“INVESTIGATION OF THE ROLE OF OXIDATIVE STRESS IN MALE INFERTILITY”

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Thesis submitted for the total fulfilment for the degree of
Doctor of Philosophy (PhD)

November 2010
DECLARATION

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Ozlem Tunc
November 2010
ACKNOWLEDGMENT

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PUBLICATIONS AND ABSTRACTS ARISING FROM THIS THESIS

PUBLICATIONS

1 - "Development of the NBT assay as a marker of sperm oxidative stress"
   Ozlem Tunc, Jeremy Thompson, Kelton Tremellen

2 - “Improvement in sperm DNA quality using an oral antioxidant therapy”
   Ozlem Tunc, Jeremy Thompson, Kelton Tremellen

3 - "Oxidative DNA damage impairs global sperm DNA methylation in infertile men"
   Ozlem Tunc, Kelton Tremellen

4 - "Macrophage activity in semen significantly correlated with sperm quality in infertile Men"
   Kelton Tremellen, Ozlem Tunc
   International Journal of Andrology 2010 Dec; 33(6):823-31

5 - “Impact of Body Mass Index on sperm oxidative stress”
   Ozlem Tunc, Hassan Bakos, Kelton Tremellen
   Andrologia (accepted in Aug 2009, anticipated online publication in December 2010)

ABSTRACTS

“A novel assay for identification of oxidative stress related male infertility”
Poster presentation  The Fertility Society of Australia 9-12 September 2007 Hobart, Tasmania

"Optimization of sperm DNA quality by using an oral antioxidant therapy”
Gene, environment, lifestyle interaction and Human reproduction 7-10 February 2008 Malmo, Sweden

“Optimization of sperm DNA quality with the use of an oral antioxidant therapy”
Oral Presentation in the Fertility Society of Australia 19-22 October 2008 Brisbane, Australia
<table>
<thead>
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<th>Full Form</th>
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<tbody>
<tr>
<td>8-OHdG</td>
<td>8-hydroxy-2-deoxyguanosine</td>
</tr>
<tr>
<td>A.U</td>
<td>Arbitrary Units</td>
</tr>
<tr>
<td>AMH</td>
<td>Anti-mullerian hormone</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of Variance</td>
</tr>
<tr>
<td>ART</td>
<td>Assisted Reproductive Technology</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>°C</td>
<td>Degrees Celsius</td>
</tr>
<tr>
<td>CK</td>
<td>Creatine kinase</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
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<tr>
<td>FSH</td>
<td>Follicle stimulating hormone</td>
</tr>
<tr>
<td>GPx</td>
<td>Glutathione peroxidise</td>
</tr>
<tr>
<td>GSR</td>
<td>Glutathione reductase</td>
</tr>
<tr>
<td>H₂O₂</td>
<td>Hydrogen peroxide</td>
</tr>
<tr>
<td>HBSS</td>
<td>Hanks buffered salt solution</td>
</tr>
<tr>
<td>hCG</td>
<td>Human chorionic gonadotrophin</td>
</tr>
<tr>
<td>Hcy</td>
<td>Homocysteine</td>
</tr>
<tr>
<td>HNE</td>
<td>Hydroxynonenal</td>
</tr>
<tr>
<td>HPLC</td>
<td>High Performance Liquid Chromatography</td>
</tr>
<tr>
<td>HRP</td>
<td>Horse Radish Peroxidase</td>
</tr>
<tr>
<td>IFNγ</td>
<td>Interferon gamma</td>
</tr>
<tr>
<td>IU</td>
<td>International unit</td>
</tr>
<tr>
<td>KOH</td>
<td>Potassium hydroxide</td>
</tr>
<tr>
<td>kg</td>
<td>Kilogram</td>
</tr>
<tr>
<td>LDH</td>
<td>Lactic acid dehydrogenase</td>
</tr>
<tr>
<td>LH</td>
<td>Luteinizing Hormone</td>
</tr>
<tr>
<td>MDA</td>
<td>Malondialdehyde</td>
</tr>
<tr>
<td>μg</td>
<td>Microgram</td>
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<tr>
<td>μl</td>
<td>Microliter</td>
</tr>
<tr>
<td>mL</td>
<td>Milliliter</td>
</tr>
<tr>
<td>mg</td>
<td>Milligram</td>
</tr>
<tr>
<td>NaCl</td>
<td>Sodium Chloride</td>
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</tbody>
</table>
NAG ................................................................. neutral alpha glucosidase
NBT ................................................................. Nitroblue Tetrazolium
NO ................................................................. Nitric oxide
NOS ................................................................. Nitric oxide synthase
PBS ................................................................. Phosphate buffered saline
PCR ................................................................. Polymerase chain reaction
PFA ................................................................. Paraformaldehyde
PMN ............................................................... Polymorphonuclear Neutrophils
Rcf ................................................................. Relative centrifugal force
ROC ................................................................. Receiver operating characteristic
ROS ............................................................... Reactive oxygen species
SD ................................................................. Standard Deviation
SDS ............................................................... Sodium dodecyl sulphate
SOD ............................................................... Superoxide dismutase
TAC ................................................................. Total antioxidant capacity
TBAR ............................................................. Thiobarbituric acid
TBARS ........................................................... Thiobarbituric acid-reacting substances
TNFα .............................................................. Tumor Necrosis Factor Alpha
X ................................................................. Xanthine
XO ................................................................. Xanthine oxidase
ABSTRACT

In recent years, there has been some suggestion of an increase in male factor infertility in the industrialized countries with a decline in sperm counts and a rise in sperm pathology. Male factor infertility is a multifactorial phenomenon that is observed in approximately half of infertile couples and affects one man in 20 in the general population. The potential causes of male infertility arise from a number of factors including genetic, lifestyle factors and chronic diseases. However, a high proportion of infertile male patients have now been shown to have defective sperm functions related to oxidative stress.

Oxidative stress in semen has been speculated as one of the major factors causing male infertility and has been identified in 30-80% of cases of male infertility. While oxidative stress is accepted as a significant pathology, there is currently an inadequate knowledge of the exact mechanisms by which oxidative stress develops in male infertility, as well as a lack of an easy and reliable method for the measurement of seminal oxidative stress in routine clinical use.

The main objective of this doctoral thesis is to investigate the underlying causes for oxidative stress in infertile men and the mechanisms by which oxidative stress develops. Furthermore it will also examine the effectiveness of an oral antioxidant therapy for treatment of seminal oxidative stress.

During these doctoral studies experiments were designed with the aims of:

- Developing a standardized protocol for the measurement of seminal oxidative stress, that can be conducted in the average clinical laboratory with minimal additional equipment (NBT Assay)
- Examining the causes for oxidative stress in semen. Obesity has previously been identified as a cause of systemic oxidative stress. Therefore I examined if obesity causes oxidative stress to sperm. Seminal inflammation and its role in oxidative damage in semen are also investigated.
- Determination if antioxidant supplementation is an effective treatment of oxidative sperm damage.
- Assessment of the relation between Oxidative stress and sperm DNA methylation. Previous studies have linked male infertility with epigenetic abnormalities of the male genome. Since oxidative stress has been shown to interfere with somatic cell epigenetic programming I investigated the possibility of a similar link in sperm.

It is hoped that advances outlined in this thesis will have made a significant contribution to the diagnosis, prevention and treatment of the male infertility.
CHAPTER 1
INTRODUCTION
1. General Overview: Free Radicals and Reactive Oxygen Species (ROS)

In order to understand the effects of oxidative stress in male infertility it is essential to understand the relevant players of oxidative stress; free radicals, reactive oxygen species (ROS) and antioxidants.

Oxygen is essential for life as it is necessary in the production of energy in the body. A molecular basis, cellular energy is produced by oxidative phosphorylation in mitochondria through electron transport from electron donors to electron acceptors such as oxygen. During these reaction steps, hydrogen is provided in the form of reducing equivalents (NADH) and energy is produced in the form of high-energy phosphates (ATP) while four-electron reduction of molecular oxygen to water produces free radicals and oxygen-derived reactive species. Therefore, the generation of ROS is an essential by-product of life giving metabolism in all individuals.

1.1 Definition of Free Radicals

The term free radicals refers to “any species capable of independent existence that contains one or more unpaired electrons” (Halliwell, 1999). These short-lived but highly reactive molecules can be formed by the loss of a single electron from a non-radical,

\[ X \leftrightarrow e^- + X^+ \]

or by the gain of a single electron by a non-radical;

\[ Y + e^- \leftrightarrow Y^+ \]

The term Reactive oxygen species (ROS) refers to all oxygen radicals, non-radical derivatives of oxygen including oxidising agents and carbon based oxygen radicals (Table 1.1).
**Table 1.1: Most common Reactive Oxygen Species including nitrogen derived free radicals and reactive oxidants. Summarised from (Halliwell, 1999).**

<table>
<thead>
<tr>
<th>REACTIVE OXYGEN SPECIES</th>
<th>GENERATION</th>
<th>EFFECT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Superoxide Anion ( (O_2^-) )</td>
<td>One electron reduction state of oxygen. Primary form of ROS. Generated in auto-oxidation reactions, Electron transport chain in mitochondria/endoplasmic reticulum</td>
<td>Oxidising and reducing agent. 1- Dismutation to hydrogen peroxide and Hydroxyl radicals 2-Formation of thyl radicals by reaction with thiol groups 3-Generation of peroxynitrite</td>
</tr>
<tr>
<td>Hydroxyl Radical ( (OH^-) )</td>
<td>Three-electron reduction state of oxygen. Formed by the reaction of superoxide and Hydrogen peroxide accompanied by metal catalyst (Haber-Weiss Reaction); ( O_2^- + H_2O_2 \rightarrow O_2 + OH^- + OH^- ) or free iron with Hydrogen Peroxide (Fenton Reaction); ( Fe^{+2} + H_2O_2 \rightarrow Fe^{+3} + OH^- + OH^- )</td>
<td>Highly reactive and very short half-life oxygen radical. The most toxic ROS. Attacks all cellular components. Main reactions of hydroxyl radical: hydrogen abstraction (initiation of lipid peroxidation), Addition Reaction(addition to purin base guanine in DNA forms an 8-hydroxyguanine), Electron-transfer reactions.</td>
</tr>
<tr>
<td>Hydrogen Peroxide ( (H_2O_2) )</td>
<td>Two electron reduced state of oxygen. Formed by dismutation of ( O_2^- ) in mitochondria during oxidative phosphorylation or produced as by-product of several oxidases. It is not radical itself.</td>
<td>High biological diffusion, easily transverse the plasma and nuclear membranes. Formation of DNA adduct, affects signalling and tissue injury processes</td>
</tr>
<tr>
<td>Singlet Oxygen ( (^3O_2) )</td>
<td>Lowest excited state of oxygen molecule. Dismutation of superoxide anion. In vitro, singlet oxygen can be produced by photosensitization.</td>
<td>Oxidising agent. Transfers its excitation energy to another molecule. Effective in carbon-carbon double bounds, such as carotenoids and fatty acids.</td>
</tr>
<tr>
<td>Peroxyl ( (ROO^-) ) and alkoxy (RO•) Radicals</td>
<td>Produced in the presence of oxygen during the breakdown of organic peroxides and reaction of carbon radicals with oxygen.</td>
<td>Oxidising agents.Oxidize ascorbate and NADH. Abstract Hydrogen ion from other molecules which is important in lipid peroxidation.</td>
</tr>
<tr>
<td>Nitric Oxide ( (NO•, NOO•) )</td>
<td>Nitric Oxide is formed from the reaction of amino acid L-arginine by NO synthase. NO is not a radical but can form radicals.</td>
<td>Transmitter substance reacts with superoxide anion to make peroxynitrite ( (OONO^-) ) which actively reacts with glutathione, cysteine, deoxyribose and thiols.</td>
</tr>
<tr>
<td>Hypochlorous acid ( (HOCl) )</td>
<td>Formed from hydrogen peroxide by myeloperoxidase</td>
<td>Highly reactive and lipid soluble. Oxidise protein components, thiol and amino groups and methionine.</td>
</tr>
</tbody>
</table>
ROS are formed by several mechanisms in vivo. The most important source of reactive oxygen species is the electron transport chain. During cellular respiration process while oxygen is reduced to CO$_2$ and ATP released as high energy molecule, oxygen is also reduced to highly reactive superoxide anion by leaking of single electron (Fridovich, 1970)(Figure 1). Some enzymes produce free radicals by reducing oxygen to the superoxide radical. Xanthine oxidase, NADPH oxidase, myeloperoxidase, thyroid peroxidase and nitric oxide synthase are well known potential producers of ROS in vivo (Halliwell, 1999).

![Figure 1.1: The pathway of oxygen reduction during cellular respiration](image)

The primary form of ROS, superoxide anion ($O_2^-$), is produced by addition of one electron to dioxygen ($O_2$). This free radical can be converted to secondary reactive oxygen species such as hydroxyl ($^0$OH), peroxyl radical (ROO$^-$), hydrogen peroxide ($H_2O_2$), nitrous oxide, peroxynitrite, nitroxy anion and peroxynitrous acid.

At physiological concentrations, reactive oxygen species have biopositive effects and take place in many chemical reactions in the cell. They also act as a cell messenger in the regulation of cellular signalling mainly by oxidatively...
modifying compounds (Joseph and Cutler, 1994). Free radicals also take part in phagocytosis process which is mediated by neutrophils and macrophages (Rosen et al., 1995).

1.2 Antioxidant Defence Systems

The body has developed antioxidant defense systems by scavenging and minimizing the formation of oxygen derived radicals to protect itself from oxidative damage. Antioxidant defense mechanisms operate by three mechanisms by using antioxidants; prevention, interception and repair (Sies, 1997). Antioxidant protection systems consist of dietary antioxidants, endogenous enzymatic and non-enzymatic constituents (Table 2).

Enzymatic antioxidants catalytically remove reactive oxygen species from biological systems. Highly reactive superoxide anion (O\(_2^-\)) is converted to hydrogen peroxide (H\(_2\)O\(_2\)) by Superoxide Dismutase (SOD) (EC 1.15.1.1) in order to prevent this radical forming highly reactive hydroxyl radical. H\(_2\)O\(_2\) is then eliminated in the presence of catalase (EC 1.11.1.6) and glutathione peroxidase (GP\(_X\)) (EC 1.11.1.9).

\[
\text{SOD} \quad 2 (O_2^-) + 2H^+ \rightarrow H_2O_2 + O_2 \\
\text{Catalase} \quad H_2O_2 \rightarrow H_2O + \frac{1}{2} O_2
\]

Glutathione peroxidase catalyses the reduction of hydrogen peroxide and lipid peroxide and removes peroxyl (ROO\(^-\)) radicals from various peroxides by using reduced glutathione (GSH) as an electron donor. Glutathione reductase regenerates reduced glutathione (GSH) from oxidized glutathione (GSSG) as shown in the following equation;

\[
\text{GP}_X \quad 2\text{GSH} + H_2O_2 \rightarrow \text{GSSG} + 2H_2O \\
\text{reductase} \quad \text{GSSG} + \text{NADPH} + H^+ \rightarrow 2\text{GSH} + \text{NADP}^+
\]

Dietary antioxidants are usually present in the form of vitamin C, vitamin E, beta-carotenes, carotenoids, and flavonoids (Sies, 1993). Metal-binding proteins such as albumin, ceruloplasmin, metallothionein, transferrin, ferritin, and myoglobin act as an antioxidant by inactivating pro-oxidant transition metal ions (Makker et al., 2009).
Antioxidant defense system differ from tissue to tissue and cell-type to cell-type (Halliwell, 1999). The antioxidant system and their importance in semen will be discussed in the following sections.

Table 1.2: Most important in vivo antioxidants

<table>
<thead>
<tr>
<th>ENZYMATIC ANTIOXIDANTS</th>
<th>NON-ENZYMATIC ANTIOXIDANTS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Superoxide Dismutase (SOD)</td>
<td>Metal Binding Proteins</td>
</tr>
<tr>
<td>Catalase (CAT)</td>
<td>Albumin</td>
</tr>
<tr>
<td>Glutathione Peroxidase (GPX)</td>
<td>Transferrin</td>
</tr>
<tr>
<td>Glutathione-S-transferase (GST)</td>
<td>Lactoferrin</td>
</tr>
<tr>
<td></td>
<td>Ceruloplasmin</td>
</tr>
<tr>
<td></td>
<td>Haptoglobin</td>
</tr>
<tr>
<td></td>
<td>Inorganic anions</td>
</tr>
<tr>
<td></td>
<td>Vitamin C (Ascorbic acid)</td>
</tr>
<tr>
<td></td>
<td>Vitamin E (alfa-tocopherol)</td>
</tr>
<tr>
<td></td>
<td>Melatonin</td>
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<tr>
<td></td>
<td>Lipoic Acid</td>
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<tr>
<td></td>
<td>Uric Acid</td>
</tr>
<tr>
<td></td>
<td>Bilirubin</td>
</tr>
<tr>
<td></td>
<td>Coenzyme Q</td>
</tr>
<tr>
<td></td>
<td>Cytochrome C</td>
</tr>
<tr>
<td></td>
<td>Glutathione</td>
</tr>
</tbody>
</table>

1.3 Oxidative Stress

Oxidative stress (OS) is a situation when there is an imbalance between production of reactive oxygen species and antioxidant defence system in favour of the former (Sies, 1997). Oxidative stress can cause damage to all types of biomolecules, including DNA, lipids and proteins. This oxidative damage can result in DNA strand breakage, cell membrane lipid peroxidation and amino acid modifications respectively (Halliwell, 1999). Oxidative stress has been linked with many degenerative processes, diseases, syndromes and ageing processes in human being (Cutler, 1991; Davies, 1995).

1.3.1 Oxidative Stress and Male Infertility

Excessive production of reactive oxygen species (ROS) are thought to be critically involved in the aetiology of male infertility (Aitken, 1989) and have been identified in 30% to 80% of infertile male’s semen (de Lamirande and Gagnon, 1995b; Tremellen, 2008). Increasing levels of oxidative stress in semen arise from high level of ROS generation or inadequate antioxidant protection capabilities of the male reproductive tract or seminal plasma (Aitken et al., 1992; Iwasaki and Gagnon, 1992).
1.3.2 The physiological role of Reactive Oxygen Species in sperm cells

At physiological concentrations, ROS participates in signal transduction mechanisms in sperm such as capacitation and the acrosome reaction (de Lamirande et al., 1997). Studies have shown that the exposure of low levels of reactive oxygen species to spermatozoa results in higher hyperactivation and capacitation than the control groups (de Lamirande and Gagnon, 1993; Griveau et al., 1994; Zini et al., 1995). Mild oxidative conditions also help promote binding of spermatozoa to the zona pellucida via lipid peroxidation of sperm membrane (Aitken, 1989). Hydrogen peroxide eases the sperm’s transit through the cumulus and zona pellucida by causing tyrosine phosphorylation and stimulating the acrosome reaction (Aitken et al., 1995b; de Lamirande et al., 1997). Therefore it is apparent that while low level of exposure to ROS are necessary for normal sperm function, high ROS exposure can be harmful.

1.4 Sources of Reactive Oxygen Species in semen

Semen consists of several different types of cells such as mature and immature spermatozoa, round cells from different stages of the spermatogenic process, leukocytes and epithelial cells. Of these, peroxidase positive leukocytes and abnormal spermatozoa are thought to be main sources of reactive oxygen species in the ejaculate (Aitken et al., 1994; Aitken and West, 1990; Cocuzza et al., 2007; Hendin et al., 1999; Mazzilli et al., 1994; Ochsendorf, 1999; Whittington and Ford, 1999).

1.4.1. Production of ROS by Leukocytes in Semen

Leukocytes in semen have been suggested to have the physiological role of removal of dead or abnormal spermatozoa (Tomlinson et al., 1992b). There are many different leukocyte subpopulations originating from different parts of the male reproductive tract. The predominant leukocyte sub-types in semen are polymorphonuclear leukocytes (50-60%), macrophages (20-30%) and T-lymphocytes (2-5%). The former two cell groups are the main producers of free radicals in semen (Fedder et al., 1993; Wolff, 1995; Wolff and Anderson, 1988). Due to the blood-testis barrier, leukocytes are excluded from direct contact with sperm except in the area where the barrier disappears in the rete testis and efferent ducts (Pollanen and Cooper, 1994). Thus the rete testis epithelium is occupied by CD8+ suppressor lymphocytes indicating active immune regulation for suppression of autoimmune attack on spermatozoa.
Lymphocytes and macrophages are mostly located in the epididymis as well as in the epithelium of the vas deferens, yet granulocytes originate from prostate and seminal tissue (Wolff, 1995). Activated leukocytes are capable of producing 100-fold higher amounts of ROS than non-activated leukocytes (Plante et al., 1994) and these activated leukocytes produce 1000-times more reactive oxygen species than spermatozoa (de Lamirande and Gagnon, 1995a). However, the relative importance of leukocytes and sperm in inducing oxidative stress in male infertility is still unclear.

1.4.2. Production of ROS by Spermatozoa

Spermatozoa were the first cell type shown to generate reactive oxygen species in human. In 1943 MacLeod observed that, when spermatozoa were incubated under high oxygen tension, they lost their motility capability and yet this impairment in sperm function was blocked by adding catalase, a hydrogen peroxide scavenger (MacLeod, 1943). Future studies in which contaminating leukocytes were separated from sperm using density gradient centrifugation and CD45 antibody absorption columns have confirmed that sperm themselves are capable of ROS generation (Baker et al., 2003). Generation of ROS by sperm cells can occur via NADH (nicotinamide adenine dinucleotide) dependent oxidase system (NOX5) at the level of plasma membrane and the NADH dependent oxido-reductase (diphorase) system at the mitochondrial level (Aitken et al., 1997). Additionally lipoxygenase (Oliw and Sprecher, 1989) and cytochrome p450 reductase (Baker et al., 2004) systems are other proposed ROS-generating systems in sperm cells. According to a recent study, the major source of ROS in sperm is mitochondrial, with excessive ROS production in sperm mitochondria being significantly correlated with a decrease in motility by inducing permanent damage in the sperm motility apparatus (Koppers et al., 2008).

The Superoxide anion is produced by sperm cells at capacitation and the sustained production of superoxide anion is necessary for the development of sperm activation and capacitation (de Lamirande and Gagnon, 1995a) The maturation development of sperm is conversely related to their production of ROS. Immature abnormal spermatozoa with the excess cytoplasm create intrinsic oxidative stress (Henkel et al., 2005). Abnormal retention of excess residual cytoplasm during the final stages of spermiogenesis can result in increase production of free radicals by the high amount of cytoplasmic enzymes
such as Creatine kinase (CK), lactic acid dehydrogenase (LDH) and Glucose 6-phosphate dehydrogenase (G-6-PDH) which produce ROS from generating NADPH (Aitken et al., 1994; Aitken et al., 1995b; Huszar and Vigue, 1993; Rao et al., 1989).

1.4.3 Susceptibility of sperm cells to Oxidative Stress

Sperm cells are more vulnerable to ROS than somatic cells due to their unique cell structure. Spermatozoa contain large number of polyunsaturated fatty acids in their plasma membrane (Jones et al., 1979), as this provides fluidity for the sperm cells membrane that is necessary for membrane fusion events such as the acrosome reaction, sperm-egg interaction and also motility. However, the unsaturated nature of these molecules predisposes them to free radical attack.

Another characteristic of sperm cells that makes them vulnerable to ROS attack is their lower amount of cytoplasmic ROS-scavenging enzymes due to their limited cytoplasmic volume (Zini et al., 1993). This restricted cytosolic space has limited capacity for protein synthesis and little antioxidant capacity, which makes them deficient of antioxidant protection enzymes such as superoxide dismutase, catalase or glutathione peroxidase in condition of excessive ROS availability (Aitken, 1989; Zini et al., 1993).

1.5 Protection of Spermatozoa from ROS

Human sperm differs from somatic cells in terms of their structure and composition (Lewis and Aitken, 2005). Being vulnerable to the exogenous factors such as oxidative stress, sperm cells have to be organized by specific protection mechanisms in order to protect their genomic integrity during transport of the paternal genome to the female. In the following section, I will outline these protection mechanisms of spermatozoa; including their unique organisation of DNA packaging and intrinsic antioxidant mechanisms.

1.5.1 Sperm Chromatin Structure

Packaging of DNA in sperm is significantly different from somatic cells because sperm have limited volume in their nucleus (Fuentes-Mascorro et al., 2000). The spermatid chromatin structure is completely reorganized throughout spermiogenesis and the DNA is repackaged by small proteins called protamines. The packaging of sperm DNA by protamines enables the DNA to be compressed into a small volume (Ward and Coffey, 1991) and this condensed structure of DNA not only provides for transferring of the very tightly
packaged genetic information to the egg but also helps protect the paternal DNA from oxidative attack (Fuentes-Mascorro et al., 2000; Ward and Coffey, 1991).

During the later stages of spermatogenesis, histone proteins are displaced with transition proteins and then protamines are added (Poccia, 1986). The replacement of histones by protamines results in the condensation of DNA into compact doughnut form (Diagram 1.1). Protamines are very basic proteins about half the size of histone proteins (Fuentes-Mascorro et al., 2000) and are rich in arginines that permit strong DNA binding. Packaging of DNA with protamines in place of somatic histones greatly condenses the DNA, preventing RNA transcription, and results in lower accessibility to DNA-damaging agents (Evenson et al., 2002). Protamines also contain a large number of cysteine residues which provide multiple intra and inter-protamine cross-links. The DNA strands are tightly wrapped around the protamine molecules and toroids are cross-linked by disulphide bond formation which provide further enhancement for the stability of the nucleus during epididymal passage and the final stages of sperm nuclear maturation (Loir and Lanneau, 1984).

