard the genetic The mitochondria are the key instigators of apoptosis in spermatozoa through the release of key effectors of the apoptotic process such as cytochrome C. In apoptotic spermatozoa, the mitochondria may be more susceptible to disruption by stressors such as exposure to oxygen free radicals. It should be emphasized however that it is probably the ultimate fate of all spermatozoa to die an apoptotic death in the female reproductive tract except, that is, for the spermatozoon that fertilized the oocyte.

Therefore, mitochondria could be the drivers of apoptosis in spermatozoa and housed in the midpiece of the spermatozoa to induce this process. Accordingly this compartment of the cell is the first to stain with caspase3 (Weng et al., 2002) and to exhibit externalised phosphatidylserine (Kotwicka et al., 2008) in the early hours of incubation. All these changes may be linked to the way in which mitochondria produce reactive oxygen species (Kopper et al 2008), for it is these molecules that appear to be responsible for a great deal of the damage associated with the apoptotic process. Immunocytochemistry of low quality spermatozoa exhibiting high levels of apoptosis revealed the highest level of DNA methylation as well as evidence of poor DNA compaction. A previous report from Weng et al., in 2002 is supportive for the present study.

In summary, apoptosis is an active process in human spermatozoa and its incidence seems to be positively correlated with the levels of DNA methylation exhibited by these cells. We suggest that the mitochondria of hypermethylated human spermatozoa are responsible for triggering the apoptosis seen in such defective sperm populations. In contrast, high quality spermatozoa are hypomethylated, giving the paternal genome maximum opportunity for expression in the zygote/early embryo. However even in these normal cells some areas of high methylation are evident in the DNA which, we speculate, might represent the paternal X chromosome which may be hypermethylated and transcriptionally silenced, in confirmation of previous reports (Khanh et al., 2003). Clearly, apoptosis plays a vital role in eliminating populations of defective human
spermatozoa exhibiting disrupted patterns of DNA methylation that might have otherwise had a devastating effect on the developmental potential of any resultant embryos.

6-4 The relation of CMA3 binding, DNA methylation and apoptosis sign in human spermatozoa.

Sperm chromatin integrity is essential for accurate transmission of male genetic information, and normal sperm chromatin structure is important for fertilization (Erenpreiss et al 2006). As previously mentioned, protamine is an important nuclear protein that has a key role in the ability of sperm to be fertile. It is responsible for sperm DNA stability and sperm DNA packing until the paternal and maternal genomes become united in the oocyte (Simoes et al., 2009). In this regard, Nasr-Esfahani et al (2001) reported that CMA3 is the most sensitive and specific of nuclear maturity tests for the prediction of in vitro fertilization rates and sperm morphology. Sperm morphology and protamine deficiency were not only claimed to be two key factors in fertilization but were both found to be reflected in the quality of CMA3 staining. Our aim has been to investigate DNA integrity, degree of DNA methylation and evidence of apoptosis in sperm populations of differing quality and to compare these attributes with measures of sperm chromatin protamination using chromomycin A3 (CMA3) staining as a diagnostic criterion.

ChromomycinA3-bandimg has been used to study the position of chromosomes in somatic cells as reflected by the studies of Gornung et al (1997) on zebra fish. Similarly, CMA3 staining of human sperm DNA has, in addition to providing important information on the state of chromatin protamination between good and poor quality spermatozoa, generated some possible insights into the arrangement of chromosomes within the head of human sperm (Fig 5.2). The images of sperm nuclear DNA obtained by CMA3 staining suggests that there are some unique and specific arrangements of chromatids in the sperm nucleus resulting in a non-random localization of chromosomes (Figure 5.2). In this regard Zalenskaya and Zalensky (2004) have reported some evidence for the non-random spatial localization of chromosomes within the nuclei of
human spermatozoa and Luetjens et al. (1999) and Hazzouri et al. (2000) have also provided data on the specific location of individual chromosomes in these cells.