All these interactions play a regulatory role in making sperm DNA the most condensed eukaryotic DNA (Ward and Coffey, 1991). This nuclear compaction and stabilization is important to protect sperm genome from free radical attack. Incomplete protamine packaging of the sperm chromatin will increase the sperm DNA’s vulnerability to damage.
1.5.2 Antioxidant Mechanisms in semen

Seminal plasma and spermatozoa themselves possess endogenous antioxidants for protecting spermatozoa from oxidative damage (Gagnon et al., 1991; Zini et al., 1993). These antioxidants are broadly divided into enzymatic and non enzymatic groups.

1.5.2.1 Enzymatic Antioxidants

Three main enzymatic antioxidants exist in seminal plasma: superoxide dismutase (SOD), catalase, and glutathione peroxidase/glutathione reductase (GPX/GRD). Spermatozoa themselves predominantly possess these enzymatic antioxidants (Zini et al., 1993).

SOD protects spermatozoa against spontaneous $O_2$ toxicity and lipid peroxidation (Alvarez et al., 1987). Seminal SOD and catalase remove ($O_2^-$) which is generated by NADPH oxidase in neutrophils and may play an
important role in decreasing lipid peroxidation as well as protecting spermatozoa during genitourinary inflammation (Aitken et al., 1995b).

Glutathione peroxidase (GPx) acts as an antioxidant by converting hydrogen peroxide to H$_2$O using glutathione as electron donor and is found in spermatozoa, testis, prostate and the epididymis (Vernet et al., 2004). GPX is mostly concentrated in the mitochondria, nucleus and acrosomal domain of differentiating spermatozoa (Vaisberg et al., 2005). There are 5 different isoforms of GPx in the different anatomical sites (Meseguer et al., 2004). GPx1 is found in sperm and the genital tract and is related with sperm motility (Dandekar et al., 2002). GPx4 is a selenium dependent form found in testicular tissue and acts as a peroxidase to protect sperm from oxidative attack (Foresta et al., 2002). Gpx4 is essential to build up the mitochondrial capsule for midpiece structure of sperm by protamine disulfide bridging (Foresta et al., 2002). Another form of Glutathione peroxidase, GPx5, has also been found in epididymis (Chabory et al.) GPx5 is particularly seen in the caudal luminal compartment in the epididymis and the lack of expression or secretion of this isoform causes an oxidative stress that alters integrity of spermatozoa (Chabory et al., 2009). GPx5, unlike the other isoforms of GPx does not require selenium for activity.

**1.5.2.2 Non-enzymatic Antioxidants**

Total seminal antioxidant activity is also supplemented by many non-enzymatic antioxidants in semen (Zini et al., 1993). These include minerals, vitamins, aminoacids and protein compounds such as zinc, vitamin E and C, albumin, glutathione, taurine, hypotaurine, carnitine, carotenoids, urate and prostasomes (Makker et al., 2009). These antioxidants act by directly neutralising free radical activity chemically either by becoming oxidized themselves or reducing free radical production directly (Tremellen, 2008).

Zinc is one of the most important antioxidant mineral in seminal plasma. Firstly, zinc is the core constituent of Superoxide dismutase enzyme. Secondly, zinc can also protect against oxidation by inhibition of the production of reactive oxygen with displacing transition metals (Bray and Bettger, 1990). Finally, zinc is bound to protamine 2 in the sperm cell and therefore it is essential for stabilizing sperm DNA against oxidative attack by augmenting chromatin packaging (Gatewood et al., 1990). According to some in vivo experiments, sperm with low chromatin zinc content express improper
chromatin condensation (Bjorndahl and Kvist, 1985; Kjellberg et al., 1992), with dietary zinc deficiency being responsible for altered protamine process in sperm cells (Evenson et al., 1993).

Vitamin E (α-tocopherol) is a powerful “chain breaking “free radical scavenger that plays an important role in preventing sperm membrane lipid peroxidation damage (Johnson, 1979).

Vitamin C exists in sertoli cells and spermatozoa at high amount. Vitamin C recycles Vitamin E, helping keep it in its active state (Yoganathan et al., 1989). Vitamin C also neutralizes the oxidative effects of pro-oxidants such as cadmium, arsenic and alcohol (Chang et al., 2007; Maneesh et al., 2005).

Prostasomes are secreted from the prostate into the seminal fluid and have important effects in regulating sperm viability and function such as motility, acrosome reaction and zona penetration. Prostasomes are rich in cholesterol and fuse with sperm cells which affect sperm fluidity (Kravets et al., 2000).

Carotenoids (beta-carotene) and ubiquinols may also play a role in quenching singlet oxygen and reducing lipid derived free-radicals with detrimental effects on sperm lipid peroxidation (Sikka et al., 1995).

Lycopene is a lipophilic carotenoid with antioxidant and pro-oxidant properties (Stahl and Sies, 1996; Young and Lowe, 2001). A recent study has shown that lycopene supplementation in vitro can protect sperm from oxidative DNA damage and has beneficial effects on sperm motility (Zini et al.).

Urate is another chain-breaking antioxidant existing in seminal plasma. It prevents metal (Fe\(^{2+}\)) driven free radical reactions by binding transition metals and scavenge peroxyl and OH\(^{-}\) radicals (Lewis et al., 1997).

Carnitine is an antioxidant which is biosynthesized from lysine and methionine amino acids (Steiber et al., 2004). It is required for the utilization of long chain fatty acids to produce energy. Carnitine is found in epididymal plasma at high concentrations and therefore has an important role preventing the formation of lipid peroxidation. Carnitine plays a role for the maturation of spermatozoa and provides readily available energy within the male reproductive tract (Dokmeci, 2005). The use of carnitine for treatment of male infertility has been conducted.
in a number of controlled and uncontrolled studies and shown beneficial effects in improvement in sperm quality (Agarwal and Said, 2004; Lenzi et al., 2003).

1.6 Oxidative Stress in Male Infertility

1.6.1 Pathophysiology of Oxidative Stress in Spermatozoa

Increased seminal ROS production has been associated with impaired sperm motility, (Griveau and Le Lannou, 1997; Plante et al., 1994), membrane function including the acrosomal reaction (Sharma and Agarwal, 1996; Zalata et al., 2004) and integrity of the sperm genome (Lewis and Aitken, 2005; Ollero et al., 2001). (Figure 1.2)

Effects of ROS in sperm cells may vary depend on factors such as the amount of ROS produced, oxygen tension, site of interaction with ROS and duration of exposure to ROS, as well as surrounding environmental factors such as activation of leukocytes, concentration of molecular components and available antioxidative systems in the seminal plasma (Agarwal and Saleh, 2002; Cocuzza et al., 2007).

It has been reported that a low concentration of hydrogen peroxide has not shown any adverse effect on sperm motility but inhibits sperm-oocyte interaction (Aitken et al., 1993a). This may explain why some patients with normal sperm parameters can still have impaired fertility. In such patients, the ROS levels are not high enough to impair basic semen analysis parameters but can cause defects in other functioning which is required for fertilization.

At higher concentrations of ROS, oxidative damage to polyunsaturated fatty acid in the sperm plasma membrane occurs and this then initiates a lipid peroxidation cascade (Storey, 1997). When lipid peroxidation occurs, lipid peroxides accumulate on the sperm surface and this reduces the fluidity of the sperm phospholipid membrane making the sperm flagella work less effectively, thereby reducing motility (Alvarez et al., 1987; Twigg et al., 1998a).

Excessive leakage of ROS from mitochondria in the midpiece may also damage the mitochondrial power source of sperm motility hence cause decreased motility. Oxidative stress therefore has the ability to produce infertility impairing sperm transit through the female reproductive tract. Furthermore, damage to the acrosomal membrane diminishes acrosin activity (Zalata et al.,
and impedes the capacity of the sperm to fuse with the oocyte, consequently induce poor fertilisation capacity (Aitken, 1989; Jedrzejczak et al., 2005).

Figure 1.2: Reactive Oxygen induced damage in sperm cell
In addition, reactive oxygen species cause depletion of intracellular ATP. In turn, due to insufficient protein phosphorylation, a decrease in beat frequency and an increase in axonemal damage lead to sperm immobilization (de Lamirande and Gagnon, 1992a; de Lamirande and Gagnon, 1992b; Griveau and Le Lannou, 1997).

Oxidative stress can also have a harmful effect on sperm DNA integrity (Hughes et al., 1996; Kodama et al., 1997; Lopes et al., 1998). Oxidative DNA damage can occur through either oxidation of DNA bases primarily by direct attack on the purine and pyrimidine bases or through causing strand breaks and cross-linking in sperm DNA (Barroso et al., 2000; Kunsch and Medford, 1999; Olinski et al., 1992; Twigg et al., 1998a; Twigg et al., 1998c). In vitro induced oxidative stress significantly increases DNA fragmentation, modification in base structure, deletions and frame shifts in sperm chromatin (Aitken et al., 1998; Dizdaroglu, 1992; Hughes et al., 1996; Kemal Duru et al., 2000).

Mitochondrial exposure to ROS provokes apoptotic process through release of apoptosis inducing factor (AIF) which also cause DNA fragmentation (Cande et al., 2002). High level of ROS agitates mitochondrial membrane polarization and activates release of the cytochrome-C protein as a trigger of apoptosis (Wang et al., 2003).

Oxidative stress has been indirectly connected to the clinical consequences of sperm DNA damage such as increased miscarriage risk (Benchaib et al., 2007; Borini et al., 2006; Virro et al., 2004). Oxidative DNA damage has also been linked with poor embryonic development (Sakkas et al., 1998) and possibly even an increase risk in childhood cancer (Lewis and Aitken, 2005).

The effects of oxidative DNA damage in sperm on the health and wellbeing of the offspring have not yet been fully clarified but still there is a possibility that spermatozoa significant with DNA damage can achieve fertilization and full term pregnancy (Ahmadi and Ng, 1999; Gandini et al., 2004; Twigg et al., 1998c).
1.7 Origins of Oxidative Stress

Oxidative stress can possibly arise from a number of endogenous and exogenous factors or interaction between each. The common causes of oxidative stress include lifestyle and environmental factors, infection, varicocele, autoimmune related conditions, elevated testicular temperature, chronic diseases and drugs as summarized in Figure 1.3.

Figure 1.3 The origins of Oxidative Stress in semen
1.7.1. Lifestyle Factors

1.7.1.1. Smoking

Smoking has adverse effect on sperm quality parameters with lower sperm counts, decreased motility and increased abnormal sperm morphology (Kunzle et al., 2003; Saleh et al., 2002; Sofikitis et al., 1995). Studies have shown that smokers have also higher degree of oxidative stress in their semen, as reflected by an increase in ROS production and a reduction in seminal plasma antioxidants (Saleh et al., 2002; Sepaniak et al., 2006). Smokers have lower seminal plasma antioxidant capacity compare to non-smokers (Fraga et al., 1996). The adverse effect of smoking on the developing and mature sperm cells may be mediated by increasing seminal leukocyte derived ROS production (Agarwal et al., 2003; Saleh et al., 2002). By increasing ROS levels, it has been postulated that smoking damages the chromatin structure and produces endogenous DNA strand breaks in sperm (Potts et al., 1999; Sepaniak et al., 2004). These alterations may result in sperm DNA mutations, which predispose offspring to greater risk of malformations, cancer and genetic diseases. Paternal smoking has been reported to be responsible for 15% of childhood cancer (Sorahan et al., 1997).

Oxidative DNA damage in sperm can be related to reduction in antioxidant capacity and decreased level of vitamin C (Mostafa et al., 2006; Song et al., 2006) and Vitamin E (Fraga et al., 1996). Smokers have also 50% of increased level of 8-OHdG in their seminal plasma compared to non-smokers (Fraga et al., 1996).

1.7.1.2. Alcohol Consumption

Alcohol is known as a promoter of ROS production and interferes with the body’s antioxidant defence mechanisms especially in the liver (Wu and Cederbaum, 2003). By stimulating the activity of cytochrome P450s, alcohol contributes to excess ROS production and increases systemic oxidative stress as well as causing deficiency in protective antioxidant level in the body (Koch et al., 2004; Wu and Cederbaum, 2003).

Studies have linked excess alcohol consumption with decreased sperm motility (Gaur et al.; Villalta et al., 1997). Although the link between alcohol intake and oxidative damage in sperm has not been fully clarified yet, it is likely that
alcohol causes systemic oxidative stress, which would extend to the male reproductive tract causing decreased sperm motility.

1.7.1.3. Advanced Paternal Age

According to many studies, systemic oxidative stress increases with age (Junqueira et al., 2004). One study has been shown that oxidative stress is more common in men older than 40 years of age compared to men younger than 40 years of age (Cocuzza et al., 2008).

There is suggestive epidemiological evidence that increased paternal age is related with the abnormal reproductive outcomes and defects in resulting children (de la Rochebrochard and Thonneau, 2002; Tarin et al., 1998). Men of advanced age seem to produce more sperm with certain type of gene mutations (Wyrobek et al., 2006) and increased DNA fragmentation (Morris et al., 2002; Moskovtsev et al., 2006; Singh et al., 2003; Spano et al., 1998).

It has been reported that there is a link between increasing paternal age and pregnancy loss (de la Rochebrochard and Thonneau, 2002; Risch et al., 1987), birth defects (Lian et al., 1986), gene mutations such as achondroplasia (FGFR3 gene mutation) (Crow, 2000; Tiemann-Boege et al., 2002); various aneuploidy and chromosomal syndromes (Sloter et al., 2004) and also the possibility of an increased risk of prostate cancer in next generation (Zhang et al., 1999). While these gene mutations in older men might be due to replication errors during germ cell division, it is possible that the observed increase in DNA mutations with increasing age are due to the concomitant increase in oxidative stress with age (Junqueira et al., 2004). Furthermore, chromatin packaging in spermatozoa is impaired with advanced age, thereby increasing the susceptibility of spermatozoal DNA to oxidative damage (Zubkova et al., 2005).

1.7.1.4. Psychological Stress

Psychological stress can also affect sperm quality. The underlying mechanism of psychological stress related semen quality alterations has not yet been clarified, but a recent study has reported that psychological stress can cause an increase of NO production and a decrease of arginase activity in the L-arginine-NO pathway (Eskiocak et al., 2006). According to these researchers there is a decreased antioxidant protection due to loss of glutathione and free sulphydryl
content of seminal plasma in males with physiological stress. Also this study showed a significant decrease in sperm motility and an increase in the percentage of abnormal morphology during the stress period, suggesting that oxidative stress may be the underlying cause for abnormal sperm parameters in the stress state (Eskiocak et al., 2005).

Extreme exercise activity can also cause reduced sperm quality. This may be due to increased oxidative stress in the general body system after exercise. During exercise, high amounts of ROS are produced via increased aerobic metabolism and muscle damage (Peake et al., 2007). A rodent study has reported that increased muscle activity result in reduced sperm count and motility with the biochemical signs of increased testicular oxidative stress (Manna et al., 2004).

1.7.2. Infections

1.7.2.1. Male Accessory Sex Gland Infection (MAGI)

Male accessory sex gland infections (MAGI) are seen in 5-12 % of cases of male infertility and associated with biological and biochemical changes in the seminal plasma (Comhaire et al., 1999). These alterations can originate from seminal white blood cells (WBC) and their secretory products which have possible adverse effects on the motility and fertilizing potential of spermatozoa. Leukocytospermia is an important indicator of inflammation in the male genital tract (Krause et al., 2003). Bacteria and viruses cause an acute inflammation and induce oxidative stress through the inflow of activated leukocytes into the affected area (Depuydt et al., 1996). According to the WHO criteria, male genital tract inflammation is defined if the patient has more than 1x10^6 leukocyte in per millilitre in the ejaculate (WHO, 2001).

Many studies have been reported that obvious decrease of semen parameters and fertility can be seen as a result of a high level of leukocytes in semen (Gonzales et al., 1992; Henkel et al., 2003; Hill et al., 1994) while others have reported no correlation between leukocytospermia and semen quality (Eggert-Kruse et al., 2001; Harrison et al., 1991). Moreover, many cases of accessory sex gland infection are an asymptomatic and clinical symptoms are not always correlated with the presence of leukocytes (Hochreiter, 2003).
Presently, the prevalence and clinical significance of leukocytes in semen is currently a matter of controversy. As main producers of ROS, leukocytes make a significant contribution to overall levels of ROS in human semen (Aitken and West, 1990). Furthermore, infiltrating leukocytes can lower the antioxidant capacity of seminal plasma by consuming antioxidants (Aitken and Baker, 1995; Sharma et al., 2001).

The leukocyte induced oxidative damage in sperm cells may depend on the number and subtype of leukocyte, as well as their activation status. Leukocytes and their involvement to the oxidative stress related infertility will be discussed further in Chapter 4.

1.7.3. Environmental Factors

1.7.3.1. Toxins and Chemicals

There are many epidemiologic studies examining the reproductive effects of environmental toxin exposure on sperm and testicular function. The existence and increase incidence of spontaneous abortions has been linked to paternal exposures to some chemical agents. Some of these reproductive toxins cause sperm oxidative damage and DNA fragmentation. These chemical agents include many commonly present occupational hazards such as anaesthetic gases, metals (mercury, lead and cadmium), solvents, pesticides, and hydrocarbons.

In vivo studies suggest that exposure to lead and cadmium causes generation of ROS and alteration of antioxidant defence systems in animals and occupationally exposed workers (Acharya et al., 2003; Benoff et al., 2000; Hsu and Guo, 2002). Cadmium (Cd) is known to produce oxidative stress and is mainly used in the battery, metal-coating, and alloy industries. In addition to these industrial uses, it also presents in cigarette (Spruill et al., 2002). Occupational exposure to lead and cadmium has genotoxic effect in somatic cells of workers in battery manufacturing area by promoting DNA damage (Palus et al., 2003).

It has been demonstrated that air pollution causes male-mediated infertility, miscarriage, and other adverse reproductive outcomes which is due to increased sperm DNA fragmentation (Evenson and Wixon, 2005; Rubes et al., 2005).
1.7.3.2. Electromagnetic Radiation

An exposure to electromagnetic radiation has a negative impact on semen quality (Agarwal et al., 2009; Aitken et al., 2005). In vitro studies have shown that electromagnetic radiation including using regular mobile phone use induces reactive oxygen species production and DNA damage in human spermatozoa (De Iuliis et al., 2009). Use of cell phones decreases motility and vitality of sperm cells as well as the concentration depending the duration of exposure to radiation (Agarwal et al., 2008). Electromagnetic radiation enhances mitochondrial ROS generation, while stimulating DNA base adduct formation (De Iuliis et al., 2009).

There is also data linking with exposure to electromagnetic radiation from regular mobile phone use and a genotoxic effect on epididymal spermatozoa that can cause DNA damage in male germ cells (Aitken et al., 2005).

1.7.3.3. Drugs and Chemotherapy

There are hundreds of chemicals, including chemotherapeutics (eg. fludarabine, cyclophosphamide, busulphan) which are known to have detrimental effects on sperm morphology, number, and motility (Chatterjee et al., 2000; Wyrobek et al., 2005).

Commonly used drugs such as aspirin and paracetamol can cause oxidative stress by producing excessive reactive oxygen species through increased cytochrome p450 activity (Tremellen, 2008). Some chemotherapeutic drugs like cyclosporine and cyclophosphamide has been linked to increased level of oxidative stress in sperm cells and decreased testicular catalase level in rats (Das et al., 2002; Ghosh et al., 2002). Allopurinol used to treat gout reduces seminal plasma levels of the antioxidant uric acid and may also cause oxidative stress.

Cocaine has also been demonstrated to induce increase in apoptosis and in turn oxidative DNA damage (Li et al., 1999).

1.7.4. Iatrogenic

The increasing use of ART (Assisted Reproductive Technologies) and handling of sperm for insemination in in vitro conditions expose them to oxidative damage for two reasons (Alvarez, 2003). Firstly, centrifugation and cryopreservation during ART procedures has been reported to increase sperm ROS production (Agarwal et al., 2006). Secondly, removal of protective antioxidants in seminal plasma during sperm preparation can also be
hazardous to sperm health (Zorn et al., 2003b). It has been demonstrated that samples prepared by swim-up semen rather than sperm cells isolated by density gradient prepared semen has reduced levels of ROS and sperm DNA damage, without exhibiting any major change in sperm motility (Twigg et al., 1998b).

1.7.5. Varicocele

Varicocele is a dilation of the spermatic vein/pampiniform venous plexus and is present in about 40% of the infertile male population but only 15% of the fertile population. While varicocele are known to cause testicular dysfunction and consequently sperm DNA damage and infertility (Saleh et al., 2003), the exact pathogenetic mechanism for this is not conclusively known. The proposed mechanisms are reflux of toxic metabolites of adrenal or renal origin, elevation of testicular temperature and elevated reactive oxygen species. While several reports has conclusively linked varicocele with elevated levels of free radicals (Agarwal et al., 2003; Zini et al., 2000), it is not understood what activates this increase in free radical production. Men with varicoceles have been found to have elevated levels of sperm DNA fragmentation compared to fertile men without varicocele (Saleh et al., 2003). Presumably the elevation in seminal free radical levels is responsible for this varicocele related sperm DNA fragmentation.

1.8 Assessment of Seminal Oxidative Stress

If we accept that oxidative stress is a major cause of male infertility, measurement of ROS in semen becomes necessary for proper investigation of male factor infertility. However, routine semen analysis is the only test that has been used for male factor evaluation in most clinics and provides limited information about the status of sperm oxidative stress. This is a major deficiency in modern clinical andrology.

While there are over 30 published assays to measure oxidative stress, all are complex and time consuming assays, often requiring expensive equipment and therefore are not practical for the average small andrology laboratory. Direct measurement of reactive oxygen species in vivo is difficult due to their short life span and can not be used for clinical use since they need relatively expensive technologies (Cocuzza et al., 2007). Direct methods such as electron-spin resonance spectroscopy (Buettner and Mason, 1990) and pulse
radiolysis (Asmus, 1984) can only be performed for research purposes with the necessity of high technical experience.

The most commonly used indirect method for measurement of ROS in sperm cells are global ROS measurement by chemiluminescence (Sharma and Agarwal, 1996) and membrane lipid peroxidation determination using Malondialdehyde (MDA) and thiobarbituric acid (TBA) (Aitken et al., 1993b; Gutteridge and Quinlan, 1983).

### 1.8.1 ROS Measurement by Chemiluminescence

The chemiluminescence method quantifies both intracellular and extracellular ROS by using sensitive probes such as luminol (5-amino-2, 3, dihydro 1, 4, phthalazinedione) and lucigenin for quantification of redox activities of spermatozoa (Aitken et al., 2004). The reaction of these probes with ROS results in production of a signal which is converted to an electrical signal and measured as counted photons per minute by luminometer (Agarwal et al., 2004). Luminol probe is an extremely sensitive and oxidisable substrate and reacts various ROS at neutral pH, thereby allowing for the measurement of intracellular and extracellular ROS. Lucigenin is superoxide anion sensitive and can only measure extracellular ROS generation (McKinney et al., 1996).

Measurement of ROS using chemiluminescence is relatively sensitive and has well established ranges in infertile and fertile population (Athayde et al., 2007; Tremellen, 2008; Williams and Ford, 2005). On the other hand, this technique has some disadvantages for clinical use, such as the need for expensive equipment and difficulties in quality control due to leukocyte and seminal plasma contamination (Aitken et al., 2004; Fingerova et al., 2009; Kobayashi et al., 2001)

### 1.8.2 Lipid Peroxidation Markers

Markers for lipid peroxidation have been widely used for oxidative stress measurement in sperm cells. Oxidative stress causes an accumulation of lipid peroxides in the spermatozoa and generates of a variety of decomposition end-products of unsaturated lipids such as Malondialdehyde (MDA), HNE (Hydroxynonenal), 2-propanal (acrolein) and isoprostanes, all which can be measured as indicators of oxidative stress (Dalle-Donne et al., 2006). Malondialdehyde is the most common used marker for the lipid peroxidation and can be quantitatively assessed by the thiobarbituric acid-reacting substances (TBARS) Assay. In this assay, MDA forms a 1:2 adduct with
thiobarbituric acid (TBA) and produces the coloured substance which can be measured by fluorometry or spectrophotometry (Aitken et al., 1993b). The TBARS assay is considered to be a nonspecific marker of membrane lipid peroxidation, likely due to the reaction of thiobarbituric acid with non-lipid moieties (Yeo et al., 1994). MDA level can also be directly measured in both sperm cells and seminal plasma, but the measurement of MDA in sperm cells needs to be performed by sensitive high performance liquid chromatography (HPLC) due to its low existency (Li et al., 2004).

The recent assays for measurement of lipid peroxidation such as isoprostane 8-iso-PGF2a and c11-BODIPY are promising but need more research to confirm their usefulness as a marker of sperm oxidative stress status. (Drummen et al., 2002; Pratico et al., 2001)

1.8.3 Measurement of Oxidative DNA Damage

Free radicals attacks DNA structure by generating base and sugar modification products (Dizdaroglu et al., 2002). The most commonly studied product for oxidative DNA damage is 8-hydroxy-20-deoxyguanosine (8-OHdG) (Halliwell and Whiteman, 2004). This oxidative DNA adduct has been used as an index of oxidative DNA damage due to its high sensitivity and abundance in DNA. 8-OHdG can be assessed by HPLC, Gas chromatography (GC) or liquid chromatography (LC), Mass Spectrometry (GC) and antibody based techniques (Dizdaroglu et al., 2002). The analytical methods to detect these markers are technically challenging and artifactual DNA damage may occur during isolation (Collins et al., 2004; Halliwell and Whiteman, 2004). Immunochemical methods visualise of the damage but they are limited for being only semi quantitative (Halliwell and Whiteman, 2004). Oxidative DNA damage can be measured indirectly by assessing DNA damage in sperm. Several tests are available for the measurement of sperm DNA integrity such as SCSA (Sperm Chromatin Structural Assay), terminal deoxynucleotidyl transferase-mediated nick end labeling (TUNEL Assay) and Chromomycin A3 Assays. However, all of these assays measure DNA damage that is not necessarily directly related to oxidative attack

1.8.4 Other Markers for ROS Measurement

Measurement of the Total Antioxidant capacity (TAC) of seminal plasma gives an indirect measurement of oxidative stress. Total antioxidant capacity measurement can be performed by several methods. The most commonly used
A method for measuring TAC in seminal fluid is enhanced chemiluminescence assay (Kobayashi et al., 2001; Sharma et al., 1999). In this assay; to produce ROS, a signal reagent which contains luminol is mixed with Horseradish Peroxidase-attached immunoglobulin. The amount of antioxidant in the seminal plasma reduces the signal of chemiluminescence and the antioxidant capacity is compared with Trolox, a water soluble tocopherol analog as a reference standard (Said et al., 2003). Although this method is accurate, it needs expensive instrumentation and has some technical difficulties.