In addition, Zalensky et al (1993) have concluded that the human sperm nucleus features a compact gathering of centromeres in a chromocenter that is located centrally and shifted towards the apical aspect of the sperm head. The images presented in Fig 5.2 demonstrate clearly that chromatids in human spermatozoa are configured within the nucleus in specific 3-dimensional patterns. We confirm the fact that the long chromatids have a tendency to be looped with the telomeres directed to the nuclear periphery as illustrated in the upper left quadrant of the sperm head depicted in Fig 5.2 a. However, this is not necessarily the case for the shorter chromatids as revealed in Fig 5.2 b, c. In addition our finding does not demonstrated a compact gathering of centromers that could be supportive by the report of Nagele and et al., (1995, 1998). We have hypothesized that the relative positioning of chromosomes in spermatozoa is similar to that observed in somatic cells. In this context, Nagele et al., (1998) have reported a common chromosome distribution pattern in several cell lines derived from different adult tissues and different individuals. Our unpublished photos have demonstrated that chromosome distribution in human cells are located around the head of sperm and the could be the subject of genetic influences inherited from the parents however the longer chromatids have placed in the upper and left sides of the photo.

In another respect, CMA3 can be used for the detection of protamine deficiency in higher-order chromatin structures, indirectly assessing the efficiency with which histones have been removed and replaced by protamines. It is suggested that these dynamic processes have a key role to play in defining the fertility of human spermatozoa. The evidence presented in this thesis reveals that the level of CMA3 positivity in spermatozoa recovered from the low-density regions of Percoll gradients is higher than in good quality sperm collected from the high-density Percoll fractions. Overall there is a highly significant difference in the levels of CMA3 fluorescence detected in these two cell populations \( (P<0.0001) \). We have also demonstrated a direct relationship between protamination deficiency and the level of DNA methylation in a small group of semen donors \( (P<0.001; r=0.90) \). It is therefore possible that the hypermethylation of DNA seen in defective spermatozoa is associated with elevated
histone levels, while protamines are associated with the unmethylated sperm DNA. There may even be causal relationships between, hypermethylation, nucleohistone retention and poor protamination that will have to be explored in future studies.

Our data also revealed a strong correlation between the levels of protamine deficiency (CMA3) and apoptosis as indicated by a strong correlation with AnnexinV expression ($P < 0.028 \ r=0.66$) in Figure 5.5 and a fair correlation to caspase activation ($P<0.005 \ r=0.48$) in Figure 5.6 respectively. These data suggest that defective spermiogenesis generates spermatozoa that exhibit deficient chromatin remodeling, as indicated by abnormally high levels of DNA methylation and poor protamination, and are also vulnerable to apoptosis, leading to a loss of DNA integrity, impaired motility and ultimately, cell death. CMA3 is indeed an excellent probe for examining the suitability of spermatozoa for ART since it reflects the efficiency of spermiogenesis and, in the end; it is the quality this process that defines the fertilizing capacity of the spermatozoa.

### 6-5 Final conclusion

In conclusion, our data strongly suggest that defective sperm function has its origins during spermiogenesis. The differentiation of round spermatids into spermatozoa is associated with an extensive remodelling of the chromatin such that all gene expression in the male gene line is silenced and the haploid genome becomes compacted through the progressive replacement of nucleohistones with protamines. Deficiencies in this process lead to spermatozoa that possess poorly protaminated DNA that is relatively hypermethylated, while the cell as a whole is vulnerable to apoptosis. Immunocytochemistry with the 5-methylcytosine antibody also revealed that the mitochondrial genome is relatively hypermethylated, possibly as a strategy to prevent expression of the paternal mitochondrial genome in the zygote. Thus the ideal spermatozoa for ART would be those exhibiting low levels of methylation in the nuclear genome and high levels of methylation in the mitochondrial genome. Having established these descriptive correlations, it is now important to resolve the underlying mechanisms. Questions that might be posed include:

Is there a causal relationship between hypermethylation of the nuclear genome and the aberrant retention of nuclear histones in defective spermatozoa?
Is the methylation of the mitochondrial genome a critical factor in preventing expression of the paternal mitochondrial genotype in the fertilized egg?

Is the DNA damage seen in defective spermatozoa entirely mediated by aberrant apoptosis?
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APPENDIX A

Reagent and Suppliers

A.1 General Reagents

Acetic acid: BDH Laboratory Suppliers, Dorset, UK
Cetonitrile: Sigma Chemical Co. Ltd., St Louis, MO, USA
Acrylamide(bis) 40%: bio-Rad Laboratories, Herts, UK
Agarose(DNA grade): Progen Biotechnology, Archerfields, QLD, AU
Ammonium bicarbonate: Sigma Chemical Co. Ltd., St Louis, MO, USA
Ampicillin: Research Organics, Cleveland, OH, USA
BSA: Sigma cat# A9056
goat serum: Sigma cat# A2007
methanol: Fronine cat# JJ023
PBS tablets: Sigma cat# P4417
Calcium chloride: BDH Laboratory Suppliers, Dorset, UK
Chloroform: BDH Laboratory Suppliers, Dorset, UK
DMSO: Sigma Chemical Co. Ltd., St Louis, MO, USA
DTT: Sigma Chemical Co. Ltd., St Louis, MO, USA
Dynabeads protein G: Dynal, Oslo, Norway
EDTA: Sigma Chemical Co. Ltd., St Louis, MO, USA
Ethanol: Fronine Supplies, Riverstone, NSW, Australia
Ethidium bromide: Sigma Chemical Co. Ltd., St Louis, MO, USA
Formalin: ProSciTech, Thuringowa, NSW, Australia

formaldehyde: ProSciTech, Thuringowa, NSW, Australia
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<th>Chemical</th>
<th>Supplier</th>
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<td>Sigma chemical Co. Ltd., St Louis, MO, USA</td>
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Appendix

Tween-20 supermarket

A.2 Materials EQUIPMENT

Axoivert 200 Fluorescent Zeiss
Biomax MS cassette Kodak
Centrifuge
Incubator oven contherm
Mini-PROTEAN 3-Cell electrophoresis unit Biorad, cat#165-3301
Mini Trans-Blot Electrophoretic Transfer Cell Biorad, cat#170-3930
Power supply, ie BioRad PowerPac200 Biorad, cat#165-5052
Glass gelplates Bio-Rad Laboratories, herts, UK
92 well plate
Orbital shaker Ratek
Water bath Thermoline

A.3 Anti bodies and kits

AnnexinV0 Invitrogen, mount waverly VIC
ECL kit GE Healthcare, Uppsala, Sweden
FLIKA
5mcytosinne antibody abcam, Saphire bioscience, Redfern
Goat anti- mouse IgG, FITC conjugate Santa Crusz, Biothech nology, CA
APPENDIX: B

BUFFERS AND SOLUTION

B.1 Sperm Preparation

BWW

915 mm NaCl
44 mM Sodium lactate
25 mM NaHCO3
20 mm HEPES
5.6 mM D-glucose
4.6 mM KCl
1.7 mM CaCl2
1.2 mM MgSO4
1.2 mM KH2PO4
0.27 mM Sodium pyruvate
5 U/ml pencillin
5 µg/ml streptomycin
PH 7.4

Sperm Diluting Fluid

0.6 M Sodium Hydrogen Carbonate,
1 % (v/v) Formaldehyde

Eosin (0.1%)

1mg Eosinl
10ml Distilled Water

Percoll 100%

1X HAMS F10 Solution
90% (v/v) Percoll
1mg/ml Polyvinyl Alcohol (PVA)
0.27mM Sodium Pyruvate
44mM Sodium Lactate
25mM Sodium Hydrogen Carbonate
PH 7
B.2 DNA investigation

Infiltration solution
100% Isopropanolol 70% Ethanol

DTT
1M Dithiothreitol in MQ water

STE buffer
500 mM NaCl
10 mM EDTA (Disodium Ethylene Diamine TetrAcetate, pH 8.0)
10mM Tris-Hcl (PH 8)