The another commonly used method for measurement of antioxidant capacity is based on colorimetric assay which uses 2,2’-azinobis-(3-ethyl-benzothiazoline-6-sulphonic acid) (ABTS). In this assay, hydrogen peroxide ABTS is oxidized to ABTS+ which produces a coloured substance that can be measured spectrophotometrically. Antioxidants within the seminal plasma compete with this reaction proportionally according to their concentration.

The research goal of this thesis can be summarised as;

1- Development of a reliable assay for measurement of sperm oxidative stress that can be easily used in an average clinical andrology laboratory with minimal additional equipment.
2- Investigate novel cause of oxidative stress in male infertility
3- Investigate the consequence of oxidative stress on sperm genome function
4- Investigate the use of antioxidant therapy in male infertility
CHAPTER 2

MATERIALS AND METHODS
2.1 INTRODUCTION

In order to achieve the aims of this thesis, I utilized the following tests related with free radical production and oxidative stress in semen:

1. Quantification of free radical production in semen was made using the NBT assay.
2. The concentration of leukocytes was measured because leukocytes are significant producers of free radicals in semen. For measuring leukocyte number, an immunocytochemistry procedure (CD45) was used. The bioactivity of leukocytes within semen was also measured using PMN Elastase (neutrophil) and Neopterin (macrophage) measurements.
3. Measurement of free radical related peroxidative damage in sperm membrane was analysed by LPO-586.
4. Determination of DNA damage was performed by using the Tdt-mediated Terminal dUTP Nick-end Labeling (TUNEL) assay
5. Quantification of sperm apoptosis was measured by immunocytochemical analysis of Annexin V assay.
6. Determination of sperm global DNA methylation was assessed by immunohistochemical 5-methylcytosine staining.
7. Determination of the protamine packaging quality of the sperm nucleus DNA was performed using CMA3 assay.
8. Epididymal and Testicular function was quantified by the measurement of α-glucosidase and blood reproductive hormone levels, respectively.
2.2 Study Subjects

The University of Adelaide’s Ethics committee was notified of the study once ethical clearance had been obtained from Women's and Children's Hospital, Human Research and Ethics Committee (HREC), as per University policy. Semen samples were obtained from an academic affiliated assisted reproductive technology unit in Adelaide, South Australia (REPROMED) after providing written informed consent for the participation in the study from all participants.

The participants in this study were drawn from two groups. The fertile control group consisted of men in the sperm donation program at Repromed who had proven paternity in the last twelve months, were in general good health, aged 18-50 years of age, with normal sperm parameters. The infertile patient cohort consisted of men with abnormal routine sperm parameters who were seeking reproductive treatment at Repromed.

All semen samples were collected and coded with a unique identifier number. All results were collated using this unique identifier rather than the patient’s name, so as to maintain patient confidentiality.

2.3 Sample collection and Semen Analysis

All semen samples were produced by masturbation into a clean container after 3-5 days of abstinence. Semen samples were allowed to liquefy at least for 30 minutes at room temperature before analysis.

Semen analysis was performed according to WHO guidelines (1999) to obtain volume, pH, sperm concentration, motility, and morphology by staff in the Andrology laboratory in Repromed (WHO, 1999a). Morphology smears were scored using WHO criteria. Sperm concentration was expressed as $10^6$ per millilitre of semen, whereas motility and morphology were expressed as percentage. The motility results reported as the sum of rapidly progressive (WHO type "a") and low progressive (WHO type "b") motility. Sperm sample was classified as normospermic if sperm concentration was $\geq 20 \times 10^6$ per ml, sperm motility was $\geq 50$ % and normal sperm morphology $\geq 15$ %.
2.4 Sample Preparation

After routine semen analysis, the remaining portion of semen was divided into different portions to analyse for research parameters (Figure 2.1). One portion of semen was washed with Dulbecco’s Phosphate buffered solution (PBS) (JRH Biosciences, Lenexa, Kansas, USA Cat No:59321C) and smeared on poly-L-Lysine coated slide (Polysine™, Menzel-Glaser GmbH&Co KG, Braunschweig, Germany) for fixation. The other part of remaining liquefied neat semen was centrifuged for 10 min at 3000 x g to separate sperm cells from plasma. Seminal plasma was aspirated by pipette and aliquoted into four separate eppendorf tubes which were stored at -70 °C until assayed. Aliquoted seminal plasmas were used to analyse LPO, a-Glucosidase, Neopterin and PMN Elastase assays. Fixed slides were use for quantification of leukocytes, apoptosis and DNA fragmentation.

![Figure 2.1: Steps in sample preparation](image-url)
To conduct the sperm washing procedure of semen, 400 µL of liquefied semen was mixed with 2000 µL of Dulbecco’s Phosphate buffered solution (PBS) and centrifuged at 300xg for 10 minutes. After spinning, the supernatant was discarded without disturbing the pellet by using a glass pipette. The pellet was gently resuspended in 400 µL PBS and this mixture was centrifuged at 300 x g for 5 minutes. This last step was applied twice. After discarding all supernatant, pellets were resuspended in 400µL PBS, thereby keeping the original volume of neat semen before the washing procedure.

This washed semen was divided into two parts; one part for measurement of Annexin V and Nitroblue tetrazolium assays while the other part was used for the preparation of slides. Before applying to the slides, the washed semen was adjusted to a concentration of 1x10^6 sperm/mL by adding PBS depending on the original sperm concentration in the neat semen to provide optimal concentration in each slide.

For the fixation step, Poly-L-lysine coated glass slides were marked by DAKO marker pen (Dako North America, Inc., Carpentaria, California, USA) in two square boundaries at each slides and labelled with sample’s identifier number. 10 µL of aliquots of washed sperm suspensions were applied to squares on the glass slides and allowed to dry at room temperature. The air dried cells were fixed for an hour at room temperature in fixative solution (96 % Ethanol) for CD45 immunoassay and sperm global DNA methylation assays and in 3:1 methanol (95%)/Glacial acetic acid fixative for DNA fragmentation (TUNEL) and sperm protamination (CMA3) assays. The slides were removed from fixative solution and allowed to dry at room temperature in a fume hood. For subsequent analysis, the slides were wrapped in aluminium foil one by one and stored at -70 °C until assayed.

2.4.1 Sperm Swim-up Procedure

For experiments requiring pure sperm without the presence of contaminating leukocytes, sperm “swim-up” procedure was used. To apply this procedure, 1 mL of warmed sperm culture media (G-Sperm™, Vitrolife, Kungsbacka, Sweden) was transferred into a 10 mL Falcon tube (Becton Dickinson Biosciences, San Jose, CA USA). 1 mL of liquefied neat semen was placed
gently beneath the media and after incubation for 1h at 37 °C, the upper third of the sperm suspension was aspirated carefully and centrifuged at 300 x g for 10 minutes.

2.4.2 Isolation of Leukocytes from Peripheral Blood

Peripheral blood leukocytes were isolated by density gradient centrifugation with subsequent erythrocyte lysis (Kovalski et al., 1991). 2 mL of whole blood was collected into an EDTA tube and then was mixed with 10 mL 0.87% NH₄Cl solution to produce osmotic lysis of the erythrocytes. The mixture was allowed to stand for 20 minutes at room temperature and then the haemolysed blood was centrifuged at 2200 rpm for 15 minutes. The pellet contains leukocytes was resuspend in 2 mL 0.87% NH₄Cl solution. The cell suspension was layered onto a single discontinuous Percoll™ Gradient (Amersham Biosciences, Uppsala, Sweden). To prepare Percoll gradients, first 20% Percoll solution in PBS was added to the tube and then 30%, 40% and 55% Percol solutions were layered underneath of each layer, respectively. 2 mL of leukocytes suspension was layered onto the top of the percoll gradient and then centrifuged at 1300 x g for 30 minutes. Neutrophils are collected from the interface between 40% and 55% Percoll gradient. Monocytes and lymphocytes are collected from the interface between 30% and 40% Percoll gradient. The harvested leukocyte suspension was washed once in phosphate buffered saline (PBS) and then resuspended at a concentration of 1 x 10⁶/mL.

2.5 Determination of Leukocytes in Semen

The existence of seminal leukocytes was analysed by immunohistochemical leukocyte common antigen marker CD 45. The quantification of neutrophil activity in semen was performed using the PMN Elastase assay.

2.5.1 Immunocytochemistry Procedure for CD-45

In this procedure, streptavidin-biotin system against the common leukocyte antigen CD45 was used. This monoclonal antibody was applied to detect granulocytes, lymphocytes and macrophages in semen simultaneously.

To apply fluorescence staining procedure, all cells were washed and fixed according to the procedure described at 2.4
The slides were taken from freezer and stood at room temperature for thawing. After thawing, the slides were washed in PBS for 5 minutes. From this point the slides were not allowed to dry out and kept in a humidified container.

**Protein Blocking Step:**
The sections in each slide were incubated for 20 minutes with 50 µL of 5% normal goat serum. This protein blocking solution was freshly prepared in each assay. To prepare the protein blocking solution (PBS/BSA Solution), 150µL of normal Goat Serum (Vector Laboratories, CA) was diluted in 2850µL PBS. After incubation with protein blocking solution, the slides were washed once with PBS for 5 min.

**Avidin-Biotin Blocking Step:**
Avidin/Biotin Blocking Kit (Vector Laboratories, CA) were used to block nonspecific binding of Biotin/Avidin System reagents. This kit contains Avidin D and Biotin Solutions and these solutions were used directly as supplied. Each section of the slides was pre-treated with the Avidin D solution for 15 min at room temperature firstly. After washing with PBS solution in 5 minutes, the slides were incubated for 15 minutes with the Biotin Solution and then washed in PBS for 5 minutes as a last step.

**Primary Antibody Step:**
The sections were incubated for 60 minutes with 50 µL. primary antibody (Rat Anti-human CD45) (Serotec Ltd,UK,Code: MCA 345) at room temperature in a humidified container. Working solution of the Primary Antibody concentration was 4µg/mL. To achieve this concentration, 10µL primary antibody solution was diluted in 4990µL PBS-BSA solution. This dilution was equal to 1/500. After treatment with primary antibody, the slides were washed twice for 5 minutes in PBS.

**Secondary Antibody Step:**
The sections were incubated for 30 minutes with 50 µL. diluted biotinylated secondary antibody solution (Goat anti-rat IgG) (Jackson Immuno Research Laboratories, Inc, USA) at room temperature in a humidified container. Stock solution of the Secondary Antibody concentration was 2 mg/mL. To prepare Working Solution from the stock solution, 2µL secondary antibody solution was diluted in 4990µL PBS-BSA solution. The slides were washed 3 times for 5 minutes each wash in PBS.

**Avidin Conjugate Step:**
The sections were incubated for 30 min with 50µL Texas Red Avidin D conjugate (Vector Laboratories Inc., CA) at room temperature in a humidified container. The concentration of the Texas Red Avidin Conjugate was 25 µg/mL.
This stock solution was diluted by the PBS in the ratio of 1/100 before starting the experiment.

After Avidin conjugate step, slides were washed 3 times in PBS for 5 minutes in each wash. A 1:1 mixture of ProLong® Gold anti-fade reagent (Invitrogen Molecular Probes, Eugene, Oregon, USA) and 10% glycerol in PBS was applied to the each section as a mounting medium and was covered by 18 x 18 mm cover slips. Subsequently, the leukocyte cells were visualized by using on Olympus BX51 fluorescence microscope with the 595nm filter for Texas Red Dye. Leukocyte concentration was determined by analysing the relative ratio of CD45 positive cells to sperm in at least 300 high power fields and expressed as $10^6$ leukocytes per milliliter.

### 2.5.2 PMN Elastase Assay

PMN Elastase is a proteinase enzyme which is secreted by activated granulocytes (Reinhardt et al., 1997). During phagocytosis PMN elastase is excreted into the extracellular matrix to phagocytise and destroy non-natural agents (Zorn et al., 2000). This enzyme can be used as a measure activity of granulocytes during an inflammatory response in seminal plasma (Reinhardt et al., 1997). Latex particles were coated with antibody fragments F (ab‘)$_2$ against human PMN elastase; changes in turbidity were measured photometrically. The extent of turbidity was proportional to PMN elastase concentration in the test sample.

Granulocyte elastase concentration in seminal plasma was measured by the homogeneous immunoassay ECOLINE® PMN elastase (Merck, Darmstadt, Germany). The kit detected PMN elastase concentrations of >4 µg/L.

To determine PMN Elastase concentration, stored seminal plasma at -70°C was taken from the freezer and stood at room temperature until thawed. While waiting for thawing of the samples, standard solutions were prepared. The concentration range of these standards was 10 - 0.16 ng/mL.

Thawed seminal plasma samples were diluted 1:100 with sample diluent which is provided by the supplier. 100 µL of each 1:100 diluted sample was added to the designated wells, in duplicate. The microplate was covered with a plate cover and incubated at room temperature for 1 hour. After removing the plate cover, the microwell strips were washed four times with 300 µL wash buffer.
After the last wash, microwell strips were tapped on an absorbent pad to remove excess wash buffer. The wells were not allowed to dry.

150 µL of HRP-conjugate was added to all wells and covered by a plate cover again and incubated for 1 hour at room temperature on a rotator. Then, microwell strips were washed four times as previously described and the procedure were carried on to the next step immediately. 200 µL of TMB substrate solution was added to all wells and incubated at room temperature for 20 min on a rotator. Microplate wells were protected from direct exposure to intense light. After the incubation, 50 µL of Stop solution which is included in the kit was pipetted into each well to stop the enzyme reaction. Immediately after addition of the stop solution, the colour reaction was measured spectrophotometrically at 450 nm by using a microplate reader (Bio-Tek Instruments, Inc. Winooski, VT, USA, Model ELx800). The concentration of PMN Elastase in the sample was calculated against to standard curve in the assay and expressed as ng/mL.

2.5.3 Determination of macrophage activity in semen (Neopterin Assay)

Neopterin is a catabolic product of guanosine triphosphate (GTP), a purine nucleotide and synthesised by macrophages undergoing immune activation. Measurement of neopterin level provides information about the immune activation status of macrophages.

Determination of Neopterin in seminal plasma was conducted by using a Neopterin screening ELISA kit following the manufacturer’s instructions (BRAHMS Aktiengesellschaft, Ennigsdorf, Germany). Neopterin ELISA Screening kit provides quantitative determination of neopterin, based on the basic principle of a competitive enzyme-linked immunosorbent assay.

An unknown amount of antigen in the sample and a fixed amount of neopterine/enzyme conjugate compete for the antibody-binding sites (polyclonal sheep-anti-neopterin) of these antibodies, thus forming an immune complex bound to solid phase (anti-neopterin antibody / neopterin or neopterin/enzyme conjugate). Unbound antigen is removed by washing. The intensity of the colour developed after the substrate incubation was inversely proportional to the amount of antigen in the sample.
Procedure of the assay

Thawed seminal plasma was first diluted 1/10 in PBS to allow quantification of seminal plasma neopterin within the ELISA’s detectable range. 50 µL of Neopterin standards (at the concentration of 2, 5, 10, 25 and 50 nmol/L) which is provided by the manufacturer and 50 µL of diluted seminal plasma were pipetted into the clean tubes with 150 µL of the enzyme conjugate. 150 µL of this mixture was transferred into the neopterin-antibody coated microtitre plate within 5 minutes and incubated for 2 hours at room temperature in the dark using black cover sheet. After incubation, the wells were washed 4 times with 350 µL of washing solution which was prepared freshly from concentrate washing solution by diluting 1/50. 100 µL of substrate (para-nitrophenyl phosphate) solution was pipetted into the wells and incubated for 30 minutes at room temperature. Then, 100 µL of stop solution (2N Sodium Hydroxide) was added to stop enzyme reaction and the formation of colour. The optical density of colour was measured in a microplate reader (Bio-Tek Instruments, Inc. Winooski, VT, USA, Model ELx800) at a wavelength of 405 nm. The results were calculated by plotting against a standard curve and expressed as nmol Neopterin per ml seminal plasma.

2.6 Measurement of Reactive Oxygen Species

2.6.1 Lipid Peroxidation Test (LPO Test)

Production of ROS level was measured by their lipid peroxidation formation using Bioxytech LPO-586 assay kit (Oxis ResearchJ, Oxis International, USA). Lipid peroxidation is quantified by measuring malondialdehyde (MDA) and 4-hydroxyalkenals, the degradation products of polyunsaturated fatty acids (PUFA) hydroperoxides (Esterbauer et al., 1991). The LPO-586 assay is based on the reaction of a chromogenic reagent (N-methyl-2-phenylindole) with MDA and 4-hydroxyalkenals to yield a stable chromophore at 586 nm absorbance spectrophotometrically.

Procedure of the LPO Test

Frozen seminal plasma samples were taken from freezer and stood on the room temperature to allow for thawing. While waiting in this procedure, standard solutions were prepared according to Table 1. Standards were run in duplicate in each assay.
Table 2.1: Standard Curve Dilution Volumes

<table>
<thead>
<tr>
<th>Standard Concentration (µM MDA)</th>
<th>0</th>
<th>0.5</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Volume of 20 µM standard to add (mL)</td>
<td>0</td>
<td>25</td>
<td>50</td>
<td>100</td>
<td>150</td>
<td>200</td>
</tr>
<tr>
<td>Volume of PBS used for diluting (mL)</td>
<td>200</td>
<td>175</td>
<td>150</td>
<td>100</td>
<td>50</td>
<td>0</td>
</tr>
</tbody>
</table>

200 µL of thawed seminal plasma were added to clean glass test tubes. Samples also were run in duplicate in each assay.

Reagent 1 (N-methyl-2-phenylindole in acetonitrile), was diluted to 1/3 by the Diluent (Ferric Iron in Methanol) which were both provided in the kit. Depending on the number of samples in each assay, one volume of Diluent was mixed with three volumes of Reagent 1 and this solution was prepared immediately before use.

650 µL of diluted R1 reagent was added to each sample tube and mixed gently by vortex. Then, 150 µL of concentrated (12 N) HCl was added to tubes and mixed well. Tubes were tightly closed and incubated at 45°C for 60 minutes. To obtain a clear supernatant, samples were centrifuged at 15000 x g for 10 minutes. The supernatants were transferred to the reading cuvettes and measured at the absorbance at 586 nm by a microplate reader (Bio-Tek Instruments, Inc. Winooski, VT, USA, Model ELx800).

By using the standard data, the net A586 for each standard was calculated by subtracting the blank (Ao) value from each of the standard A\textsubscript{586} values. The concentration of analyte in each sample was calculated by using standard curve.

2.6.2 Nitro Blue Tetrazolium Assay (NBT Assay)

A modified colorimetric Nitro Blue Tetrazolium (NBT) test was used to evaluate reactive oxygen species (ROS) production of both leukocytes and sperm within semen (Choi et al., 2006; Esfandiari et al., 2003).

Nitroblue tetrazolium test depends on the reaction of aromatic tetrazolium compound with cellular superoxide ions (Baehner et al., 1976). Metabolically active cells reduce the yellow water-soluble tetrazolium salt to blue water-insoluble formazan derivative. The composition of formazan serves as an estimate of contribution of spermatozoa and leukocytes toward the amount of ROS production in semen.
**Figure 2.2: Schematic chemical formula of the NBT assay**

**Procedure of the NBT Assay**

100 µL of washed semen, which was prepared the procedure described at 2.4, was incubated with an equal volume of NBT Working Reagent at 37°C for 45 minutes. For each sample, the assay was run in duplicate using eppendorf tubes away from light exposure. The NBT working solution was prepared from 0.01% NBT stock, (Sigma-Aldrich, St. Louis, MO, USA Cat No: N5514) at the dilution of 1/10 with PBS. The stock solution was prepared by dissolving one tablet of NBT reagent into the 1000 µL of distilled water. Following the incubation the tubes were centrifuged at 500 x g for 10 minutes to concentrate the formazan crystals and then washed in 1000 µL PBS twice in order to remove all residual NBT solution. To quantify the formazan product, the intracellular formazan was solubilised in 60 µL of 2 M KOH and shaken vigorously. 60 µL of Dimethyl Sulphoxide (DMSO) (Sigma-Aldrich, St. Louis, MO, USA Cat.No: D2650) was added to tubes and tubes were mixed with vortex. The resulting colour density was measured spectrophotometrically on the aide of a microplate reader (Bio-Tek Instruments, Inc. Winooski, VT, USA, Model ELx800) at 630 nm, as previously reported for leukocytes (Choi et al., 2006; Rook et al., 1985).

ROS production was expressed as µg formazan per 10⁷ sperm, derived from a standard curve of absorbance values for known amounts of formazan substrate.

**2.6.2.1 Formazan Production for Standard Curve in NBT Assay**

A standard curve for the NBT assay was performed after producing formazan crystals in our laboratory. Since formazan crystals are no longer commercially available, they were produced from NBT solution by the action of superoxide anions generated by the Xanthine/ Xanthine Oxidase system. Xanthine oxidase with xanthine solution generates significant amounts of superoxide anions with the resulting production of formazan crystals when added to NBT solution.
To perform the procedure, 1 mmol/L Xanthine (Sigma-Aldrich, St. Louis, USA, Cat No: X7375) solution was prepared by dissolving in 150 mL NaOH and subsequently in deionised water. 25 U/mL Xanthine oxidase solution (Sigma-Aldrich, St. Louis, USA, Cat No: X2252) was prepared from 3.2 mg Xanthine oxidase solid and aliquoted into small amounts to freeze until the next assay run.

Before the assay, the weight of half-dozen 2mL eppendorf tubes was recorded for the measurement of the formazan crystals after reaction with NBT reagent. 500 µL of Xanthine solution was added to tubes following the addition of 500 µL NBT working solution. After pipetting 10 µL of Xanthine oxidase, tubes were incubated at 37 °C for 30 minutes to produce formazan crystals. Formazan crystals were washed twice in deionised water and centrifuged at 500 x g for 10 minutes. All supernatant was taken from tubes and let dry for overnight. To measure how much formazan was produced; tubes were re-weighed and then solubilised in 300 µL DMSO and KOH. A standard curve was derived by plotting measured formazan mass versus absorbance value. This assay was repeated in 5 different days to provide accurate measurement for optimizing the test.

2.7 Detection of Defective Sperm Chromatin Packaging

2.7.1 Chromomycin A3 Assay

Principle of the Assay
Chromatin packaging assessment was quantified using the chromomycin A3 (CMA3) fluorochrome which indirectly demonstrates a decreased presence of protamine.

CMA3 is a fluorochrome which is specific for GC-rich sequences in DNA (Franken et al., 1999). CMA3 competes with protamines for the same binding sites in the minor groove of DNA (Bizzaro et al., 1998). Therefore, high CMA3 fluorescence is a strong indicator of poor protamination of the mature spermatozoa, suggesting increased vulnerability to sperm DNA fragmentation (Bianchi et al., 1996).

Procedure of the Chromomycin A3 Assay
The slides which have been fixed in methanol-glacial acetic acid (3:1) and frozen at – 70 °C were taken from the freezer and stood at room temperature for thawing. After thawing, slides were washed in PBS for 5 minutes and treated for 20 minutes with 100 µL. CMA3 solution which was prepared at the
concentration of 0.25 mg/mL in McIlvaine Buffer [3.53 mL of 0.2 M anhydrous citric acid and 16.47 mL of 0.2 M anhydrous sodium phosphate dibasic (Na₂HPO₄)]. The slides were rinsed in PBS buffer and mounted with buffered glycerol (1:1 v/v PBS-glycerol). Microscopic evaluation was performed by using fluorescent microscope with the appropriate filters (460 nm - 470 nm).

A total of 200 spermatozoa were randomly evaluated by distinguishing spermatozoa that stain bright yellow as CMA₃ positive from those that stain a dull yellow as CMA₃ negative (Picture 2.1). CMA₃ positive cells were identified microscopically and quantified using IP Lab 3.6.1 software. The samples contain more than 30 % CMA₃ positive cells were assessed as pathologic.

The protamination level of sperm is given by the formula;

\[
\text{Protamination} \% = 100 \text{ - CMA}_3 \text{ result}
\]

Normally sperm have a level of protamination >85%.

![Picture 2.1: Representative image of CMA3 staining under the fluorescence microscope. Bright cells represents poor protamination stained by CMA3. Normal protaminated sperm appear dull yellow](image)

### 2.8 Assessment of DNA Damage

#### 2.8.1 TUNEL (Tdt-mediated Terminal dUTP Nick-end Labeling) assay

**Principle of the assay**

DNA fragmentation induced in spermatozoa was assessed using the TdT-mediated-dUTP nick-end labeling (TUNEL) technique. This assay identifies single and double stranded DNA breaks by labelling free 3’-OH termini of the DNA in an enzymatic reaction with terminal deoxynucleotidyl transferase(TdT).
followed by fluorescein labelling with propidium iodide (Heatwole, 1999). In this study, the modified microscopic TUNEL technique first described by Lopes et al (Benchaib et al., 2003b; Gandini et al., 2000; Lopes et al., 1998) was performed.

**Procedure of the TUNEL Assay**

The slides which have been fixed in methanol-glacial acetic acid (3:1) and kept in the -70°C freezer were taken out and stood at room temperature until thawed. After soaking the slides in PBS for 5 minutes at room temperature, sperm cells were permeabilised in permeabilisation solution (0.1% Triton X-100 in 0.1% sodium citrate). To perform this, 100 µL permeabilisation solution was applied to each sections in the glass slides and incubated for 30 minutes at room temperature in a humidified chamber. After washing twice with PBS, cells treated with 20 µL TUNEL reaction mixture which was prepared by diluting 1 part enzyme solution (FITC-labelled terminal deoxynucleotidyl transferase-TdT) in 9 part Label solution (Nucleotide mixture), ie 10 µL enzyme solution in 90 µL label solution for each sample. The slides were incubated for 1 hour at 37°C incubator and labelled with 50 µL Propidium Iodide (10 µg/mL) for 30 minutes at room temperature in the dark. Slides were rinsed twice in 50 µL PBS buffer for two minutes and mounted in a 1:1 mixture of ProLong® Gold antifade reagent (Invitrogen Molecular Probes, Oregon, USA) and glycerol. Stained cells were quantified on Olympus BX51 fluorescence microscope, with a minimum of 300 sperm per slide being assessed using image analysis software (MacProbe V 4.3, Perceptive Scientific Instruments, League, Texas). DNA fragmentation in sperm cells was evaluated as negative or positive on the basis of the presence or absence of head staining. The percentage of sperm DNA fragmentation was calculated as the number of TUNEL positive nuclei (FITC-labeled, green) per total number of sperm nuclei (Propidium Iodide, red) in approximately 300 cells (Picture 2.2). For a positive control sperm cells were incubated with 3 U/µL DNase prior to incubation with the TUNEL reagents and for a negative control the terminal transferase was omitted from the reaction.
Picture 2.2: Representative examples of a typical microscopic TUNEL result for both a low (A) and high (B) DNA fragmentation semen sample.

2.9 Assessment of Aberrant Apoptosis

2.9.1 Annexin V Assay

Annexin V is a fluorescence compound which binds to phospholipids in the presence of calcium and helps to detect apoptotic cells (Oosterhuis et al., 2000). At the beginning of the apoptosis, phospholipids, which are found normally on the internal part of the plasma membrane, are translocated to external part of the membrane (Figure 2.3). Binding of Annexin V to phospholipids in plasma membrane shows early apoptotic process.
Figure 2.3: Schematic diagram of the Annexin V assay

Procedure of the assay

The procedure was conducted according to the protocol recommended by the manufacturer. As explained in section 2.4, washed neat semen was used for Annexin V assay. To carry out the procedure, 400µL of semen was mixed with 2000µL PBS and centrifuged at $300 \times g$ for 10 minutes. After taking the supernatant, the washing procedure was repeated once and the pellet was resuspended in 400µL PBS. According to the sperm concentration of the sample, the mixture was diluted at a concentration of $1 \times 10^6$ cell/mL with the relevant amount of binding buffer which consists of 100 mM HEPES/NaOH Buffer. 1µL Annexin V conjugate and 0.5µL Propidium Iodide were added to the cell suspension. After the incubation of the tubes at room temperature for 10 minutes under light-proof conditions, the suspension was centrifuged at $400 \times g$ for 5 min and the pellet was mounted on a poly-L lysine coated slide by using an 18 x 18 mm cover slip. The cells were examined immediately with an Olympus fluorescence microscope equipped with an Olympus digital camera. At least 200 sperm cells were counted on per slide.

Three different patterns of fluorescence were observed:
1- ) Cells which were early in the apoptotic process were stained with the Annexin V and seen as green colour.
2- ) Necrotic cells were stained with both Propidium Iodide and Annexin V and seen in red and green colour respectively.
3- ) The live cells showed no staining by either Propidium Iodide or Annexin V and were seen only by phase contrast microscopy.

The division of non-staining cells (live cells) to green coloured cells (apoptotic cells) was evaluated as an apoptotic live cells (Picture 3.2).
2.10 Assesment of sperm global DNA Methylation

2.10.1 Immunohistochemical 5-methylcytosine Staining

Measurement of sperm global DNA methylation was made using the immunohistochemical 5-methylcytosine staining technique (Benchaib et al., 2003a) which is based on labelling of methylation sites of CpG dinucleotides.

Procedure of the assay

After removing slides from freezer fixation of cells with ethanol (96%) the slides were rinsed twice in PBS containing 0.25% Triton X (Sigma-Aldrich, St. Louis, USA, Cat No: T9284). Decondensation of sperm DNA was carried out by decondensing buffer (1M HCL, 10 mM Tris Buffer, pH 9.5 containing dithiotretiol) (Sigma-Aldrich, St. Louis, USA) at room temperature for 20 min. Sperm were then washed twice in PBS / Tween 0.5 % buffer and then the sperm DNA was denatured by 6 N HCL to ensure access to methylation bases. The denaturation process was followed by neutralisation with 100 mM TRIS HCL (pH 8.5) for 30 min at room temperature. Cells were then incubated with a dilution of 1:50 monoclonal primary specific antibody against 5-methylcytidine (5mC) (Eurogentec S.A., BI-MECY-1000, Seraig, Belgium) for 1 hour at 37C. After rinsing twice in PBS, sperm were incubated with Fluorescein
Affinipure goat anti-mouse IgG (Jackson Immunoresearch Laboratories Inc, West Grove, PA, USA) at a dilution of 1:100 in 0.05% Tween 20 in PBS for 20 min. The slides were then rinsed in PBS buffer and mounted in ProLong® Gold antifade reagent (Invitrogen Molecular Probes, Oregon, USA). Negative controls were performed via the application of secondary antibody by omitting the primary 5-methylcytidine antibody. The stained cells were visualized on Olympus BX51 fluorescence microscope and the degree of global DNA methylation was determined by measuring mean value of intensity of the fluorescence and expressed as Arbitrary Unit (A.U.) on at least 300 cells. The primary antibody step was omitted on the negative control slides.

In order to minimize qualitative error related to fading of fluorescent staining intensity over time, all slides were captured for later image analysis within 1 hour of completion of the immunochemistry process. Furthermore, all measurements were conducted on a single microscope at a set fluorescent light exposure intensity. Finally, individual infertile patient’s entry and exit methylation slides were always analysed in the same assay run, so as to minimize skewing of results by inter-assay variation. Using these precautions the intra and inter-assay CV was 5 and 7% respectively.

2.11 Assessment of epididymal function

2.11.1 α-Glucosidase assay

α-Glucosidase enzyme (EC.3.2.1.20) activity is a marker of human epididymal secretory function (WHO, 1999b). α-glucosidase is found in two isoenzyme forms and mostly originates from epididymis (Paquin et al., 1984). The neutral isoenzyme form of α-glucosidase is released from the epididymis and contributes 80% of total activity. The acid isoenzyme form of α-glucosidase provides 20% of total activity and is secreted by the accessory reproductive glands (Bedford, 1994).

α-glucosidase activity can be measured photometrically in seminal plasma. In this study colorimetric neutral α-Glucosidase kit was used (Roche α-Glucosidase Assay Kit Cat No: 11742027001, Roche Applied Science, Mannheim, Germany).

The principle of the assay was dependent on neutral α-glucosidase activity in the sample liberating yellow 4-nitrophenol from the substrate solution of 4-nitrophenyl-α-D-glucopyranoside according the equation below;
4-Nitrophenyl- α -D-glucopyranoside

\[ \text{α-glucosidase} \]

4-Nitrophenol + α -D-glucopyranoside

The kit contains the substrate and all buffers for the measurement of neutral α -glucosidase in seminal plasma including sodium dodecyl sulfate (SDS) and castanospermine substrate which provides the specific measurement of epididymal origin of neutral α-glucosidase.

**Procedure of the assay**

In a first step the stored frozen semen samples were thawed and centrifuged at 1000 \( x \) g for 10 minutes. The clear supernatant was taken by using positive displacement pipette for avoiding higher variances because of the possibility of existence of high viscosity in some samples. Before the assay, the reaction solution was prepared according to manufacturer instructions. To prepare the reaction solution, substrate concentrate solution containing 4-nitrophenyl-α -D-glucopyranoside was warmed to 37 °C until the content of the bottle is entirely dissolved. For each sample, 10 µL of substrate solution was prepared by dissolving in 90 µL reaction buffer which contains pH 6.8 phosphate buffer with 1% SDS to maintain a neutral pH.

Standard samples were prepared from 100 µM standard concentrate solution (para-nitrophenol) by diluting with stopping buffer in serial. The end concentrations of standard solution were 0, 20, 40, 60, 80, 1000 µM para-nitrophenol. Standards were run in duplicate in each assay.

100 µL of reaction solution was pre-warmed at 37°C and mixed with 15 µL seminal plasma. The mixture was vortexed and incubated at 37°C for 2 hours. A blank control sample was set up by mixing the specific inhibitor castanospermine to the sample before adding the substrate. After adding substrate solution into the sample the reaction was started. During 2 hour incubation at +37°C the α-glucosidase containing sample will liberate coloured 4-nitrophenol from the substrate 4- nitrophenyl- α -D-glucopyranoside.

The resulting colour reaction was stopped by adding 1ml. stop buffer. 250 µL of aliquot was transferred into a 96 well microplate for measurement at 405
nm on microplate reader (Bio-Tek Instruments, Inc. Winooski, VT, USA, Model ELx800). The final result was then derived after subtracting the castanospermine inhibited semen blank absorbance results from each well. The enzyme activity (mU/mL) was determined from an absorbance versus enzyme activity standard curve.

2.12 Assessment of testicular function

2.12.1 Measurement of Serum Reproductive Hormone Levels

The assessment of testicular function was done by measuring serum reproductive hormone levels. The level of serum testosterone and LH measurement was applied for evaluation of Leydig cell function, while FSH, Estradiol and AMH (Anti-Mullerian Hormone) assays were applied for Sertoli cells function.

Venous blood samples were collected into gel separator tubes (BD Vacutainer®, Becton-Dickinson Company, Plymouth, UK) in the morning and centrifuged at 4000rpm for 10 minutes. Serum was separated within 1 hour of collection and frozen at –70°C until assayed. All hormone measurements, with the exception of AMH (see below), were conducted using the automated ADVIA Centaur chemiluminescent immunoassay system obtained from Bayer Australia Ltd. (Pymble, NSW, Australia).

2.12.2 Measurement of AMH (Anti Mullerian Hormone) level in serum

Serum AMH levels, a measure of sertoli cell function (Rajpert-De Meyts et al., 1999), were measured using the Immunotech high-sensitivity immunoenzymetric assay kit (Beckman Coulter, Marseille, France). In the first step the Antimullerian Hormone is captured by a monoclonal antibody bound to the wells of a microtiter plate. In the second step a biotinylated antibody binds to the solid phase antibody-antigen complex following streptavidin-horse radish peroxidase (HRP) conjugate binding. After incubation and washing the wells, the antigen complex bound to the well was detected by addition of a chromogenic TMB (tetramethylbenzidine) substrate. In the presence of HRP, the TMB and peroxide contained in the substrate solution react to produce a blue byproduct. The colour intensity is proportional to the amount of HRP activity, which in turn is related to the levels of AMH. Addition of 2 N sulfuric acid stop solution changes the color to yellow, enabling accurate measurement of the intensity at 450 nm using a plate reader.
Procedure of the assay
Serial standard solution was prepared prior to the assay according to the manufacturer’s instruction. The concentration of standards was 0, 3, 9, 27, 81 and 150 pM and provided by the manufacturer in the kit. 25 µL of serum sample and standard solutions were pipetted in duplicate into anti-AMH coated plate following addition 100 µL of buffer solution to each well. The wells were incubated for 2 hours at room temperature on plate shaker and then washed with freshly prepared wash buffer three times. After adding 50 µL of biotinylated antibody and 50 µL of conjugate, the wells were incubated for 30 minutes at room temperature with shaking. After the conjugate binding step, the wells were washed three times with wash solution and the remain wash solution was completely removed by firmly tapping the inverted microplate on to the absorbent paper. After pipetting 100 µL of TMB substrate, wells were incubated for 30 minutes at room temperature away from light on the plate shaker. The colour reaction was stopped by adding 50 µL stop solution to each well and the intensity of colour was measured using microplate reader at 450 nm. The concentration of AMH was calculated using standard curve and expressed as pmol/L.

2.13 Serum Homocysteine assay
Homocysteine (Hcy) is a thiol-containing amino acid produced by the intracellular demethylation of methionine (Finkelstein, 1990). Homocysteine level in serum was measured by using Bio-Rad Microplate Enzyme Immunoassay Homocysteine Kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA) which is based on competition between S-adenosyl-L-homocysteine (SAH) in the sample and immobilized SAH bound to the walls of the microtitre plate for binding sites on a monoclonal anti-SAH antibody. All samples including calibrators and controls for the assay were pretreated with pretreatment solution which contains Dithiothreitol (DTT) to reduce the mixed sulfide and protein-bound forms of homocysteine to free homocysteine. Homocysteine in the sample was also converted to S-adenosyl-L-homocysteine (SAH) by the use of SAH hydrolase in pretreatment solution.

Procedure of the assay
25 µL of sample and standard were pre-treated with 500 µL pretreatment solution and incubated for 30 minutes at 37°C in a tightly capped tube. 500 µL enzyme inhibitor (<0.2% Thiomersal in phosphate buffer) and 500 µL
Adenosine Deaminase was added to tubes and incubated at room temperature for 15 minutes respectively. 25 μL diluted standard and sample from the tubes was pipetted into the SAH-coated wells accompanying with 200 μL α-SAH antibody (Monoclonal mouse-anti-S-adenosyl-L-homocysteine antibody). After the incubation for 30 minutes at room temperature, wells were washed with wash buffer 4 times. After washing the wells was emptied on paper towels and 100 μL enzyme conjugate (Rabbit anti-mouse-antibody with horseradish peroxidase-HRP) was pipetted. Then 100μL 10% N-methyl-2-pyrolidone was added as substrate and incubated at for 10 min at room temperature following the addition of 100 μL 0.8 M sulphuric acid as stop solution.

The colour reaction was occurred depending on horseradish peroxidase activity after addition of substrate solution and was inversely related to homocysteine concentration in the sample. Measurement was done spectrophotometrically in microplate reader (Bio-Tek Instruments, Inc. Winooski, VT, USA, Model ELx800) at the wavelength of 450 nm. The result for the homocysteine concentration in the sample was derived according the standard curve and expressed as μmol/L. The reference range for homocysteine was accepted as <11.4 mmol/L at 95th percentile in folate replete men aged 20-45 years (Selhub et al., 1999).
CHAPTER 3
Development of the Nitro Blue Tetrazolium (NBT) Assay as a marker of sperm Oxidative Stress
3.1 Abstract

Oxidative stress is a well established cause of male infertility, with Reactive Oxygen Species (ROS) causing infertility principally by impairing sperm motility and DNA integrity. Currently most clinics do not test their infertile patients for the presence of oxidative stress because the available tests are expensive or difficult to conduct. Since antioxidant therapy may improve sperm DNA integrity and pregnancy outcomes, it has become apparent that there is an unmet clinical need for an inexpensive and easy to conduct assay to identify sperm oxidative stress. The aim of this study was to develop a standardized protocol for the conduct of a photometric Nitro Blue Tetrazolium (NBT) assay for the measurement of seminal ROS production via production of coloured formazan, while correlating these results with impaired sperm function (motility and DNA integrity). Semen samples from 21 fertile and 36 male aetiology infertile men were assessed for ROS production (NBT assay), sperm DNA integrity (TUNEL), apoptosis (Annexin V), and sperm motility. Infertile men's semen contained on average four-fold higher levels of ROS than fertile men. The production of ROS by sperm was positively correlated with sperm DNA fragmentation and apoptosis, while being negatively correlated with sperm motility.

Receiver-operating characteristic plot analysis established a cut-off point of 24 µg formazan / 10⁷ sperm as having a sensitivity of 91.7% and a specificity of 81% for determining the fertility status of an individual. This study has been successful in establishing a standardized protocol for the conduct of a photometric seminal NBT assay that has significant clinical utility in identifying men with impaired fertility due to oxidative stress.
3.2 Introduction

While oxidative stress is a common and potentially treatable pathology, the vast majority of clinical andrology laboratories do not test for its presence. Currently there are over 30 different assays to quantify sperm oxidative stress (Tremellen, 2008). Chemo-luminescent assays using either Luminol or Lucigenin are the most commonly described technique for detection of ROS within semen. While these assays are sensitive, have relatively well established normal ranges (Athayde et al., 2007; Williams and Ford, 2005) and are the only assay of oxidative stress described in the WHO semen analysis manual (WHO, 2001), they are rarely used by clinical andrology laboratories. This is probably because of significant set up costs (purchase of a luminometer) and difficulties with quality control created by assay confounders such as incubation time, leukocyte contamination and the presence of seminal plasma contamination (Kobayashi et al., 2001). Simpler, less expensive assays with well defined normal ranges based on sperm function must be established before clinical andrology laboratories will begin to test for the presence of oxidative stress.

The Nitro Blue Tetrazolium (NBT) assay is a well established laboratory technique used to quantify neutrophil function and cellular oxidative metabolism (Baehner et al., 1976). The principal behind the NBT assay is relatively simple. Cells incubated in a water soluble tetrazolium salt (NBT) solution will take up NBT into their cytoplasm where it is converted by the action of superoxide anions to a water insoluble blue formazan crystal (Baehner et al., 1976). These crystals are trapped within the cell but can be released by solubilisation in a solvent solution and then quantified by measuring absorbance of the resulting purple-blue solution (Choi et al., 2006; Rook et al., 1985). Previous NBT assay protocols had used wavelengths ranging from 530 nm (Mercado-Pichardo et al., 1981) to 630 nm (Choi et al., 2006; Rook et al., 1985) for determination of formazan production. The NBT test has been shown to be useful in quantifying both leukocyte (Choi et al., 2006; Rook et al., 1985) as well as sperm ROS production (Esfandiari et al., 2003; Mercado-Pichardo et al., 1981).

Furthermore, the measurement of formazan by histochemical staining within sperm and seminal leukocytes has been reported to be closely related to ROS
quantification by the well established chemo-luminescent ROS assay (Esfandiari et al., 2003).

While the NBT is relatively easy and inexpensive to conduct, its use within clinical andrology laboratories is hampered by a lack of published normal ranges. The aim of the present study was to develop standardized protocols for the conduct of the semen NBT assay and identify the associated normal ranges. An integral part of this study will be to analyse the correlation between NBT quantified ROS production and features of sperm oxidative attack (reduced motility, increase in sperm apoptosis and DNA fragmentation).

3.3 Materials and Methods

3.3.1 Subjects and Study Design

Participants were recruited from men undergoing infertility assessment or fertile sperm donors at an academic affiliated ART unit (Repromed, Dulwich, South Australia). The only entry criteria for participants was the requirement to have a minimum sperm concentration of $2 \times 10^6$/mL, as this was the minimum amount of sperm needed to reliably conduct the various sperm assays. Men with proven past paternity or men in an infertile relationship but who had normal semen parameters (count, motility morphology) and their partner had a well defined female cause for their infertility (anovulation, endometriosis, tubal obstruction, diminished ovarian reserve) were considered “fertile”. Those men in an infertile relationship who had at least one defect either in count, motility or morphology were considered to be have male factor infertility (‘infertile group’). A total of 21 men were classified as fertile. They consisted of 12 sperm donors of proven fertility, 2 men who initiated infertility investigation but whose partner then conceived without medical assistance, and 7 men in an infertile relationship of purely female aetiology. The remaining 36 participants had at least one defect in routine sperm parameters and were considered as having male infertility.

The study was prospectively approved by the Human Research and Ethics Committee, Women’s and Children’s Hospital, with all participants giving written informed consent for their involvement.
3.3.2 Sample Preparation

Sample preparation and the assays used in this study was discussed in Chapter 2.4

3.3.3 Statistical Analysis

Data were analysed using the statistical software Sigmastat (Systat Software Inc, California, USA) and presented as median values (inter-quartile ranges) as sperm parameters were not normally distributed. The normality of the distribution was assessed automatically by the statistical software itself. Correlation analysis was conducted using the Spearman Rank Order correlation test and between group comparisons were analysed using the Mann-Whitney Rank Sum test. A $P$ value $< 0.05$ was considered statistically significant.

Receiver operating characteristic (ROC) curve analysis was used to develop an optimal “cut off” point for formazan production that would best classify individuals as being fertile or infertile. ROC analysis and the associated sensitivity, specificity, positive and negative predictive value calculations were all performed using the SAS Version 9.1 statistical package (SAS Institute Inc., Cary, NC, USA).

3.4 Results

3.4.1 Standardization of assay conditions

In order to determine the ideal wavelength for quantifying sperm ROS production, the absorbance of a formazan solution was measured between the range of 240 and 1100 nm using a continuous spectrophotometer (Model UV-160, Shimadzu Corporation, Kyoto, Japan). As peak absorbance occurred between 620 and 740 nm, it was decided to make all future assay measurements at 630 nm since this is a common wavelength filter available for most ELISA plate readers. Furthermore, measurement of formazan absorbance on an ELISA plate reader, rather than using a spectrometer, was favoured as most laboratories have access to an ELISA plate reader and this technique allowed for duplicate measurements of multiple samples and standards in a simultaneous fashion.
A previous publication describing the use of the NBT assay to quantify sperm metabolic activity had determined that NBT reduction by sperm was optimally observed at a pH of 7.4, temperature of 37 degrees Celsius, with the reaction being complete within 30-40 minutes (Mercado-Pichardo et al., 1981). In our lab I measured formazan production at various concentrations of sperm following incubation for between 5 and 120 minutes and found that formazan production was effectively complete by 45 minutes, with very minimal additional formazan being produced between 45 and 120 minutes (Figure 3.1). As the aim of this study was to develop a rapid, easy to conduct assay suitable for clinical use it was terminated all further NBT assays would be read at 45 minutes.

![Formazan production at various concentrations of sperm at different incubation periods](image)

**Figure 3.1: Formazan production at various concentrations of sperm at different incubation period.**

Initial experiments in which the NBT solution was added to neat semen proved unsuccessful as the working solution immediately turned blue, even when using fertile men’s semen containing presumably low levels of ROS. Follow up experiments in which serial dilutions of seminal plasma were added to NBT solution revealed that the seminal plasma itself had the capacity to reduce NBT to formazan. Therefore, in order to properly quantify cellular ROS production it
was necessary to remove seminal plasma from the sperm/seminal leukocyte sample by a double wash in PBS before adding the NBT working solution.

The chemo-luminescent assays most widely used to quantify sperm ROS production are typically analysed at a standard concentration of $20 \times 10^6$ sperm per ml, with ROS production being expressed as $10^4$ counted photons per minute per $20 \times 10^6$ sperm (Athayde et al., 2007; Williams and Ford, 2005). The use of such a high starting concentration of sperm makes determination of ROS difficult in oligospermic men as they may have insufficient sperm to meet the $20 \times 10^6$ concentration criteria, especially if samples are assayed in duplicate. Furthermore, dilution of clinical samples to a standard concentration of $20 \times 10^6$ sperm may lead to dilution errors. Therefore it was decided to measure sperm ROS production at the sperm concentration that existed in neat semen, but then mathematically standardize the results as $\mu g$ formazan produced per $10^7$ sperm. Measurement of formazan production by individual men’s sperm diluted over a range of concentrations from 2 to 500 million sperm showed a good linear response (Figure 3.2A), supporting the utility of such an approach.

Figure 3.2A: ROS production for a single infertile individual upon serial dilution of a sperm suspension in the range of 2 to 500x10^6 sperm/mL. ROS production is represented as absorbance values (630 nm) on the right y axis and calculated formazan production ($\mu g$) on the left y axis.
The chemical reaction of xanthine oxidase with xanthine solution to generate variable amounts of superoxide anions in solution (Fridovich, 1970) was performed to confirm the NBT assays ability to accurately quantify changes in ROS production. As can be seen from Figure 3.2B, an increasing concentration of Xanthine substrate reacting with a fixed concentration of Xanthine Oxidase produced a linear increase in formazan production. The calculated intra and inter-assay coefficients of variation for the NBT assay using Xanthine/ Xanthine Oxidase as a ROS generator were 9.3 and 7.4 % respectively.

Figure 3.2B: Formazan production using the chemical Xanthine / Xanthine Oxidase (X/XO) ROS production system. A fixed concentration of Xanthine Oxidase (2µL 25 U/L) was added to an increasing concentration of Xanthine solution to stimulate increasing levels of ROS production. Mean values ± SD plotted in each graph.
Table 3.1: Summary statistics of routine semen parameters, reactive oxygen species (ROS) production and sperm DNA quality in fertile and infertile men. Values are represented as mean ± SD and median (inter-quartile range). Mann-Whitney Rank Sum test was used for statistical analysis.