SDS
20% SDS

Proteinase K
20mg proteinase K/ml buffer
Proteinase K Buffer
10mM Tris HCl, pH 8.0, 50mM NaCl, 5mM EDTA, 1mM DTT, 0.5% SDS 50mM Tris-Hcl, pH 8.0

Ammonium orthophosphate
50 mM diammonium orthophosphate
50 mM orthophosphoric acid
pH to 4.1 with 1 M orthophosphoric acid.
RNAase cocktail
Chloroform

B.3 proteine extraction

SDS extraction buffer
2% (w/v) SDS
50%(v/v) 0.375 M Tris (PH 6.8)
10%(w/v)sucrose
Protease inhibitor cocktail tablet
4% v/v 2-mercaptoethano

Coomassie blue
1% Coomassie
40%(v/v) methanol
10%(v/v) acetic acid

DMSO
(v/v 7%) DSMO added to Milli-Q H2O and autoclaved

B.4 Gel electrophoresis

Agarose gel
1% w/v agarose in TAE

TAE buffer
Appendix

40mM Tris
50mM EDTA (PH 8.0)
0.1% (v/v) acetic acid

8% polyacryl amide gel

0.4% (w/v) SDS
0.5 M Tris base (6.8)PH
85 acrylamide
0.1% (w/v) ammonium per sulfate
0.2% (v/v) TEMED

SDS-PAGE resolving

0.4% w/v SDS
0.5 M Tris base, PH 8.

SDS- PAGE running buffer

0.1% (w/v) SDS
25mM Tris base
192 mM glycine

SDS-PAGE sample buffer(2x)

4% (v/v) mercaptoetanol
2% (w/v) SDS
50% (v/v) 0.375 MTris (PH 6.8)
10% (w/v)sucrose
0.5 mg/ml bromophenol blue

SDS-PAGE stacking buffer

0.5 % w/v SDS
1.5 M Tris base, Ph 6.8

SDS-PAGE stacking gel

0.49 ml 40% acrylamide
1.25 ml stacking buffer
3.05 ml mili-q H2O
30µ 10% ammonium persulfate
7µ TEMED

TBS

M Tris base
0.14 M sodium chloride

B.5 Western blotting

TBS-T

TBS containing 0.1% (v/v) Tween-20

Anti body buffer

1% (w/v) BSA
20% (V/v) Tween-20 in TBS
Appendix

### B.6 Immunocytochemistry

**PBS**

- 0.14 M NaCl
- 2.7 mm KCl
- 8.1 mM Na2HPO4
- 1.7 mM KH2PO4
- PH 7.4

**TBS**

- 25 mM Tris base
- 137mM NaCl
- 2.7 mM KCl
- PH 7.4

Paraformaldehyde (4% w/v)

- 4g paraformaldehyde in 100ml xPBS( adjust to PH=11 with NaOH), then adjust PH=7 with H2SO4

**MOWOIL**

- 2.4g MOWIOL 4-88
- 6 g glycerin
- 6 ml dH2O
- 12ml 0.2 M Tris (PH 8.5)

**CMA3**

- 0.2 mM CMA3 make up in MClivanes Buffer

**MClivanes Buffer (10mM)**

- 41ml NaH2PO4(200mM)
- 9 ml Citric acid(100mM)
- 50.75 mg MgCl2 .6H2O2 (10mM)
- PH 7.0

### B.7 Flowcytometry

Propidium iodide
Appendix

Buffer: 1 X PBS
2% newborn calf serum (or 0.2% BSA)
0.1% sodium azide

PI buffer:

Dissolve PI in buffer at a concentration of 1 microgram/ml. Keep the solution tightly closed at 4°C protected from light. Discard after 1 month.

PI stock buffer:

Dissolve PI in buffer at a concentration of 500 micrograms/ml. stored in 4°C protected from light.