<table>
<thead>
<tr>
<th></th>
<th>Fertile Group (n = 21)</th>
<th>Infertile Group (n = 36)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age (years)</strong></td>
<td>38 (33.5 – 41.5)</td>
<td>38 (35 – 42)</td>
<td>0.904</td>
</tr>
<tr>
<td><strong>ROS Production (µg Formazan/10⁷ sperm)</strong></td>
<td>16 (5.5 – 22.5)</td>
<td>65.5 (28.0 – 99.8)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td><strong>TUNEL positivity (%)</strong></td>
<td>9.6 (8.2 – 12.6)</td>
<td>20.7 (14.4 – 25.9)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td><strong>Early apoptosis (%)</strong></td>
<td>12.3 (7.0 – 15.5)</td>
<td>22.3 (14.9 – 34.4)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td><strong>Motility (%)</strong></td>
<td>52 (46 – 56)</td>
<td>38 (30 – 45.8)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td><strong>Morphology (%)</strong></td>
<td>19.0 (15.0 – 22.0)</td>
<td>7.0 (2.0 – 10.8)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td><strong>Sperm Concentration (x10⁶ cell/mL)</strong></td>
<td>107 (58.3–205.3)</td>
<td>26.6 (10.5 – 47.9)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td><strong>PMN Elastase (ng/mL)</strong></td>
<td>86.8 (36.5–169.0)</td>
<td>45.5 (15.5– 202.3)</td>
<td>0.529</td>
</tr>
</tbody>
</table>
3.4.2 Relationship between ROS production and measures of sperm health

The mean production of formazan by infertile men was considerably higher than that observed for their fertile counterparts (72.6 ± 58.5 µg v 17.8 ± 12.6 µg per 10⁷ sperm, p < 0.0001, Table 3.1), confirming a well established observation that oxidative stress is more prominent in the infertile population.

Figure 3.3 illustrate the correlation between levels of sperm oxidative stress (formazan production) and impairment of sperm function. A negative correlation exists between formazan production and sperm motility (r= -0.479, p= 0.002), suggesting that production of ROS by sperm and seminal leukocytes does impair sperm motility (Figure 3.3A). Furthermore, a very strong correlation was seen between initiation of apoptosis (Annexin V staining) and increasing levels of seminal ROS production (r= 0.669, p<0.0001, Figure 3.3B). Linked with this increase in sperm apoptosis is the observation of a significant increase in sperm DNA fragmentation with increasing levels of production of ROS (r = 0.591, p<0.0001, Figure 3.3C).

Seminal plasma elastase is secreted by activated neutrophils and is an acknowledged marker of both leukocyte number and activity within semen (Zorn et al., 2000). As neutrophils are potent producers of ROS, it was anticipated that seminal elastase concentration would be positively correlated with formazan production. Surprisingly, no significant correlation was observed between seminal plasma elastase and formazan production (Figure 3.4, r =0.006, p= 0.965). However, only 4 out of 36 participants (11%) exhibited signs of elevated leukocyte activity (PMN elastase > 290 ng/mL), which may have limited this studies ability to correlate seminal leukocyte activity with formazan production.

Furthermore, while infertile men’s sperm produce less than half the amount of formazan than peripheral leukocytes on a per cell basis (Figure 3.5), the production of ROS by sperm appears to be the dominant determinant of net seminal ROS production because of their vast numerical superiority over leukocytes.
Figure 3.3: Linear regression analysis depicting the relationship between ROS production (µg formazan per 10^7 sperm) and sperm motility (A), early apoptosis (Annexin V staining) (B) and sperm DNA fragmentation (TUNEL Assay) (C) for the combined fertile and infertile groups. Spearman regression correlations and p values are recorded for each observation.
Figure 3.4: Correlation between ROS production and seminal PMN Elastase concentrations in the combined fertile and infertile groups. No significant relationship was observed on spearman correlation analysis ($p = 0.719$).

$r = 0.006$
$P = 0.965$

Figure 3.5: Depiction of the relative ability of a pure “swim up” infertile sperm preparation and peripheral blood leukocytes to reduce NBT to formazan. This graph depicts two separate experiments at different concentrations of cells (1x10$^6$ cells and 4x10$^6$ cells/mL), with all experiments being conducted in triplicate.
3.5 Development of normal ranges

The ROC curve illustrated in Figure 3.6 depicts changes in the NBT assay sensitivity and specificity over the entire threshold range. From this graph two key determinants of the NBT assay’s clinical utility can be determined. Firstly, the optimal cut off point to maximize sensitivity and specificity is 24 µg formazan / 10^7 sperm, resulting in a sensitivity of 91.7% and a specificity of 81.0%. The positive and negative predictive values for this cut off point are 89.2% and 85% respectively. The calculated area under the curve (AUC) is 0.88, suggesting that the NBT assay has an excellent ability to distinguish between fertile and infertile individuals.

![ROC Curve](image)

Figure 3.6: ROC (receiver operating characteristic) curve for the assessment of ROS production in semen using NBT assay. The area under the curve (AUC) for this assay was 0.88, indicating that the assay was very good at distinguishing between fertile/infertile individuals.
3.6 Discussion

The results presented in this study confirm spermatozoa’s ability to reduce NBT to formazan, as has been suggested by most (Esfandiari et al., 2003; Mercado-Pichardo et al., 1981), but not all previous investigators (Armstrong et al., 2002). The sperm cytoplasm contains glucose-6-phosphate dehydrogenase which uses glucose to produce β-nicotinamide adenine dinucleotide phosphate (NADPH) via the hexose monophosphate shunt. NADPH is then used to fuel the generation of superoxide anions via NADPH oxidase contained within sperm, which in turn converts NBT into formazan. Seminal leukocytes are also known to contain NADPH oxidase and therefore are also responsible for some of the formazan production within each semen sample. However, a “swim up” generated pure sperm fraction was capable of reducing NBT to formazan, irrefutably confirming the capacity of sperm themselves to generate formazan even in the absence of contaminating leukocytes. This observation contradicts Armstrong’s earlier finding that sperm could not reduce NBT to formazan, even in the presence of PMA stimulation (Armstrong et al., 2002). It is unclear why this discrepancy exists, yet these results are consistent with the observations of two other groups who have confirmed sperm’s ability to reduce NBT to formazan (Esfandiari et al., 2003; Mercado-Pichardo et al., 1981).

To the best of my knowledge, this study is the first to correlate seminal ROS production using the NBT assay with markers of sperm quality such as sperm DNA fragmentation and motility. The production of formazan was significantly correlated with a decline in sperm motility. This result is consistent with previous studies that had shown seminal ROS production, quantified by chemiluminescent techniques, to be negatively correlated with sperm motility (Aitken, 1989; Whittington and Ford, 1998). It is likely that increasing production of ROS inhibit sperm motility by two different mechanisms. Firstly, ROS induce peroxidation of the sperm membrane, decreasing its flexibility and tail motion. Sperm membranes are vulnerable to this type of damage as they contain large amounts of unsaturated fatty acids. Secondly, ROS can directly damage sperm mitochondria, thereby decreasing energy availability and impeding motility.
A significant positive correlation between formazan production and an increase in sperm DNA fragmentation was noted, consistent with the well established capacity of ROS to damage sperm DNA (Aitken et al., 1998; Meseguer et al., 2008; Ozmen et al., 2007; Zorn et al., 2003b). Free radicals are able to induce sperm DNA fragmentation by two mechanisms. Firstly, ROS can directly attack the purine and pyrimidine bases and deoxyribose backbone of sperm DNA. Secondly, free radical damage will initiate apoptotic endonuclease fragmentation of the sperm DNA (Moustafa et al., 2004). It has been reported that infertile men producing high levels of ROS exhibit significantly greater levels of sperm early apoptosis (Annexin V+, PI -) compared to infertile men with low levels of ROS production (Moustafa et al., 2004). As formazan production was strongly correlated with early apoptosis in this study, it is likely that the observed increase in sperm DNA fragmentation seen with increasing oxidative stress is the net result of both direct ROS attack and endonuclease mediated apoptotic digestion of DNA.

Seminal plasma elastase was measured as it is a well recognised marker of leukocyte number and activity within semen. Zorn et al. have reported that a seminal plasma elastase level > 290 ng/mL has an 80% sensitivity for identifying leukocytospermia (Zorn et al., 2000). Since leukocytes are potent producers of ROS, and several researchers have correlated leukocyte number with seminal ROS production, it was anticipated that a significant positive correlation would exist between seminal plasma elastase concentrations and formazan production. Surprisingly, no such correlation was seen. The most likely reason for this was that only 11 % of the infertile participants had evidence for leukocytospermia (elastase > 290 ng/mL), with the vast majority of subjects being non-leukocytospermic, thereby weakening the study’s ability to correlate leukocyte activity with ROS output. Interestingly, Zorn et al. also found no significant correlation between elastase concentration and ROS production using chemo-luminescent techniques, with 25% of his study cohort having an elastase concentration exceeding 290 ng/mL (Zorn et al., 2000). Finally, seminal plasma elastase did not correlate with sperm DNA quality or motility in the study cohort (data not shown). My interpretation of these findings is that at least in non-leukocytospermic patients, sperm cells are by far the dominant producer of ROS and the key determinant of sperm DNA integrity, not seminal leukocytes. This finding is consistent with the observations of Henkel et al. (Henkel et al., 2005) who reported a much stronger correlation existed between sperm DNA integrity and intrinsic (sperm
derived) ROS production compared to extrinsic (leukocyte derived) ROS production.

The use of Receiver-operating characteristic (ROC) plots provide a pure index of accuracy by demonstrating a test’s ability to discriminate between alternative states of health (fertile v infertile) over the complete spectrum of operating conditions (Zweig and Campbell, 1993). This ROC analysis of the NBT assay has shown the assay to have a very good ability to determine fertility status for an individual. The sensitivity (91.7%) and specificity (81.0%) of the NBT assay, together with the recorded ROC AUC of 0.88, are all within the range associated as a highly useful clinical diagnostic test performance (Zweig and Campbell, 1993). However, before definitive comment can be passed on the clinical usefulness of the NBT assay larger scale clinical trials will need to be conducted.

Levels of sperm DNA damage exceeding 15% have now been linked with a reduction in embryo quality, an increase in miscarriage and a reduction in live births on the IVF program (Benchaib et al., 2007; Chohan et al., 2006). A large majority (84.3 %) of infertile men had levels of sperm DNA damage exceeding 15% when their NBT result exceeded the optimal cut off point of 24 µg / 10^7 sperm. Conversely no infertile man with an NBT result below 24 µg /10^7 sperm had sperm DNA fragmentation levels exceeding the clinically relevant 15% TUNEL threshold. Therefore it appears that the chosen a cut off point of 24 µg / 10^7 sperm is clinically appropriate.

Recently it has been reported that antioxidant therapies have the ability to improve infertile men’s sperm DNA integrity (Comhaire et al., 2000; Greco et al., 2005a; Menezo et al., 2007), while also improving pregnancy outcomes during both IVF and natural conception (Greco et al., 2005b; Suleiman et al., 1996; Tremellen et al., 2007). Despite this, the majority of clinicians still do not routinely prescribe antioxidants to their infertile patients. The current lack of availability of an inexpensive, easy to conduct assay for sperm oxidative stress is impeding optimal clinical care as many physicians are reluctant to offer empirical antioxidant treatments without laboratory evidence for the existence of oxidative pathology. I believe that this study provides support for the NBT assay to be further developed as an easy to perform assessment of sperm oxidative stress. I acknowledge that this study is relatively small and needs to be replicated by others, preferably in large scale multi-centred trials.
However, the NBT assay holds considerable promise because of its accuracy in identifying oxidatively damaged sperm and its relatively inexpensive and ease of conduct.
CHAPTER 4

Seminal Inflammation as a Cause of Oxidative Stress and Macrophage Activity in Infertile Men’s semen
4.1 Abstract

The presence of leukocytes within semen has the potential to impair sperm function. Neutrophils and macrophages make up 95% of seminal leukocytes, with both having the ability to damage sperm via the generation of reactive oxygen species (ROS), proteases and the induction of apoptosis. Existing cytological techniques for quantifying leukocyte activity within semen (peroxidase, CD45) are less than ideal as they merely count the number of leukocytes, rather than assess their activity.

Seminal plasma elastase effectively determines neutrophil activity, yet gives no insight into macrophage activity. Neopterin, a molecule released from activated macrophages, may be a useful marker for macrophage activity in the male reproductive tract. In order to examine this possibility a total of 63 asymptomatic subjects with male factor infertility and 11 fertile controls provided semen samples for measurement of various inflammatory markers. This study was able to confirm for the first time that seminal plasma does indeed contain neopterin and that the levels of this macrophage activity marker are 3-fold higher in infertile than fertile men.

Furthermore, seminal plasma neopterin concentration was significantly correlated with sperm oxidative stress, DNA fragmentation (TUNEL) and apoptosis (Annexin V), making it a useful marker of sperm health. In contrast, seminal plasma elastase showed no correlation with any marker of sperm health.
4.2 Introduction

Currently there is considerable controversy regarding the role that seminal leukocytes play in male infertility. Leukocytes generally represent a very small proportion of cells within semen (Eggert-Kruse et al., 1992), yet they have the potential to very significantly alter the function of adjacent spermatozoa (Aitken and Baker, 1995; Aitken et al., 1995a; Fraczek et al., 2008; Henkel et al., 2005; Plante et al., 1994). Polymorphonuclear (PMN) granulocytes account for 50% to 60% of seminal leukocytes, macrophages 20% to 30% and T lymphocytes for the remaining 5% (Smith et al., 1989; Wang and Holstein, 1983; Wolff, 1995). Histological analysis of the male reproductive tract suggests that macrophages primarily originate from the testicular interstitium and epididymis, as they are generally absent from the ejaculate of vasectomised men (Pelliccione et al., 2008; Wang and Holstein, 1983). Conversely, PMN granulocytes (principally neutrophils) are present in the ejaculate of vasectomised men, suggesting a seminal vesicle or prostatic origin for these cells (Anderson, 1990). Both neutrophils and macrophages have the potential to produce significant damage to sperm through their generation of reactive oxygen and nitrogen species, the release of hydrolytic enzymes and cytotoxic peptides (defensins) and via cytokine triggered sperm apoptosis.

It is interesting to note that the majority of semen samples, even those from fertile men with no evidence of genitourinary tract inflammation or infection, do contain leukocytes. There is general agreement that leukocyte counts between 1-5 x 10⁴/mL are normally present within fertile men’s semen and have no negative effect on sperm function (Aitken et al., 1995a; Tomlinson et al., 1993; Wolff and Anderson, 1988). It has even been postulated that low numbers of leukocytes may actually play a beneficial role by removing abnormal and degenerative spermatozoa, while generating hydrogen peroxide that may assist the process of sperm capacitation (Bize et al., 1991; Tomlinson et al., 1993). In support of such a positive role is the recent observation that the presence of a moderate number of leukocytes within semen (0.5 -1.0 x 10⁶/mL) is associated with improved sperm motility and epididymal function compared with a seminal leukocyte concentration of less 0.5 x 10⁶ (Ziyyat et al., 2008).

While low numbers of seminal leukocytes may play a positive role, the traditional view has been that once seminal leukocyte concentration rises above a threshold of 1 x 10⁵/mL (leukocytospermia), they have significant
potential to damage sperm and cause infertility. This negative impact of leukocytospermia on sperm function was suggested by studies linking the presence of greater than $1 \times 10^6$ leukocytes per ml of semen with poor sperm function (Berger et al., 1982; Wolff et al., 1990) and is the basis of the WHO definition of pathological seminal leukocyte content (WHO, 1999b). Furthermore, several studies have reported that leukocyte counts are significantly higher in infertile men’s semen than fertile controls (Ochsendorf, 1999; Plante et al., 1994; Sharma et al., 2001; Wolff, 1995), with up to 20% of infertile men exhibiting WHO defined leukocytospermia (Henkel et al., 2003).

Interestingly not all investigators have identified a link between semen leukocyte concentration and male fertility potential. For example, Aitken et al. (1995) found no link between seminal leukocyte count and sperm function while Tomlinson et al. (1993) reported no link between seminal leukocyte count and male fertility (Tomlinson et al., 1993). Aitken and Baker (1995) suggest that the role seminal leukocytes play in male fertility runs deeper than mere concentration due to two significant variables. Firstly, active leukocytes are more likely to create damage to sperm than dormant leukocytes. Secondly, the site of contact between seminal leukocytes and sperm may play a critical role in determining sperm function. For example, epididymal macrophages are in close proximity to sperm for prolonged periods of time during epididymal transit and storage, placing them in a favourable position to damage sperm (Barratt et al., 1990; Wilton et al., 1988). Conversely, neutrophils from the seminal vesicles and prostate only get an opportunity to damage sperm post-ejaculation when these accessory sex gland secretions come in direct contact with sperm. Assessment of leukocyte activity in the male reproductive tract, rather than mere determination of leukocyte concentration using traditional peroxidase or immunocytochemical means (Villegas et al., 2002; WHO, 1999b; Wolff, 1998), is therefore more likely to accurately reflect the immune systems influence on sperm function.

Currently the most widely used marker of male genital tract inflammation is seminal plasma elastase. When neutrophils become activated by inflammatory changes they undergo a “respiratory burst” with the release of reactive oxygen species (ROS) and proteases such as elastase. Seminal plasma elastase levels therefore directly reflect neutrophil inflammatory activity. The enzyme elastase has the potential to create proteolytic cell damage and DNA fragmentation in sperm and is easily quantifiable in seminal plasma using a widely available
commercial ELISA (Zorn et al., 2003a). The seminal plasma of infertile men contains higher levels of elastase than that observed in fertile men (Jochum et al., 1986; Reinhardt et al., 1997; Wolff and Anderson, 1988; Zopfgen et al., 2000; Zorn et al., 2000), with increasing seminal elastase levels being correlated with a reduction in sperm motility (Wolff et al., 1991; Wolff et al., 1990) and DNA integrity (Zorn et al., 2000).

No study to date has investigated the link between macrophage activity and sperm quality. This is a serious omission given that macrophages are the second most prolific leukocyte within semen, making up almost one third of all seminal leukocytes. Surprisingly, the WHO manuals own standard method for determination of leukocytospermia, the peroxidase staining technique, fails to identify macrophages and as a result significantly underestimates true leukocyte numbers within semen (Villegas et al., 2002; WHO, 1999b). While immunocytochemical methods can reliably quantify macrophage numbers, they do not directly measure macrophage inflammatory activity. As several groups have published evidence that macrophages play an important role in male reproductive dysfunction (Haidl et al., 2008; Jones, 2004; Pelliccione et al., 2008; Wilton et al., 1988), there is clearly an unmet need for an easily performed non-invasive test that can quantify macrophage inflammatory activity within the male genital tract.

Neopterin is an established marker of macrophage activity that has been studied for over 30 years. Neopterin is a low molecular mass pteridine molecule released from macrophages / monocytes primarily upon stimulation by the cytokine interferon gamma, and to a lesser extent other pro-inflammatory cytokines (Hamerlinck, 1999). There is a close relationship between the amount of neopterin released from macrophages and their capacity to produce reactive oxygen species (Berdowska and Zwirska-Korczala, 2001; Weiss et al., 1998). While neopterin has been reported to be present in various biological fluids such as serum, cerebrospinal fluid, synovial fluid, urine, saliva, ascitic fluid and pancreatic juices (Berdowska and Zwirska-Korczala, 2001; Hamerlinck, 1999), no study to date has reported the presence of neopterin in seminal plasma. Therefore, the aim of this study was to determine if neopterin is present in seminal plasma and if present, correlate neopterin levels with various markers of sperm health.
4.3 Materials and Methods

4.3.1 Subjects and Study Design

Participants in the study were recruited from men with known male factor infertility ("infertile subjects") defined as the presence of abnormal WHO semen quality criteria (WHO, 1999b) and the inability to conceive despite more than 12 months of unprotected intercourse. “Fertile controls” were men who were acting as sperm donors at an academic affiliated ART unit (Repromed, South Australia) and who had past proven fertility with normal semen parameters according to WHO criteria. None of the participants had any history suggestive of past or present infection of the male genital tract. All fertile sperm donors were assessed for active infection by semen culture and PCR analysis (Chlamydia, gonorrhoea), as is legally required. However, infertile men were not formally screened for infection our experience and that of others (Barratt et al., 1990) have found a very low positive yield for such tests in the asymptomatic population. At the time of producing the study semen sample no participant was taking any antioxidant supplements or medications that could affect their immune response.

The study was prospectively approved by the Human Research and Ethics Committee, Women’s and Children’s Hospital, with all participants giving written informed consent for their involvement.

4.3.2 Sample Collection and Preparation

Sample preparation and assays used in this study was discussed in Chapter 2.4

4.4 Results

As expected, standard semen parameters (concentration, motility and morphology) were significantly inferior in the infertile than the fertile cohort (Table 4.1). Furthermore, production of ROS (formazan) and the resulting oxidative damage to the sperm membrane (LPO-586) was significantly higher in the infertile than the fertile cohort. Sperm DNA damage (TUNEL) and apoptosis (Annexin V) were also significantly higher in infertile men’s sperm.
Table 4.1: Sperm Quality Parameters in infertile and fertile groups.

<table>
<thead>
<tr>
<th></th>
<th>Infertile Group (n=63)</th>
<th>Fertile Group (n=11)</th>
<th>P</th>
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<tr>
<td><strong>Sperm Concentration</strong> (x10^6 cell/ml)</td>
<td>25.8 (11.65–52.23)</td>
<td>97.5 (53 – 201)</td>
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<td><strong>Motility</strong> (%)</td>
<td>42 (30 – 48)</td>
<td>55 (49 – 57)</td>
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<td><strong>Morphology</strong> (%)</td>
<td>6 ( 2– 11)</td>
<td>21 (15 – 25)</td>
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<td><strong>Semen Volume</strong> (mL)</td>
<td>2.8 (1.8 – 4.0)</td>
<td>3.3 (2.7 – 4.5)</td>
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<td><strong>ROS Production</strong> (µg Formazan/10^7 sperm)</td>
<td>64.0 (31.8 – 88.0)</td>
<td>11.9(4.7–20.0)</td>
<td>&lt;0.0001</td>
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<td><strong>Lipid Peroxidation</strong> (µM MDA x10^7/sperm)</td>
<td>1.3 ( 0.4– 3.5)</td>
<td>0.05(0.01–0.14)</td>
<td>&lt;0.0001</td>
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<td><strong>TUNEL</strong> (%)</td>
<td>20.2 (13.9 – 25.8)</td>
<td>9.6 (8.1– 13.6)</td>
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<td><strong>Apoptosis</strong> (Annexin V) (%)</td>
<td>23.5 (18.2 – 33.9)</td>
<td>8.6 (5.4– 13.9)</td>
<td>&lt;0.0001</td>
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<td><strong>CD 45</strong> (x10^6 cell/ml)</td>
<td>1.0 (0.6 – 1.9)</td>
<td>0.9 (0.6 – 1.2)</td>
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<td><strong>PMN Elastase</strong> (ng/mL)</td>
<td>123 (22.8–275.3)</td>
<td>49 (10.5–118.5)</td>
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<td><strong>Neopterin</strong> (ng/mL)</td>
<td>96.9 (53 – 186)</td>
<td>42 (28 - 99)</td>
<td>0.013</td>
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All values are expressed as Median (inter-quartile range). Statistical analysis was performed using Mann-Whitney Rank Sum Test.

An excellent correlation was observed between the levels of production of ROS and resulting sperm membrane lipid peroxidation (r=0.812, p <0.001, Table 4.2), supporting the use of these assays as measures of sperm oxidative stress. Seminal ROS production was also strongly correlated with sperm DNA damage (TUNEL, r= 0.631, p <0.001) and apoptosis (Annexin V, r= 0.688, p < 0.001); while sperm DNA damage and apoptosis were also significantly positively correlated (r = 0.806, p<0.001).
Seminal leukocyte concentration was not significantly correlated with ROS production, sperm lipid peroxidation, motility, apoptosis or DNA fragmentation (Table 4.2). Seminal plasma elastase and neopterin were present at significantly higher concentrations in infertile than fertile men (Table 4.1). A highly significant correlation was seen between total leukocyte concentration (CD45) and neutrophil activity (elastase, $r= 0.472$, $p< 0.0004$), yet no significant correlation was observed between leukocyte concentration and macrophage activity (neopterin, $r= -0.019$, $p > 0.05$, Table 4.2). Furthermore, no significant correlation was observed between neopterin and elastase concentration in seminal plasma ($r = 0.028$, $p >0.05$).

While seminal plasma PMN elastase levels were significantly elevated in the infertile cohort, elastase concentration was not significantly correlated with any marker of semen quality aside from CD45 concentration (Table 4.2). Conversely, seminal plasma neopterin levels were predictive of many aspects of semen quality. Seminal plasma neopterin was significantly correlated with sperm concentration ($r=-0.309$, $p= 0.008$), morphology ($r = -0.325$, $p=0.008$), sperm DNA fragmentation ($r=0.340$, $p=0.003$) and apoptosis (annexin V, $r=0.481$, $p< 0.001$). There was also a strong positive correlation between neopterin concentration and markers of sperm oxidative stress such as LPO and NBT assay ($r = 0.425$, $p< 0.0001$ and $r = 0.477$, $p< 0.001$, respectively).
Table 4.2: Comparison between sperm quality between and inflammatory markers in all groups  \( t = \) statistically significant correlation

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In order to help locate the site of macrophage activity within the infertile men’s reproductive tract, seminal plasma neopterin concentration was analysed in relation to markers of epididymal (neutral alpha-glucosidase- NAG) and testicular function (testosterone, LH, FSH, AMH). Neopterin levels were not significantly correlated with any marker of testicular or epididymal function (Table 4.3).

Table 4.3: Correlation between macrophage activity (Neopterin) and epididymal/testicular function

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<td>Testosterone</td>
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<td>0.559</td>
</tr>
<tr>
<td>AMH</td>
<td>0.004</td>
<td>0.976</td>
</tr>
</tbody>
</table>

4.5 Discussion

The results of this study clearly show that genital tract inflammation is more commonly present in infertile than fertile men. This observation is in agreement with previous reports linking increased seminal leukocyte numbers (Plante et al., 1994; Wolff, 1995) (Ochsendorf, 1999; Sharma et al., 2001) and elastase concentration (Jochum et al., 1986; Reinhardt et al., 1997; Wolff and Anderson, 1988; Zopfgen et al., 2000; Zorn et al., 2000) with infertility. However, this study is the first to report the presence of the macrophage activity marker neopterin in human seminal plasma.

The observation that seminal plasma neopterin levels are three-fold higher in infertile than fertile men is consistent with the small number of histological studies reported in the literature. Frungieri et al (2002) examined macrophage numbers/ activity using immunocytochemistry and RT-PCR techniques in testicular biopsy samples from men with normal and abnormal
spermatogenesis (Frungieri et al., 2002). They found that macrophages were mainly localized to the testicular interstitium, with others also being located within the wall and lumen of seminiferous tubules. The density of testicular macrophages in men with spermatogenic defects was increased between two and six times compared to men with normal spermatogenesis, depending on the particular nature of the spermatogenic defect.

Hussein et al (2005) compared various leukocyte densities between azoospermic men with normal (obstructive) spermatogenesis and those with sertoli cell-only or germ cell maturation arrest (Hussein et al., 2005). This group also observed the presence of macrophages in the interstitium and seminiferous tubules, with the density of macrophages in the seminiferous tubule wall and lumen of men with abnormal spermatogenesis being double that seen in men with normal spermatogenesis. The presence of macrophages within the epididymis has also been confirmed by histological studies (Wang and Holstein, 1983; Yeung and Cooper, 1994). It has been suggested that epididymal macrophages may actually be present under normal physiological conditions to allow for the phagocytic elimination of dead or dying sperm (Holstein, 1978; Tomlinson et al., 1992a). Conversely, elevated numbers of macrophages have also been described in pathological conditions such as epididymitis and post vasectomy (Flickinger et al., 1995). As the majority of cases of chronic epididymitis are clinically silent (Haidl et al., 2008), it is possible that many of these infertile subjects may have experienced epididymitis despite being totally asymptomatic.

One of the most significant findings of this study was that measurement of macrophage activity by seminal plasma neopterin has proven to be a more useful marker of sperm quality than the more traditionally used inflammatory markers seminal plasma elastase and leukocyte concentration (CD45). No correlation was observed between seminal plasma elastase and any marker of sperm quality. This result is consistent with much of the published literature. While some observers have linked elevated seminal plasma elastase levels with a reduction in sperm count, motility (Wolff et al., 1991; Wolff et al., 1990) and an increase in sperm DNA damage (Zorn et al., 2000), many other studies have found no such link (Eggert-Kruse et al., 1997; Eggert-Kruse et al., 2009; Henkel et al., 2003; Maegawa et al., 2001). More importantly, seminal plasma elastase concentrations in asymptomatic males has been reported to have
absolutely no bearing on subsequent in vivo (Eggert-Kruse et al., 2009) or in vitro pregnancy rates (Henkel et al., 2003). These observations and studies failure to link seminal elastase concentration with sperm function in an asymptomatic population should not be interpreted as suggesting that neutrophils have no role in causing male infertility. A weak non-significant trend was observed between elastase concentration and ROS formation ($r=0.20$, $p=0.089$). As none of the study population had any symptoms of active infection, it is likely that most of the neutrophils present within semen were relatively inactive. Therefore, the presence of active infection could possibly result in a significant correlation between seminal plasma elastase and ROS production, resulting in impaired sperm function. What seems more certain is that neutrophils do not appear to play an important role in impairing sperm function in men without symptoms of active genital tract infection.

Leukocyte concentration (CD45) was only significantly linked with sperm concentration and semen volume, showing absolutely no correlation with all other measures of sperm function. It is possible that past infection of the prostate and seminal vesicle glands, the two principal determinants of semen volume, has resulted in this significant correlation. Infective damage to either the prostate and seminal vesicle will reduce semen volume (Jequier, 2000), increasing the sperm concentration per ml of semen, even when the rate of sperm production remains unchanged. A history of previous genital tract infection is a good predictor of current genital tract inflammation, but such a history is only found in 5-14% of cases (Comhaire et al., 1995; Zorn et al., 2003a). Furthermore, chronic genital tract inflammation is significantly more common in infertile men and the majority of cases are clinically asymptomatic (Haidl et al., 2008; Schuppe et al., 2008). This would appear to be the case in my study cohort.

The finding that only macrophage (neopterin), not neutrophil activity (elastase), was correlated with sperm functional capacity makes perfect sense when one considers the origins of these different types of leukocytes. Neutrophils are believed to be primarily derived from the prostate and seminal vesicle, while macrophages have a testicular or epididymal origin. Sperm only comes in contact with the secretions of the prostate and seminal vesicles at the time of ejaculation, giving neutrophils very limited time to damage sperm. Conversely, sperm are likely to be in direct contact with macrophages within
the seminiferous tubules and epididymis for many days during spermatogenesis, giving these activated macrophages ample opportunity to alter sperm function (Aitken and Baker, 1995; Barratt et al., 1990). As this study was unable to find any correlation between seminal plasma neopterin and markers of testicular or epididymal function, it is uncertain at which point in the male reproductive tract that macrophages primarily impact sperm functional capacity.

Activated macrophages are capable of harming cells such as sperm through three principal mechanisms (Anderson, 1990). Firstly, they are capable of producing large amounts of reactive oxygen and nitrogen species that will lead to sperm membrane lipid peroxidation and oxidative DNA damage. Secondly, they can destroy cells through the release of enzymes such as lysosomes and hydrolytic enzymes or cytotoxic peptides commonly referred to as defensins. Finally, macrophages can induce apoptosis in adjacent cells directly by Fas ligand or indirectly through the release of cytokines such as Tumor Necrosis Factor alpha (TNFα). Testicular macrophages have been confirmed to produce significant amounts of TNFα (Frungieri et al., 2002); with in vitro studies suggesting that TNFα has the ability to reduce sperm motility and genomic integrity (Said et al., 2005). Since I observed that neopterin levels were significantly correlated with LPO-586 and formazan production (NBT assay) plus annexin V expression, it is likely that macrophages damage sperm by both apoptotic and oxidative stress pathways. Furthermore, these results are in agreement with studies conducted by Solis et al, who also reported a positive correlation between macrophage numbers in semen and sperm DNA denaturation (Solis et al., 2003).

The measurement of seminal plasma neopterin has created a new non-invasive tool for measuring macrophage activity within the male reproductive tract. The observation that seminal plasma of infertile men contains significantly increased levels of neopterin, suggestive of an increased state of macrophage activation, has two potential interpretations in regards to the underlying aetiology of male infertility (Pelliccione et al., 2008). Firstly, it is possible that a silent infection or an auto-immune response to sperm antigens, akin to that seen following vasectomy (Flickinger et al., 1995), may lead to activation and recruitment of macrophages into the male reproductive tract where they could induce sperm damage. Alternatively, the activation of macrophages within the male reproductive tract may represent a normal physiological response in
which macrophages are involved in the removal of dead or defective sperm (Holstein, 1978; Tomlinson et al., 1992a), both more commonly produced by infertile men. In this second scenario macrophages are simply associated with defective spermatogenesis, rather than being the underlying cause. My observation of a significant link between seminal plasma neopterin and abnormal sperm morphology and apoptosis could support such a “physiological clearance” role for macrophages. However, it should be understood that even if macrophage clearance of dead and dying sperm is a normal physiological process, it may still pose a threat to male fertility as good quality live sperm may be damaged “in the cross fire” by proteases and ROS released from activated macrophages. Therefore, it is possible that immuno-suppressive therapies such as Pentoxifylline and non-steroidal anti-inflammatory drugs may prove beneficial in improving sperm quality in a select group of infertile men with high macrophage activity in their reproductive tract.

In summary, in an asymptomatic population the measurement of seminal plasma neopterin appears to be a significantly better marker of sperm health than the more traditional markers of male genital tract inflammation such as seminal plasma elastase or leukocyte concentration. Larger studies in the future will help develop normal “cut-off” values for seminal plasma neopterin that best define sperm health. While it has yet to be conclusively proven that macrophage activity is actually responsible for impaired sperm function, elevated seminal plasma neopterin levels may prove in the future to be a useful clinical indicator for initiating anti-inflammatory and antioxidant therapy so as to maximize sperm genomic integrity.
CHAPTER 5

Obesity as a Cause of Oxidative Stress
5.1 Abstract

Male obesity has been linked with a reduction in sperm concentration and motility, an increase in sperm DNA damage and changes in reproductive hormones. Recent large observational studies have linked male obesity with a reduced chance of becoming a father. One of the potential underlying pathological mechanisms behind diminished reproductive performance in obese men is sperm oxidative stress. The primary aim of this study was to determine if sperm oxidative stress was more common in obese/overweight men. A total of 81 men had their Body Mass Index (BMI) correlated with seminal Reactive Oxygen Species (ROS) production (NBT assay), sperm DNA damage (TUNEL), markers of semen inflammation (CD45, seminal plasma PMN elastase and neopterin concentration) and routine sperm parameters, together with reproductive hormones. The principal finding from this study was that oxidative stress did increase with an increase in BMI, primarily due to an increase in seminal macrophage activation. However, the magnitude of this increase was small and of only minor clinical significance as there was no associated decline in sperm DNA integrity or sperm motility with increasing Reactive Oxygen Species (ROS) production. Increased BMI was also found to be significantly linked with a fall in sperm concentration and serum testosterone, and an increase in serum estradiol.
5.2 Introduction

Obesity has become an increasing public health concern in Western society over the last few decades due to the ready availability of high calorie foods and a sedentary lifestyle. It is currently estimated that one third of adult males are obese and a further third are overweight (Hedley et al., 2004). Obesity is now recognized as an established cause of female sub-fertility, with increasing Body Mass Index (BMI) being linked with anovulation and an increased risk of miscarriage (Rich-Edwards et al., 2002; Wang et al., 2002). However, more recent data suggests that obesity outcomes for six thousand men over 30 years concluded that even after adjustment for marital status, obese men were 22% less likely to have fathered a child than their normal weight counterparts by the time they had reached their late 40’s (Jokela et al., 2008). Similarly, the Norwegian Mother and Child cohort study reported that overweight men (BMI 25- 29.9 kg/m\(^2\)) had an OR for infertility of 1.19 (95% CI 1.03-1.62) and obese men (BMI > 30 kg/m\(^2\)) had an OR for infertility of 1.36 (95% CI 1.12-1.62) (Nguyen et al., 2007).

The literature examining the link between BMI and routine sperm quality parameters is unfortunately confusing due to the presence of several conflicting observations (Hammoud et al., 2008). While many studies have reported a reduction in sperm concentration with increasing BMI (Fejes et al., 2005; Jensen et al., 2004; Magnusdottir et al., 2005), others have reported no link (Aggerholm et al., 2008; Pauli et al., 2008; Qin et al., 2007). Similarly, the link between BMI and sperm motility is also uncertain, with some studies reporting a significant negative correlation (Fejes et al., 2005; Kort et al., 2006), while others report no correlation (Aggerholm et al., 2008; Jensen et al., 2004; Pauli et al., 2008; Qin et al., 2007). No study to date has reported any significant relationship between BMI and the percentage normal sperm morphology (Jensen et al., 2004; Pauli et al., 2008; Qin et al., 2007).

Several studies have reported that obese men have abnormal reproductive hormone profiles which may impair spermatogenesis (Hammoud et al., 2008). Obese men consistently have been found to have lower serum testosterone and higher estrogen levels than their normal weight counterparts (Aggerholm et al., 2008; Jensen et al., 2004; Pauli et al., 2008; Schneider et al., 1979; Strain et al., 1988). The peripheral conversion of androgens to estrogen by aromatase action in adipose tissue is likely to be responsible for this altered
hormone profile. The relative high levels of estrogen and increased concentrations of hypothalamic opioids seen in obese men are hypothesised to create a negative feedback on the pituitary, impairing the release of LH and FSH (Hammoud et al., 2008). However, while some studies have identified a relative hypogonadotrophic state in obese men (de Boer et al., 2005; Giagulli et al., 1994; Pauli et al., 2008), others have reported serum LH and FSH levels compatible with normal weight controls (Aggerholm et al., 2008; Jensen et al., 2004).

Testicular heat stress may play a role in obesity related impaired spermatogenesis. The testicular temperature in obese individuals is likely to be higher than ideal due to two factors. Firstly, with an increase in BMI there is an increase in the deposition of fat in the suprapubic and inner thigh regions, enveloping the scrotum and impairing normal testicular heat transfer. Secondly, obese individuals tend to lead a more sedentary lifestyle, with the sitting position known to produce up to a 2 degree Celsius increase in scrotal temperature compared to a standing position (Jung et al., 2005). It is therefore probable that obesity related heating of the testicle may reduce sperm quality as spermatogenesis is very sensitive to increases in temperature (Jung and Schuppe, 2007).

A final mechanism by which obesity may impair male fertility is oxidative stress initiated damage to sperm. While no study to date has linked obesity with sperm oxidative DNA damage, there are several reasons to suspect such a link may exist. Firstly, obesity has been reported to create a state of systemic oxidative stress (Furukawa et al., 2004; Ozata et al., 2002), a situation that is likely to extend to the testicular micro-environment. Obese individuals are in a chronic state of inflammation due to adipose tissues production of pro-inflammatory cytokines such as IL-6 and TNFα which can stimulate leukocytes production of ROS (Das, 2001). Secondly, the production of Leptin by adipose tissue has been reported to increase sperm oxidative metabolism (Lampiao and du Plessis, 2008). Therefore, the primary aim of this study was to determine if increases in BMI above the healthy weight range (20-25 kg/m²) are associated with changes in seminal oxidative stress and sperm DNA damage. The secondary aims were to further analyse any possible correlations between BMI and other markers of male reproductive health (sperm count, motility, morphology and reproductive hormones) and signs of semen inflammation.
(semen leukocyte count and seminal plasma elastase/ neopterin concentration).

5.3 Materials and Methods

5.3.1 Subjects and Study Design

Study participants consisted of 81 men presenting for semen analysis at the Adelaide Fertility Centre (Repromed) as part of their assessment of male fertility potential. None of the men had any significant symptoms or signs of andrological dysfunction. Patient height (meters) and weight (kilograms) were recorded and converted into a Body Mass Index (BMI = kg/ m$^2$). Participants were classified according to the following published BMI ranges: normal weight BMI 20-25 kg/m$^2$, overweight 25.1-30 kg/m$^2$ and obese > 30 kg/m$^2$. The study was prospectively approved by the Human Research and Ethics Committee, Women’s and Children’s Hospital, with all participants giving informed written consent for their involvement.

5.3.2 Sample Collection and Preparation

Sample preparation and assays used in this study was discussed in Chapter 2.4

5.3.3 Statistical Analysis

Data were analysed using GraphPad Software (GraphPad Software Inc., La Jolla, CA, USA) and presented as median (inter-quartile ranges) due to the non-Gaussian distribution of the data. Statistical comparisons between groups (normal and high BMI groups) were made using the non-parametric Mann Whitney U test. Analysis of the correlation between continuous variables (BMI and various semen parameters/ reproductive hormones) was conducted using the Pearson’s correlation coefficient test. A $P$ value $< 0.05$ was considered statistically significant.

5.4 Results

A weak, yet statistically significant positive correlation was observed between BMI and seminal oxidative stress ($r = 0.23$, $p=0.039$, Figure 5.1). As BMI increased beyond the normal weight range (BMI > 25 kg/m$^2$) an increasing number of individuals exhibited quite marked oxidative stress. While many normal weight individuals exhibited ROS production exceeding the upper limit of ideal (24 µg formazan per $10^7$ sperm (Tunc et al., 2008), a disproportionate larger number of overweight/obese individuals exhibited very high levels of ROS production (Figure 5.1).
Analysis of the relationship between BMI and sperm morphology, a key determinant of sperm ROS production (Aziz et al., 2004), identified no significant relationship ($r = -0.08, p=0.53$). Furthermore, no significant correlations were identified between BMI and seminal CD45 leukocyte count or seminal plasma elastase activity. However, a weak yet statistically significant positive correlation was observed between seminal plasma neopterin and BMI (Figure 5.2), suggesting an enhanced state of macrophage activation with obesity.

**Figure 5.1:** Correlation between ROS production ($\mu$g formazan per $10^7$ sperm) and BMI.

**Figure 5.2:** Correlation between seminal plasma neopterin concentration (nmol/L) and BMI.
Figure 5.3 depicts the relationship between sperm DNA damage (TUNEL) and BMI. While there appears to be the possibility of a weak positive relationship between sperm DNA damage and BMI, this relationship was not statistically significant ($r = 0.13$, $p = 0.26$). As was found for seminal ROS production, extremes of sperm DNA damage were much more commonly seen in the high BMI group than those with a BMI in the normal 20-25 kg/m$^2$ range. The median sperm DNA fragmentation in the normal weight group was 12.4 % compared to 17.6 % in the group with a BMI > 25 kg/m$^2$, a result that bordered on significant ($p = 0.064$). When the high BMI group (BMI > 25 kg/m$^2$) was sub-divided into two groups depending on whether individuals had normal or high degrees of sperm DNA fragmentation (TUNEL < or ≥ 15%, respectively), the low DNA damage group had a median ROS output of only 23µg formazan per 10$^7$ sperm, yet the high DNA damage group produced a median output of 68.6 µg formazan per 10$^7$ sperm ($p < 0.001$).

![Figure 5.3: Linear regression analysis between sperm DNA damage (TUNEL positivity %) and Body Mass Index (BMI).](image)

$\text{r} = 0.13$

$p = 0.260$
A significant negative correlation was found between BMI and sperm concentration ($r = -0.33$, $p = 0.002$, Figure 5.4), but no significant relationship was noted between BMI and sperm motility. A significant correlation was also seen between BMI and serum oestradiol/testosterone concentrations. As depicted in Figure 5, serum testosterone decreased ($r = -0.29$, $p = 0.039$) and serum oestradiol increased ($r = 0.35$, $p = 0.026$) with increases in BMI. No significant correlation was seen between BMI and gonadotrophin (LH, FSH) concentrations.

*Figure 5.4: Correlation between sperm concentration and BMI.*

*Figure 5.5: Depiction of the correlation between BMI and serum testosterone and estradiol concentrations.*
5.5 Discussion

The results of this study suggest that sperm oxidative stress is increased in overweight (BMI >25 kg/m$^2$) compared to normal weight individuals. However, the link between increasing BMI and oxidative stress is a relatively weak one and only likely to be of limited reproductive importance. This study found no significant correlation between increasing BMI and sperm DNA damage or motility, both likely targets for ROS attack. A previous large study of 520 men did observe a moderate increase in sperm DNA damage with increasing BMI (Kort et al., 2006). It is acknowledged that this study was relatively small and therefore it is possible that the weak positive trend observed between BMI and sperm DNA fragmentation may have become statistically significant with a larger sample size.

The mechanism by which obesity may increase sperm oxidative stress is now becoming clearer. It is believed that the principal sperm source of ROS production in infertile men comes from morphologically abnormal immature sperm containing excess cytoplasm (Aziz et al., 2004). It is postulated that glucose-6-phosphate dehydrogenase contained in the excess residual cytoplasm fuels the enhanced generation of NADPH that in turn, stimulates the production of ROS. As this study did not identify any significant increase in abnormal sperm morphology with increasing BMI, it is uncertain how sperm themselves could be responsible for the observed increase in seminal ROS production associated with obesity. However, a recent report has shown that leptin and insulin, both elevated in the obese individual, can increase sperm metabolic activity and nitric oxide production (Lampiao and du Plessis, 2008). It is therefore still possible that morphologically normal sperm may generate increased amounts of ROS in obese men through mechanisms unrelated to sperm morphology.

Traditionally neutrophils have been considered the primary leukocyte source of ROS production in semen as they account for up to 70% of seminal leukocyte numbers and are potent producers of ROS during infection / inflammation. However, in this study no correlation between seminal plasma elastase concentration and BMI, making neutrophils an unlikely source for obesity
related oxidative stress. Conversely, seminal plasma neopterin levels were significantly positively correlated with BMI, suggesting that obesity creates a state of macrophage immune activation within the male reproductive tract. Neopterin is a low molecular mass pteridine molecule released specifically from macrophages / monocytes primarily upon stimulation by the cytokine interferon gamma and to a lesser extent other pro-inflammatory cytokines (Hamerlinck, 1999). It has been well established that there is a close relationship between the amount of neopterin released from macrophages and their capacity to produce reactive oxygen species (Berdowska and Zwirska-Korczala, 2001; Weiss et al., 1998). A significant relationship between seminal macrophage activation (seminal plasma neopterin) and ROS production (Tremellen and Tunc), supporting macrophages as an important source of oxidative stress. While previous papers have described a link between obesity and elevated serum neopterin levels (Ledochowski et al 1999, Brandacher et al 2006), this study is the first to describe the relationship between obesity and elevated seminal plasma neopterin concentrations. It would therefore appear that the previously described state of systemic immune activation seen in obese individuals (Bozdemir et al., 2006; Das, 2001; Ledochowski et al., 1999) also extends to the male reproductive tract, resulting in an enhanced state of macrophage activity and seminal oxidative stress.

In regards to my secondary objectives, I identified a significant negative correlation between sperm concentration and BMI, yet no link between BMI and sperm motility or morphology. While the published literature contains many conflicting reports regarding the relationship between BMI and routine sperm parameters, the finding of a significant negative correlation between BMI and sperm concentration is consistent with the bulk of this literature (Fejes et al., 2005; Jensen et al., 2004; Magnusdottir et al., 2005). My observations of a significant fall in serum testosterone and an increase in oestradiol with increasing BMI are also entirely consistent with the published literature (Aggerholm et al., 2008; Jensen et al., 2004; Pauli et al., 2008; Schneider et al., 1979; Strain et al., 1988).

This study is the first to report a significant correlation between increasing BMI and seminal oxidative stress, primarily related to an increase in macrophage activity within the male reproductive tract. I acknowledge that this link is a relatively weak one, with obesity likely to play only a minor role in the aetiology of oxidative sperm damage in the majority of individuals. However,
as obesity has been shown to reduce sperm concentration by this and other studies, it would appear prudent to advise all men of reproductive age to maintain a normal body weight to maximize their reproductive potential. As weight loss has been shown to reduce systemic oxidative stress (Melissas et al., 2006; Uzun et al., 2004), additional studies are warranted to determine if weight loss in obese individuals can result in a reduction in sperm oxidative stress and an improvement in sperm concentration.
CHAPTER 6

Treatment of Oxidative DNA Damage in sperm using an Oral Antioxidant Therapy
6.1 Abstract

Oxidative stress is now recognized as a common pathology affecting up to half of all infertile men. One of the principal mechanisms by which oxidative stress produces infertility is by damage to sperm DNA, either through direct oxidation of the DNA by reactive oxygen species (ROS) or by the initiation of apoptosis. The objective of this study was to determine if an oral antioxidant/mineral supplement could improve sperm DNA integrity in men with known oxidative stress. A total of 50 infertile men identified as exhibiting oxidative stress were administered oral antioxidant therapy for a period of 3 months. Sperm DNA integrity (TUNEL), apoptosis (Annexin V), protamination (CMA3) and reactive oxygen species (NBT Assay) production were assessed in all participants at entry and exit. Sperm concentration, motility and morphology; together with assessment of serum male reproductive hormones (LH, FSH, Testosterone, Anti-Mullerian Hormone) was also monitored. The principal finding that emerged from this study was that antioxidant therapy resulted in significant improvements in sperm DNA integrity and protamine packaging; accompanied by a reduction in seminal ROS production and apoptosis. No significant changes in routine sperm parameters (concentration, motility, morphology) or male reproductive hormones were observed.
6.2 Introduction

Elevated levels of sperm DNA damage are associated with increased time to natural conception (Spano et al., 2000; Loft et al. 2003), decreased IUI and IVF/ICSI pregnancy rates (Larson et al. 2000; Benchaib et al., 2003; Bengum et al., 2004; Virro et al., 2004; Borini et al 2006), increased miscarriage risk (Virro et al., 2004; Borini et al., 2006; Benchaib et al., 2007;) and possibly even increases in childhood cancer (Lewis and Aitken 2005). As at least 15% of men in infertile relationships have compromised sperm DNA integrity (Benchaib et al 2007), the development of new treatments to improve sperm DNA quality is of major clinical importance.

While the exact causes of sperm DNA damage have not been fully elucidated, several inter-related mechanisms have been suggested (Tesarik 2006; Aitken and De Iuliis 2007; Ozmen et al., 2007; Tarozzi 2007). Firstly, the most widely accepted cause for sperm DNA fragmentation is the presence of oxidative stress. The generation of sperm oxidative stress in vitro, either through the direct application of hydrogen peroxide (Aitken et al., 1998) or by stimulation of the sperm's own intrinsic production of free radicals (Twigg et al., 1998), has been reported to significantly increase the levels of DNA damage within sperm.

Defective protamine packaging of sperm DNA may also make infertile men’s sperm more susceptible to oxidative attack (Ozmen et al., 2007). During normal spermatogenesis 85% of nuclear histones are replaced with protamines that allow tight packaging of the sperm DNA, protecting it from oxidative attack. Approximately 15% of infertile men have been reported to exhibit deficient protamine packaging (Carrell and Liu 2001), making these men’s sperm more vulnerable to oxidative DNA damage (Aoki et al., 2005; Torregrosa et al., 2006).

The final mechanism responsible for sperm DNA fragmentation is “abortive apoptosis” (Sakkas et al 2003). Apoptosis plays two principal roles in spermatogenesis. Firstly, it is essential in limiting the population of germ cells to a number that can be adequately supported by Sertoli cells, thus ensuring normal spermatogenesis. Secondly, apoptosis is proposed to be responsible for
the selective depletion of abnormal germ cells (Sakkas et al 2003; Tarozzi 2007). However, even when good quality sperm are exposed to oxidative stress, apoptotic destruction is often triggered (Moustafa et al., 2004). If apoptosis fails to completely destroy these damaged cells ("abortive apoptosis"), endonuclease mediated digestion of the sperm DNA will occur within these still viable sperm. Therefore, oxidative damage to sperm DNA is the net effect of direct oxidative damage to the purine and pyrimidine bases of the sperm DNA, in tandem with apoptotic endonuclease digestion.

The Menevit<sup>®</sup> nutraceutical is an antioxidant preparation that has recently been shown in a placebo controlled randomized study to improve pregnancy outcomes when used in conjunction with IVF-ICSI treatment (Tremellen et al., 2007).

<table>
<thead>
<tr>
<th>Menevit&lt;sup&gt;®&lt;/sup&gt; Antioxidant Preparation</th>
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<tbody>
<tr>
<td>Lycopene 6 mg.</td>
</tr>
<tr>
<td>Vitamin E 400 IU</td>
</tr>
<tr>
<td>Vitamin C 100 mg.</td>
</tr>
<tr>
<td>Zinc 25 mg.</td>
</tr>
<tr>
<td>Selenium 26 µg.</td>
</tr>
<tr>
<td>Folate 500 µg.</td>
</tr>
<tr>
<td>Garlic oil 333 µg. (equivalent 1 g. garlic)</td>
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Table 6.1: Content of the MENEVIT<sup>®</sup> Antioxidant Preparation

The seven different components of Menevit<sup>®</sup> (Table 6.1) were identified as suitable for promoting sperm DNA health because of their capacity to impede oxidative attack through three overlapping mechanisms. Firstly, Vitamins C and E, selenium, garlic and lycopene have direct anti-oxidant effects (Heber and Lu 2002; Saravananan et al., 2004; Valko et al., 2004), assisting in the neutralization of ROS already produced by sperm or seminal leukocytes. Secondly, lycopene (Heber and Lu 2002) and garlic (Hodge et al., 2002) have
been shown to have potent anti-inflammatory activity, thereby potentially resulting in a reduction in seminal leukocyte ROS production.

Finally, zinc, folate and selenium are known to play a vital role in sperm DNA synthesis and protamine packaging (Kvist et al., 1987; Evenson et al., 1993; Pfeifer et al., 2001; Brewer et al., 2002;), possibly protecting sperm DNA from oxidative stress. The aim of the ADAM study (Assessment of DNA After Menevit®) was to determine if the Menevit® antioxidant can alter oxidative attack, protamine packaging, sperm DNA integrity and the production of male reproductive hormones.

6.3 Materials and methods

6.3.1 Subjects and Study Design

Participants in the ADAM study were recruited from men undergoing infertility assessment at an academic affiliated ART unit (Repromed, Dulwich, South Australia). To be eligible for involvement study participants were required to meet two inclusion criteria. Firstly, the entry semen analysis had to exhibit significant oxidative stress, as assessed by the Nitro Blue Tetrazolium (NBT) assay. Levels of seminal ROS production exceeding the 75\textsuperscript{th} percentile for fertile donors (19 \mu g formazan/ 10\textsuperscript{7} sperm) were considered as sufficient evidence for oxidative stress. A previous study of 12 fertile donors and 25 randomly selected infertile men had found that 68\% of infertile men had a seminal ROS score exceeding the 75\textsuperscript{th} percentile for fertile donors. Secondly, participants were required to have a minimum sperm concentration of 1 x 10\textsuperscript{6}/mL, as this was the minimum amount of sperm needed to conduct the various sperm DNA quality assays.

A total of 56 men with probable oxidative stress (poor motility, high abnormal sperm morphology, altered semen viscosity on routine analysis- Tremellen 2008) were screened to identify the target sample of 50 men with confirmed oxidative stress. Three men were excluded from the study due to low sperm count and three because of inadequate levels of oxidative stress on NBT assessment. The average age of participants was 39 ± 5.8 years (range 26 to 53 years) with an average duration of infertility of 2.5 ± 0.6 years.
Those participants who tested positive for oxidative stress were asked to take one capsule of Menevit® (Bayer Australia Ltd, Sydney, Australia) per day for a period of 3 months and to provide a semen and serum sample both at entry and exit. Four men withdrew from the study before completing their 3 months of antioxidant treatment due to a lack of continuing interest. One man withdrew as he believed the treatment aggravated his symptoms of Irritable Bowel Syndrome. The study was prospectively approved by the Human Research and Ethics Committee, Women’s and Children’s Hospital, with all participants giving written informed consent for their involvement.

6.3.2 Sample Collection and Preparation

Sample preparation and assays used in this study was discussed in Chapter 2.4

6.3.3 Statistical Analysis

Data were analysed using the statistical software Sigmastat (Systat Software Inc, California, USA). As the majority of data was not normally distributed, the results are principally expressed as median values (inter-quartile range), with statistical analysis being performed using the non-parametric Wilcoxon Signed Rank test. When the data was normally distributed, results are expressed as mean ± SD and were analyzed using the paired t test. A P value < 0.05 was considered statistically significant.

6.4 Results

After 3 months of antioxidant treatment sperm DNA integrity improved significantly, with the median sperm DNA fragmentation level dropping from 22.2% to 18.2% (Table 6.2). Recently it has been reported that short periods of abstinence can result in spontaneous improvements in sperm DNA integrity (Bakos et al., 2008). No significant differences in the period of abstinence between the entry and exit samples (mean 3.8 v 4.2 days respectively) could account for the observed improvements in sperm DNA integrity. Furthermore, the observed improvements in sperm DNA quality were mirrored by reductions in early apoptosis (Annexin V positive, PI negative), and a significant fall in seminal ROS production, suggesting that an anti-oxidant effect was primarily responsible for the observed improvement in sperm DNA integrity.

Three months of antioxidant treatment containing zinc and selenium produced a small but statistically significant improvement in median levels of sperm
protamination (69% v 73.6%, p<0.001). During normal spermatogenesis 85% of sperm histones are replaced by protamines; with impaired reproductive outcomes being observed once sperm protamination levels fall below 70% (Nasar-Esfahani et al 2004). At the commencement of the study, 26 infertile men (58%) had inadequate levels of sperm DNA protamination (< 70% sperm protamine content), yet by study completion only 14 participants (31%) exhibited protamine deficiency (<70% sperm protamine content).

Basic sperm parameters (count, motility, morphology, semen volume) did not change significantly following three months of anti-oxidant treatment (Table 6.3). All 45 trial participants had at least one defect in routine sperm quality, since only men with pre-existing male factor infertility were approached to enter the study. However, from review of Table 6.3 it can be seen that the average sperm parameters of the group were only moderately impaired.

<table>
<thead>
<tr>
<th></th>
<th>ENTRY Median (25%-75% range)</th>
<th>EXIT Median (25%-75% range)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>DNA fragmentation (%)</td>
<td>22.2 (16.5 – 26.6)</td>
<td>18.2 (13.4 – 23.1)</td>
<td>0.002</td>
</tr>
<tr>
<td>Early Apoptosis (%) (Annexin V +, PI -)</td>
<td>27.3 (20.4 – 34.7)</td>
<td>22.5 (19.3 – 28.1)</td>
<td>0.004</td>
</tr>
<tr>
<td>Protamination (%)</td>
<td>69 (63.5 – 73.1)</td>
<td>73.6 (69.3 – 77.5)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>ROS production (µg Formazan/ 10⁷ sperm)</td>
<td>66.4 (43.1 – 87.8)</td>
<td>44.4 (33.3 – 81.4)</td>
<td>0.027</td>
</tr>
</tbody>
</table>
Three months of antioxidant therapy did not appear to effect the production of any of the reproductive hormones monitored (Table 6.4). No significant changes in Leydig cell (serum LH and testosterone), nor Sertoli cell (serum FSH, AMH) derived hormones were observed. Furthermore, when a sub-group analysis examining only those participants with evidence of Sertoli or Leydig cell dysfunction at trial entry (FSH > 10 IU/L, Testosterone < 8 nmol/L) was performed, no significant differences in any reproductive hormones was observed following antioxidant therapy.
Thirty seven participants in the ADAM study underwent IVF treatment while on
the Menevit® antioxidant, although this was not a criterion for entry to the
study. The average age of the female partners was 35.7 ± 4.5 years, with an
average duration of infertility 3.0 ± 1.3 years. The majority of ADAM
participants underwent IVF-ICSI (36 cycles ICSI, 1 cycle IVF), with the
observed mean fertilization rate being 68.0 ± 26.7%. Thirty six of the 37
cycles proceed to an embryo transfer, with one transfer being cancelled due to
no genetically normal embryos being available for transfer (pre-implantation
genetic diagnosis cycle). Sixteen of the partners achieved a clinical pregnancy,
with 13 of these pregnancies (81.25%) being viable on a first trimester
ultrasound. A further 3 couples experienced a biochemical pregnancy, giving an
overall biochemical pregnancy rate of 52.8% and a clinical (ultrasound
identified) pregnancy rate of 44.4% per embryo transfer. Within this viable
pregnant cohort the median sperm DNA fragmentation reduced from 22 % at
entry to 13.3 % at exit.

<table>
<thead>
<tr>
<th></th>
<th>ENTRY</th>
<th>EXIT</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>LH (IU/L)</strong></td>
<td>3.93 ± 1.33</td>
<td>4.23 ± 1.34</td>
<td>0.078</td>
</tr>
<tr>
<td><strong>FSH (IU/L)</strong></td>
<td>5.58 ± 3.13</td>
<td>5.81 ± 3.28</td>
<td>0.160</td>
</tr>
<tr>
<td><strong>Testosterone (nmol/L)</strong></td>
<td>14.07 ± 4.1</td>
<td>14.96 ± 5.5</td>
<td>0.073</td>
</tr>
<tr>
<td><strong>AMH (pmol/L.)</strong></td>
<td>61.8 ± 28.9</td>
<td>61.4 ± 28.3</td>
<td>0.731</td>
</tr>
</tbody>
</table>

*Table 6.4 Reproductive Hormone concentrations during antioxidant treatment*
6.5 Discussion

The present data show a significant improvement in sperm DNA integrity following 3 months of antioxidant therapy. This result is consistent with the published literature. Firstly, a combination of Vitamin C and E has been shown in well conducted placebo controlled RCT’s to reduce both sperm (Greco et al., 2005) and peripheral blood leukocyte DNA damage (Moller et al., 2004). Non placebo controlled prospective studies have also shown antioxidants to improve sperm DNA integrity (Comhaire et al., 2000; Menezo et al. 2007).

The presence of high levels of sperm DNA damage was not used as a criterion for entry into the ADAM study, so as to avoid any suggestion that the primary study outcome (sperm DNA quality) was biased by the “regression to the mean” phenomenon. Baker and Kovacs (1985) had previously highlighted that when a group of subjects are selected for extreme results, on average their result tend to “normalize” on re-testing- a so called “regression to the mean”. This regression phenomenon is an important consideration when analysing semen parameters such as sperm count or motility, which are prone to wild intra-subject variability, but is of minimal importance when considering more stable parameters such as sperm DNA quality. A previous longitudinal study (Sergerie et al. 2005) has reported that sperm DNA fragmentation measured by TUNEL analysis exhibits well within individual stability over time, allowing for accurate assessment of baseline DNA damage from just a single entry sample.

The observation that antioxidant therapy was able to significantly reduce seminal ROS suggests that participants received a biologically adequate dose of antioxidant to positively influence the sperm DNA environment. Aside from a reduction in direct oxidative damage to the sperm, the results of this study suggest two other important mechanisms by which the Menevit® antioxidant may improve sperm DNA integrity. Firstly, a very significant reduction in sperm apoptosis was observed while on antioxidant therapy. Oxidative stress is known to initiate apoptosis (Moustafa et al., 2004), which in turn will ultimately lead to endonuclease fragmentation of the sperm DNA. It is likely that by reducing free radical attack on “good quality” sperm, less of these sperm will be forced down the path of abortive apoptotic DNA fragmentation. Secondly, the Menevit® antioxidant was shown to produce a small but significant improvement in sperm DNA protamination, making the sperm DNA more
impregnable to ROS attack. To the best of my knowledge, the ADAM study is the first of its kind to show that sperm protamination and apoptosis can be positively influenced by the use of an oral antioxidant/ mineral supplement.

A previous study using a combinational antioxidant supplement (Vitamin C 400 mg, Vitamin E 400 mg, β-carotene 18 mg, Zinc 500 µmol, Selenium 1 µmol) has reported that antioxidants may adversely effect sperm chromatin condensation, possibly mediated by Vitamin C interfering with inter-chain disulphide linkages in protamines (Menezo et al. 2007). These authors warn against the use of antioxidants in men with high degrees of sperm nuclear decondensation as protamine deficiency is linked with sperm premature chromosomal condensation and fertilization failure (Nasr-Esfahani 2004). It is possible that the lower dose of Vitamin C contained in the Menevit® supplement, together with the higher doses of zinc and selenium, may be responsible for the observed small improvement in sperm protamination compared to those observed in the Menezo study. A previous placebo controlled study (Tremellen et al. 2007) has reported that the use of the Menevit® antioxidant slightly improves ICSI fertilization rates when compared to placebo or patients own historical control IVF-ICSI cycles, making a significant negative effect of this antioxidant combination therapy on sperm nuclear condensation highly improbable. This is also supported by the good fertilization rates observed in the ADAM patients who did undergo IVF-ICSI treatment.

The active ingredients responsible for the observed improvement in sperm protamine content are most likely to be zinc and selenium as both have been associated with the process of sperm protamination. A link between zinc deficiency in men and abnormal sperm DNA condensation was first noted by Kvist et al., (1987). Similarly, rodents fed a zinc deficient diet have been reported to have abnormal sperm chromatin packaging and a resulting increase in sperm DNA damage (Evenson et al., 1993). Zinc enhances sperm DNA integrity by augmenting protamine 2 binding to the sperm DNA, thereby making the sperm less susceptible to oxidative attack (Brewer et al., 2002). Selenium is also an important cofactor in sperm DNA protamine condensation, with a 35 kDa seleno-protein being highly expressed late in spermatogenesis, and is felt to play an important role in disulphide cross-linking of protamines (Pfeifer et al., 2001). As a deficiency in zinc and selenium is not uncommon within infertile men (Ebisch et al., 2007), one can speculate that supplementation with zinc and selenium may have increased sperm protamine
related DNA condensation, thereby making sperm less susceptible to oxidative DNA damage.

The Menevit® anti-oxidant treatment had no significant effect on sperm count, motility or morphology. Previous small non-controlled studies have reported that lycopene (Gupta and Kumar 2002), vitamin C and E (several studies reviewed in Agarwal 2004), zinc and folate (Wong et al., 2002) can improve sperm parameters such as count, motility and morphology. Four previous double-blind RCT’s have examined the effect of anti-oxidant preparations (vitamin C, vitamin E, selenium combinations) on sperm parameters. Two (Greco et al., 2005, Rolf et al., 1999) found no significant effect of anti-oxidants on sperm count, motility or morphology which is consistent with my observations. Conversely, the remaining two RCT’s found a small but significant improvement in sperm motility but no improvement in sperm count or morphology (Suleiman et al., 1996; Keskes-Ammar et al., 2003).

Several researchers have identified a significant correlation between sperm count, motility and morphology and sperm DNA integrity (Tremellen 2008). Therefore it is uncertain why this study did not observe an improvement in these routine sperm parameters with antioxidant treatment, when sperm DNA integrity did improved. There are several possible explanations for this. Firstly, it is possible that sperm DNA is more resistant to ROS attack than the sperm membrane/ mitochondria (determinants of motility). If oxidative attack is reduced by antioxidant therapy, yet not totally resolved, the levels of available ROS may no longer be sufficient to damage the DNA but are still capable of decreasing motility. Secondly, while previous studies have linked poor routine sperm parameters (count, motility and morphology) with high levels of ROS production, an association does not prove causation. Without a cause and effect link, antioxidant therapy can not be expected to normalize all sperm parameters. For example, it has been well documented that excessive residual cytoplasm (abnormal morphology) is linked with an increase in the generation of ROS within sperm because of the availability of more cytoplasmic glucose-6-phosphate dehydrogenase activity (Said et al., 2005). Here abnormal morphology causes oxidative stress. However, while the use of antioxidants will reduce ROS within the sperm, possibly reducing DNA damage, they can not be expected to normalize sperm morphology. While the above statements are speculative, what is clear is that the majority of good quality in vivo studies do not show antioxidant therapy to have any material effect on routine sperm
parameters (Tremellen 2008), an observation which is consistent with my results.

Three months antioxidant treatment had no significant effect on serum hormone levels, suggesting no change in production by either the testicular Leydig (LH, Testosterone) or Sertoli cells (FSH, AMH). Some investigators have speculated that oxidative damage to the Leydig cell LH receptor is responsible for the gradual decline in serum testosterone observed with increasing male age (Hardy and Schlegel 2004). If this is the case, one would have expected to see some increase in serum testosterone or a drop in LH concentration, yet no such changes were observed. This lack of effect of the Menevit® antioxidant on reproductive hormones is consistent with the few examples within the existing literature. Comhaire et al., (2005) reported no change in serum LH, FSH or testosterone following 3 months therapy with a potent carotenoid antioxidant (Astaxanthin®), despite recording a very significant fall in seminal ROS production. Similarly, a double blind randomized, placebo controlled trial using a combination of zinc and folate reported no significant changes in serum testosterone or FSH (Wong et al., 2002).

In conclusion, the results of the ADAM study suggest that previously reported improvements in pregnancy outcome during IVF-ICSI treatment using the Menevit® antioxidant (Tremellen et al., 2007) was most likely mediated by significant improvements in sperm DNA integrity. The current data suggest that the observed reduction in sperm DNA fragmentation is the net effect of a reduction in ROS attack, a reduction in DNA susceptibility to ROS damage because of improved sperm DNA protamine packaging, and a decline in ROS initiated apoptosis. To the best of my knowledge, the ADAM study is the first study to describe the ability of an oral antioxidant / mineral supplement to enhance sperm DNA protamination while reducing sperm apoptosis.

This study adds to the growing body of evidence supporting the use of antioxidant combinational therapy to improve sperm DNA integrity (Comhaire et al., 2000; Greco et al., 2005; Menezo et al. 2007), especially for those men undergoing IVF-ICSI treatment. Bypassing the “quality control” of natural fertilization by the use of ICSI enables fertilization to occur even in the presence of severely damaged sperm DNA, placing patients at significantly increased risk of miscarriage (Virro et al., 2004; Borini et al., 2006; Benchaib et al., 2007). The clinical miscarriage rate observed in this study cohort was
similar to that reported for fertile couples with no underlying sperm DNA quality issues (Hassold and Chiu 1985). This observation suggests that treatment of men with high degrees of oxidative DNA damage with antioxidants before their partner commences IVF-ICSI therapy may be capable of improving pregnancy outcomes.
CHAPTER 7

Oxidative Stress as a cause of sperm DNA Hypomethylation
7.1 Abstract

Methylation of sperm DNA is impaired in many infertile men; potentially adversely effecting reproductive outcomes. In somatic cells oxidative damage to DNA and hyperhomocysteinaemia are linked with DNA hypomethylation. The objective of this study was to investigate if these pathologies also impair sperm DNA methylation. The relationship between sperm DNA quality, oxidative stress and serum homocysteine was analysed at study entry and after 3 months of antioxidant treatment.

Overall a significant negative correlation was observed between sperm DNA methylation and sperm DNA fragmentation, as well as seminal reactive oxygen species (ROS) production. Sperm DNA methylation was not significantly related to serum homocysteine concentrations. Administration of an antioxidant supplement produced a significant fall in seminal ROS levels and sperm DNA fragmentation, while increasing sperm DNA methylation.

These results suggest that oxidative stress related damage to sperm DNA impedes the process of methylation, while antioxidant supplementation appears to have the potential to reduce DNA damage and normalize sperm DNA methylation.
7.2 Introduction

It is currently widely accepted that oxidative stress is a common cause for sperm DNA damage. However, the effect of oxidative stress on the paternal genome epigenetic expression is currently unknown. As oxidative stress has been linked with altered epigenetic programming in somatic cells, it was thought to be useful to explore this possible link in sperm.

Since reports emerged linking IVF conceived pregnancies with the epigenetic disorders Angelman and Beckwith-Wiedemann Syndromes (Cox et al., 2002; DeBaun et al., 2003; Gosden et al., 2003; Lawrence and Moley, 2008), there has been intense scientific interest in epigenetic processes involved in reproduction. Disorders of genomic imprinting occur when there is a failure of the correct pattern of DNA parental-origin-dependant monoallelic gene expression (Lawrence and Moley, 2008). The methylation of cytosine residues in DNA by DNA methyltransferase is considered to be one of the major epigenetic mechanisms controlling gene expression and imprinting (Schaefer et al., 2007). Hypomethylation of DNA is associated with gene transcriptional activity while hypermethylation is associated with gene silencing.

While several studies have suggested that adverse embryo culture environments are responsible for the majority of epigenetic defects observed in IVF conceived pregnancies (DeBaun et al., 2003; Lawrence and Moley, 2008; Thompson et al., 2002), other observations have suggested that sperm abnormalities may also play a role. Firstly, investigators have reported that aberrant sperm DNA methylation, principally in the form of hypomethylation, is more commonly seen in infertile men compared to their normozoospermic counterparts (Benchaib et al., 2003a; Houshdaran et al., 2007; Kobayashi et al., 2007; Marques et al., 2004; Marques et al., 2008). Secondly, experimental inhibition of sperm epigenetic programming by exposure of male rodents to endocrine disruptors results in reduced sperm fertilization capacity, altered embryonic gene expression, an increase in pre-implantation embryonic loss (Doerksen et al., 2000; Doerksen and Trasler, 1996; Kelly et al., 2003; Oakes et al., 2007; Pathak et al., 2008) and cancer in future generations (Anway et al., 2005; Anway and Skinner, 2008). Finally, studies have also linked sperm DNA hypomethylation in men with a reduction in IVF pregnancy rates (Benchaib et al., 2005; Cisneros, 2004; Kobayashi et al., 2007). Therefore,
infertility related anomalies in sperm epigenetic programming may have serious clinical consequences and certainly warrants further investigation.

Epigenetic programming of sperm DNA occurs at several key stages in the spermatogenesis cycle. In rodents it is reported that DNA methyltransferase 1 (DMT1) mRNA and protein are expressed at high levels in mitotic and early meiotic male germ cells, with the enzyme then being translationally down regulated in pachytene spermatocytes (Jue et al., 1995; Numata et al., 1994; Trasler et al., 1992). Human studies have demonstrated that the paternally imprinted gene H19 has its methylation pattern erased in early fetal life; with remethylation being initiated as spermatogonia enter meiosis and is effectively complete by the primary spermatocyte stage of differentiation (Kerjean et al., 2000). Testicular tissue samples taken from fertile men show DMT1 gene expression is restricted to spermatogonia, pachytene spermatocytes and round spermatids; with DMT1 protein only being expressed in the nucleus of spermatogonia and in the cytoplasm of round spermatids (Omisanjo et al., 2007). Interestingly, Ariel et al reported that spermatogenesis-specific genes can also undergo quite late epigenetic re-programming while maturing within the epididymis (Ariel et al., 1994). Therefore, pathology within both the testicular and epididymal environment has the potential to disrupt the establishment of normal sperm DNA methylation patterns.

To date only one study has reported on any underlying mechanism for abnormal sperm DNA methylation seen in infertile men (Kobayashi et al., 2009). This study identified DNA sequence variations in the gene encoding the DNA methyltransferase enzyme DNMT3L in several infertile men, which in turn was associated with abnormal paternal DNA methylation. However, studies investigating the link between somatic cell DNA hypomethylation and cancer have suggested other possible mechanisms for sperm DNA hypomethylation may exist such as defects in the folate / homocysteine pathway and oxidative stress.

Oxidative stress may predispose to epigenetic abnormality as oxidative attack leads to the generation of DNA strand breaks and the formation of DNA base adducts such as 8-hydroxy-2’-deoxyguanosine (8-OH-dG) and O6-methylguanine, both reported to interfere with the DNA’s ability to act as a substrate for DNA methyltransferases (Franco et al., 2008). The presence of 8-OHdG in CpG dinucleotide sequences (Turk et al., 1995; Valinluck et al., 2004;
Weitzman et al., 1994) or O\textsuperscript{6}-methylguanine (Hepburn et al., 1991), strongly inhibits methylation of adjacent cytosine residues and ultimately leads to global DNA hypomethylation.

The folate/homocysteine pathway is responsible for the generation of methyl donors and therefore is central to the process of DNA methylation for all cells (Yi et al., 2000). Defects in the folate cycle are responsible for depleting S-adenosylmethionine (SAM) levels and have been linked with DNA hypomethylation in somatic cells (Jamaluddin et al., 2007; Yi et al., 2000).

Methylenetetrahydrofolate reductase (MTHFR) is a key folate-metabolizing enzyme which catalyses the conversion of 5,10-methylene tetrahydrofolate to 5-methyl tetrahydrofolate, the later which provides methyl groups for the methionine synthase-mediated remethylation of homocysteine to methionine. Studies in mice have linked polymorphisms in the MTHFR gene with reduced availability of methionine / SAM resulting in hypomethylation of testicular DNA (Chen et al., 2001). Interestingly, while polymorphisms in the MTHFR gene limiting its enzymatic activity are more common in infertile men (Bezold et al., 2001; Paracchini et al., 2006; Singh et al., 2005; Stuppia et al., 2003), no study has yet examined the link between defects in the folate pathway and sperm DNA methylation.

Since oxidative stress and hyper-homocysteinaemia have been linked with somatic cell DNA hypomethylation, it was proposed that similar mechanisms may operate in sperm. The principal aim of this study was to investigate the possible link between seminal oxidative stress related DNA fragmentation, serum homocysteine and methylation of sperm DNA.

### 7.3 Materials and Methods

#### 7.3.1 Subjects and Study Design

Participants in the study were recruited from men with known male factor infertility (“infertile subjects”) defined as the presence of abnormal WHO semen quality criteria (WHO 1999) and the inability to conceive despite more than 12 months of unprotected intercourse. “Fertile controls” were men who were acting as sperm donors at an academic affiliated ART unit (Repromed, South Australia) and who had proven fertility within the last 12 months and normal semen parameters according to WHO criteria. All infertile participants
were asked to take one capsule of Menevit® (Bayer Australia Ltd, Sydney, Australia) per day for a period of 3 months and to provide a semen and serum sample both at study entry and exit. Three months duration of antioxidant therapy was considered appropriate as this would cover one full spermatogenesis cycle (~ 70 days). Each capsule of Menevit contained 500 µg of folate and various anti-oxidants (Vitamin C 100 mg, Vitamin E 100 IU, Lycopene 6 mg, zinc 25 mg, selenium 26 µg, and garlic oil 333 µg). Fifty men entered the study, with 5 withdrawing over the next 3 months due to a lack of continuing interest (4) or perceived side effects (1). The twelve “fertile controls” produced only a single semen sample for the purposes of this study and were not administered any antioxidant therapy. The study was prospectively approved by the Human Research and Ethics Committee, Women’s and Children’s Hospital, with all participants giving written informed consent for their involvement.

7.3.2 Sample Collection and Preparation

Sample preparation and assays used in this study was discussed in Chapter 2.4

7.3.3 Statistical Analysis

Data were analysed using GraphPad Prism (GraphPad Software Inc.,La Jolla, CA,USA). Correlations between variables were analysed using the Spearman Rank Order correlation test. Sperm quality parameters before and after antioxidant therapy were expressed as mean ± SD or median (inter-quartile ranges) and analysed using the pair t-test or Wilcoxon Signed Rank test, depending on whether the data followed a normal distribution. A P value < 0.05 was considered statistically significant.

7.4 Results

Table 7.1 depicts sperm quality parameters for both the fertile and infertile groups. As only men with male factor infertility were recruited into the study, it was expected that the infertile group would have lower sperm quality than the fertile controls. There was a statistically significantly increased level of sperm DNA fragmentation (TUNEL) and semen reactive oxygen species production (NBT) in the infertile group compared to the fertile men. The relationship between sperm global DNA methylation and semen parameters such as sperm concentration ($r= -0.330$, $p=0.0289$) and morphology ($r= -0.394$, $p=0.008$)
was significant, but no significant relationship was observed between levels of DNA methylation and sperm motility \( (r=0.075, p=0.624) \) or leukocyte concentration \( (r= -0.21, p= 0.12) \).

**Table 7.1 Comparison of sperm quality between the infertile and fertile study groups**

<table>
<thead>
<tr>
<th>SPERM QUALITY PARAMETERS</th>
<th>FERTILE n= 12</th>
<th>INFERTILE n=45</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sperm Concentration ( (\times 10^6/\text{mL}) )</td>
<td>122.1± 79.8</td>
<td>36.17± 46.2</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Motility ( (%) )</td>
<td>52.6 ± 7.0</td>
<td>36.5 ± 12.3</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Normal Morphology ( (%) )</td>
<td>21.0 ± 6.6</td>
<td>6.7 ± 5.0</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>CD 45 ( (\times 10^6 \text{cell/ml}) )</td>
<td>0.89 ± 0.51</td>
<td>1.53 ± 1.30</td>
<td>0.136</td>
</tr>
<tr>
<td>ROS Production ( (\text{mg Formazan/mL}) )</td>
<td>15.4 ± 12.1</td>
<td>77.8 ± 58.9</td>
<td>0.0006</td>
</tr>
<tr>
<td>DNA Fragmentation ( (\text{TUNEL Positive %}) )</td>
<td>10.3 ± 3.8</td>
<td>22.9 ± 9.1</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Sperm Global DNA Methylation ( (\text{au}) )</td>
<td>104.7± 11.3</td>
<td>93.96±30.99</td>
<td>0.095</td>
</tr>
</tbody>
</table>

*All values are expressed as mean ± standard deviation and analysed using the unpaired t-test*

The results in Figure 7.1A illustrate the correlation between levels of sperm DNA methylation and DNA fragmentation (TUNEL). It can be seen that a significant negative correlation exists between these two parameters \( (r=-0.311, p=0.02) \), supporting a possible link between DNA damage and impaired capacity for methylation. The observation of a similar statistically significant negative correlation between sperm DNA methylation and seminal ROS production \( (r=-0.378, p=0.004; \text{Figure 7.1B}) \), together with a positive correlation between TUNEL and NBT results \( (r=0.336, p=0.022) \) suggests that oxidative damage to sperm DNA is at least in part responsible for modifying sperm global DNA hypomethylation.
Figure 7.1A: Relationship between Sperm DNA fragmentation (TUNEL) and Global DNA Methylation.

Figure 7.1B: Relationship between seminal ROS production and Global DNA Methylation.
No significant correlation was observed between serum homocysteine and global sperm DNA methylation in the infertile cohort ($r=0.26$, $p=0.09$). Furthermore, sperm global DNA methylation levels did not differ significantly between those infertile men with “normal” serum homocysteine range (<11.4 mmol/L - 95th percentile in folate replete men aged 20-45 years) (Selhub et al., 1999) and those with hyper-homocysteinemia (mean sperm DNA methylation 91.9 ± 29.7 v 94.8 ± 35.9 a.u. respectively, $p=0.378$).

Three months supplementation with antioxidant produced a significant fall in seminal ROS levels and DNA damage (TUNEL), combined with an increase in sperm global DNA hypomethylation (Table 7.2). Sperm count, motility and morphology did not change significantly over the 3 month supplementation period, while serum homocysteine levels decreased a small but statistically significant amount.

Table 7.2: Sperm quality before and after 3 months of anti-oxidant supplementation

<table>
<thead>
<tr>
<th>SPERM QUALITY PARAMETERS n = 45</th>
<th>Pre-antioxidant supplementation</th>
<th>On anti-oxidant supplementation</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sperm Concentration (x10^6/mL)</td>
<td>21.7(12.08 – 38.33)</td>
<td>21.4(12.75–44.1)</td>
<td>0.304</td>
</tr>
<tr>
<td>Motility (%)</td>
<td>36.5 ± 12.3</td>
<td>37.1 ± 11.5</td>
<td>0.778</td>
</tr>
<tr>
<td>Normal Morphology (%)</td>
<td>6.7 ± 5.0</td>
<td>6.71 ± 4.5</td>
<td>0.938</td>
</tr>
<tr>
<td>Homocysteine (µmol/L)</td>
<td>10.00 (9.13–11.68)</td>
<td>9.38 (8.50–10.90)</td>
<td>0.001</td>
</tr>
<tr>
<td>ROS Production (µg Formazan/mL)</td>
<td>66.4 (43.5–88.0)</td>
<td>44.4 (33.3 – 81.4)</td>
<td>0.027</td>
</tr>
<tr>
<td>DNA Fragmentation (TUNEL Positive%)</td>
<td>22.6 (16.5 – 28.8)</td>
<td>18.2 (13.4 – 23.1)</td>
<td>0.002</td>
</tr>
<tr>
<td>Sperm Global DNA Methylation (a.u.)</td>
<td>93.96 ± 30.99</td>
<td>108.61± 35.54</td>
<td>0.0017</td>
</tr>
</tbody>
</table>
7.5 Discussion

While several papers have now reported that infertile men’s sperm are more likely to express aberrant DNA methylation patterns (Benchaib et al., 2003a; Benchaib et al., 2003b; Houshdaran et al., 2007; Kobayashi et al., 2007; Marques et al., 2004; Marques et al., 2008), this study is one of the first to report on the underlying mechanisms behind such observations. The results of this study suggest that oxidative damage to sperm DNA integrity inhibits methylation, while abnormalities in the methyl donor pathway do not appear to play a significant role in sperm DNA methylation. These results are consistent with the small body of evidence already existing. Firstly, a recent report (Tavalaee et al., 2008) also found a statistically significant negative correlation between sperm DNA fragmentation, as assessed by the Sperm Chromatin dispersion Test, and sperm DNA methylation. A similar negative trend ($r = -0.45$, $P > 0.05$) had earlier been reported between sperm DNA fragmentation (TUNEL) and methylation (Benchaib et al., 2005). Unfortunately neither group of researchers measured seminal ROS levels in their study, making it impossible to confirm that oxidative stress was the underlying cause of the observed DNA damage/hypomethylation. Interestingly, Tavalaee et al. (2009) explained their results by suggesting that hypomethylated sperm may be more prone to DNA damage (Tavalaee et al., 2009). While it is possible that normally methylated DNA may be less susceptible to DNA damage, I am unaware of any evidence supporting the concept that methylation of DNA protects it from apoptotic or oxidative damage, the two principal causes of sperm DNA damage (Aitken, 2004). Furthermore, Tavalaee et al. (Tavalaee et al., 2008) observed no link between protamination of the sperm (CMA$_3$ staining) and global DNA methylation, making a non specific defect in chromatin remodelling unlikely. As oxidative stress effects a significant proportion of infertile men (Tremellen, 2008) and is widely believed to be the primary cause of sperm DNA fragmentation (Aitken, 2004; Aitken and De Iuliis, 2007), it is more likely that oxidative DNA damage played some role in sperm DNA hypomethylation reported in these earlier studies.

The link between oxidative DNA damage and hypomethylation is already established for somatic cells, with several investigators reporting a link
between the presences of oxidative DNA adducts in somatic cells and impaired DNA methyltransferase activity (Turk et al., 1995; Weitzman et al., 1994). Furthermore, incorporation of 8-OHdG in the methyl-CpG binding protein (MBP) recognition sequence has been reported to result in significant inhibition of MBP binding, further impeding the process of DNA methylation (Valinluck et al., 2004). The observation of a statistically significant negative correlation between sperm DNA methylation and seminal ROS production, together with a strong positive correlation between TUNEL and NBT results strongly suggests that oxidative damage to sperm DNA is responsible for sperm global DNA hypomethylation. Furthermore, the observed improvement in sperm DNA methylation with 3 months antioxidant therapy also suggests that oxidative damage to sperm impairs DNA methylation. However, the link between sperm oxidative DNA damage and hypomethylation may best be confirmed by studies correlating the generation of the oxidative specific DNA base adduct 8-hydroxyl-2'-deoxyguanosine (8-OH-dG) with sperm DNA methylation.

While this study did observe a very significant correlation between total semen ROS production and sperm DNA methylation, no significant correlation was observed between semen leukocyte concentration and sperm DNA methylation. This would imply that sperm themselves, not seminal leukocytes, are the primary source of ROS production interfering with the DNA methylation process. As none of the men in this study had any history suggestive of active genital tract infection, it is likely that the seminal leukocytes were relatively inactive and therefore not a dominant source of ROS production. Furthermore, it makes biological sense that intrinsic ROS production within the sperm cytoplasm is more likely to interfere with the process of sperm DNA methylation in the adjacent nucleus than extrinsic ROS released into the extracellular environment by leukocytes.

A weakness of this study was that it did not include a concurrent placebo control in the infertile subgroup. Some sperm parameters such as concentration and motility are prone to large fluctuations on different sampling occasions, even within the same individual, and therefore have a tendency for spontaneous “improvement” if subjects are recruited into a trial based on an initial low result. This non-treatment related improvement in sperm quality over time is termed “regression to the mean”. Conversely, failure to see significant fluctuations in sperm quality in a placebo control group over time suggests that regression to the mean is not an important determinant for that
particular sperm parameter. As there was not a placebo control in the infertile group, one can not state for certain that the observed improvements in sperm DNA methylation over three months of antioxidant therapy are not the result of spontaneous “regression to the mean”. However, since infertile participants were not selected for enrolment in the trial based on low initial sperm DNA methylation results, regression to the mean is unlikely to be a major cause for the observed improvement in sperm DNA methylation. Furthermore, the observed significant correlation between ROS production and sperm DNA methylation suggests a true biological cause-effect association between oxidative stress and impaired sperm DNA methylation.

The observation of no significant correlation between serum homocysteine and sperm DNA methylation does not support a significant role for abnormalities in the folate/ homocysteine pathway as a cause of sperm DNA hypomethylation. Infertile men are more prone to inefficient folate cycle reconversion of homocysteine to methionine as polymorphisms in their MTHFR gene are more common (Bezold et al., 2001; Dhillon et al., 2007; Lee et al., 2006; Paracchini et al., 2006; Singh et al., 2005; Stuppia et al., 2003), resulting in an up to 70% reduction in MTHFR activity. However, the lack of a significant negative correlation between sperm DNA methylation and homocysteine makes mutations in the MTHFR gene and hyperhomocysteinaemia unlikely candidates for causing sperm DNA hypomethylation in infertile men. A weakness of this study was that it did not specifically target men with MTHFR homozygous mutations and poor dietary folate intake who are likely to have extremely high levels of serum homocysteine. Such extreme abnormalities in homocysteine metabolism may still potentially be associated with alterations in sperm DNA methylation, despite the evidence suggesting that minor elevations in homocystine do not impact on sperm DNA methylation status.

The clinical implications of impaired sperm DNA methylation are presently uncertain. Animal studies have shown that chemically blocking sperm DNA methylation results in reduced sperm fertilization capacity, altered embryonic gene expression and an increase in pre-implantation embryonic loss (Doerksen et al., 2000; Doerksen and Trasler, 1996; Kelly et al., 2003; Oakes et al., 2007; Pathak et al., 2008). Furthermore, the creation of mouse embryos using sperm with high degrees of DNA damage has been shown to result in epigenetic abnormalities in the resulting progeny with major physical and behavioural abnormalities later in life (Fernandez-Gonzalez et al., 2008).
Aitken and De Iuliis have speculated that aberrant sperm DNA methylation may also lead to epigenetic defects that adversely affecting the health of the next generation of children (Aitken and De Iuliis, 2007). Imprinting disorders such as Beckwith-Wiedermann and Angelman syndromes are relatively rare, making epidemiological linkage between infertility and the development of these imprinting disorders extremely difficult. As such, the link between sperm DNA methylation and the development of childhood imprinting disorders is still far from proven. Russell-Silver Syndrome, a rare disorder characterized by growth restriction, limb and facial anomalies and learning difficulties has been shown to be primarily caused by hypomethylation on the paternal allele of DMR1 at 11p15 (Gicquel et al., 2005; Netchine et al., 2007). It is interesting to note that hypomethylation of the 11p15 DMR1 has also been reported to be more common in oligospermic infertile men (Marques et al., 2004), and at least one case report has linked the use of IVF-ICSI for severe male factor infertility with the development of Russell-Silver Syndrome. However, the rare association of IVF-ICSI treatment with any form of imprinting syndrome (Amor and Halliday, 2008) suggests that aberrant sperm DNA methylation is not a significant cause for any classical imprinting syndrome.

The majority of human studies have suggested a negative link between sperm DNA methylation status and chances of pregnancy (Benchaib et al., 2005; Cisneros, 2004; Kobayashi et al., 2007), with only one study failing to report such a link (Tavalaee et al., 2008). A complicating factor in determining the direct effect of sperm DNA methylation on pregnancy outcome is its positive association with sperm DNA integrity (Benchaib et al., 2005; Tavalaee et al., 2009). Since sperm DNA fragmentation is clearly linked with pregnancy outcome (Zini et al., 2008), it is virtually impossible to determine if sperm DNA fragmentation alone or hypomethylation are primarily responsible for pregnancy outcome. Future experiments correlating sperm DNA methylation status with the embryonic epigenetic profile may shed light on the role that sperm DNA methylation plays in early embryo development.

The results of this study suggest that antioxidant supplementation in infertile men can result in significant improvements in sperm DNA integrity and methylation. Improvements in sperm DNA integrity with antioxidant therapy has been reported by many previous investigators, yet the ability of antioxidants to boost pregnancy rates is still under considerable debate (Tremellen, 2008). Antioxidant supplements will not be capable of normalizing sperm DNA hypomethylation in all infertile men as in some individuals
hypomethylation of individual gene loci is related to mutations within the DNMT3L gene (Kobayashi et al., 2009), not oxidative stress. Furthermore, while the role that impaired sperm DNA integrity and methylation plays in the health of the next generation has yet to be determined, one can speculate that antioxidant mediated improvements in sperm DNA quality has at least the potential to benefit reproductive outcomes (Zini et al., 2008). Large prospective studies correlating sperm DNA quality in the insemination sample with epigenetic profiles and the health outcomes in the resulting children are urgently required. If these studies do confirm a link between poor sperm DNA quality and adverse child health outcomes, pre-conception anti-oxidant supplements may become standard clinical practice, just as pre-conception folate supplementation is the standard for women. Until these studies are conducted, the absolute value of male pre-conception antioxidant supplementation will be unknown.
CHAPTER 8

Final Discussion and Conclusions
8.1 Discussion

As seminal oxidative stress is a common pathology effecting sperm function in many infertile men, it is important to know the underlying causes of seminal oxidative stress as this may lead to more effective targeted therapy. The aim of this thesis was to advance our understanding of the causes of Oxidative stress in infertile men as well as examine the effectiveness of antioxidant treatment to boost fertility potential in the infertile male population.

Previous work by other investigators has identified many underlying causes for oxidative stress such as local infection in the reproductive tract, systemic diseases such as diabetes and lifestyle factors such as smoking. The research in this thesis has identified a new cause of seminal oxidative stress, obesity. The work described in Chapter 5 has highlighted for the first time that obesity results in an increase in seminal ROS production and an infiltration of macrophages into the semen. Obesity has previously been shown to be linked with activation of macrophages in adipose tissue and the vascular system and therefore it is not surprising that this inflammation extends to the reproductive tract. What will be interesting to see in the future is whether loss of weight can result in a decrease in seminal macrophage activity and ROS production. It is our intent to conduct these studies in the future.

The studies described in this thesis outline the development of a new method for measurement of oxidative stress in semen. While the NBT is not a new test of cell ROS production, these studies have modified the test to allow for quantitative assessment of seminal ROS production.

Sperm cells can generate reactive oxygen species as they contain glucose-6-phosphate dehydrogenase in their cytoplasm. This enzyme uses glucose to produce β-nicotinamide adenine dinucleotide phosphate (NADPH) via the hexose monophosphate shunt and generate superoxide radical. The NBT method developed in chapter 3 is based on the ability of the superoxide anion to reduce yellow water-soluble tetrazolium salt
(NBT) to a blue water-insoluble formazan derivative, which can be solubilized and quantified by spectrophotometrically at 630 nm. Seminal ROS production quantified by the NBT assay has for the first time been shown to have a significant correlation with markers of sperm quality such as motility and DNA fragmentation. In light of these findings I believe that the NBT assay has the potential to be used by many clinical andrology laboratories as a screening test for oxidative stress related male infertility, especially as the NBT assay has been shown to be accurate, easy to establish and low cost.

Sperm cells along with leukocytes are the main ROS producers in semen and their contribution to the generation of oxidative stress is dominant in the absence of infection. The studies in this thesis found no significant correlation between seminal leukocyte markers (PMN elastase and CD 45) and markers of oxidative stress (NBT, Lipid Peroxidase-LPO) in infertile men’s semen. This is not surprising as none of the men had any physical signs or symptoms of genital tract infection that would have resulted in activation of an inflammatory response. However, chronic low grade genital tract inflammation is significantly more common in the infertile male population than a fertile cohort, with many of these men being asymptomatic (Haidl et al., 2008; Schuppe et al., 2008). In my study I was anticipating to find a significant correlation between seminal plasma PMN Elastase activity and ROS production, but no such correlation was observed between these two parameters. This may result from having low numbers of leukocytospermic patients in my study cohort or it may reflect that neutrophils are not the primary leukocyte type contributing to ROS production in semen. While neutrophils are the most common leukocyte contained in semen, accounting for up to 70% of seminal leukocytes, macrophages also are prevalent in semen (~30% of leukocytes). Accordingly I aimed to examine if macrophages were a significant source of ROS production in seminal plasma by correlating the concentration of Neopterin in seminal plasma, an established marker for macrophage activity, with ROS levels. This study was the first to report
the presence of the macrophage activity marker neopterin in human seminal plasma.

Furthermore, the studies outlined in chapter 4 show for the first time that seminal plasma neopterin levels correlated better with semen ROS production than the more traditionally assessed seminal plasma elastase. Neopterin levels in seminal plasma of infertile men was nearly double that seen in the fertile cohort. Seminal plasma elastase and CD 45 levels were also present at significantly higher concentrations in the infertile group. While seminal plasma PMN elastase levels show no correlation with any marker of semen quality aside from leukocyte (CD45) concentration, neopterin level was significantly correlated with sperm concentration, morphology, sperm DNA fragmentation and apoptosis. There was also a strong positive correlation between neopterin concentration and markers of sperm oxidative stress such as LPO and NBT assay. Overall this suggests that measuring macrophage activity in seminal plasma using the neopterin assay is a more useful marker of sperm quality than the more traditionally used inflammatory markers seminal plasma elastase and leukocyte concentration (CD45).

Two possibilities exist for the underlying cause of a macrophage infiltration into semen. Firstly, it could represent a normal inflammatory response to a pathogenic stimulus such as low grade epididymal infection. Secondly, the macrophage influx may be a beneficial "physiological" response in which the body is attempting to remove dead or defective sperm. The studies in chapter 4 outline for the first time a significant correlation between seminal plasma neopterin and sperm abnormal morphology and apoptosis which suggests this second "physiological" role for macrophages may be dominant.

Oxidative stress is known to produce sperm DNA fragmentation which in turn results in increased time to natural conception and miscarriage risk. Furthermore, decreased IVF success rates and even a possible increased risk of childhood cancer have been linked with increased sperm DNA damage. Several inter-related mechanisms have been
postulated as a cause of DNA damage in sperm cells. These are oxidative stress, abortive apoptosis and abnormal sperm protamination.

During spermatogenesis, when the apoptotic destruction of sperm cells is failed, damaged cells can not be completely destroyed. This abortive apoptotic state can be triggered by oxidative stress and DNA will then be damaged by endonucleases mediated digestion. As can be seen in Chapter 3, the link between ROS and early apoptosis with DNA damage confirm this postulate. The infertile group had three times more ROS production and two times more DNA damage and apoptosis than the fertile cohort.

The replacing of nuclear histones with protamines during normal spermatogenesis allows a tight packaging of the sperm DNA which protects the defective sperm from oxidative attack. Infertile men more commonly have defective protamination of their DNA, making these sperm cells more susceptible to ROS damage. Approximately 15% of infertile men have been reported to exhibit deficient protamine packaging (Carrell and Liu, 2001). The results in chapter 6 show that sperm protamination can be improved by antioxidant treatments. While several previous studies have reported that antioxidants can reduce sperm DNA damage, the study outlined in chapter 7 has shown for the first time that a combination of antioxidants and minerals such as zinc and selenium, vital to protamination processes, can also boost sperm DNA protamine packaging, thereby improving sperm DNA integrity. These results help explain the molecular mechanisms behind how the Menevit antioxidant preparation improved pregnancy outcomes during IVF/ICSI treatment in a previously reported placebo controlled study (Tremellen et al., 2007).

The contribution of epigenetic processes in reproduction is a subject of considerable scientific and clinical interest at present. While oxidative stress has already been linked with altered epigenetic programming in somatic cells, the effect of oxidative stress on the sperm paternal
genome epigenetic expression was unknown before the conduct of experiments outlined in chapter 7.

Methylation of cytosine residues in DNA by DNA methyltransferase is considered to be one of the major epigenetic mechanisms controlling gene expression. Hypomethylation of the sperm DNA has been reported to be related with abnormal semen parameters and low fertilization capability (Benchaib et al., 2003a; Houshdaran et al., 2007). Previous reports (Benchaib et al., 2005; Tavalaee et al., 2009) have shown a negative correlation between DNA fragmentation and DNA methylation, but these investigators did not assess oxidative stress and therefore did not identify sperm oxidative stress as a cause of DNA hypomethylation.

The research outlined in chapter 7 reveals for the first time a significant negative correlation between seminal ROS production and sperm DNA methylation. This observation, together with the fact that sperm DNA methylation improved with antioxidant therapy, suggests that oxidative stress is a significant cause of sperm DNA hypomethylation seen in infertile men. While it is clear that infertile men have aberrant sperm DNA methylation, it remains to be seen how this may affect the health of the next generation.

8.2 Future Directions

In order to validate the NBT Assay developed in this thesis for use in clinical Andrology laboratories there is a need to conduct larger scale trials, preferably multi-centred. Expansion on the study sample size, especially the fertile “normal” cohort will hopefully provide more robust reference ranges. Since publication of my NBT paper in 2009, several laboratories from around the world have made contact with our research group and commenced their own testing.
Obesity has been shown to create oxidative stress in semen, primarily from activation if a macrophage inflammatory response in the male reproductive tract. In the future I would like to investigate if a weight loss program in infertile men can result in a decrease in seminal macrophage activity and ROS production, and a subsequent improvement in sperm quality.

The link observed between sperm oxidative DNA damage and hypomethylation using the NBT assay will be confirmed by a future study correlating the generation of the oxidative specific DNA base adduct 8-hydroxyl-2’-deoxyguanosine (8-OH-dG) with sperm DNA methylation. Furthermore, correlation between embryonic epigenetic profiles with sperm DNA methylation will hopefully help shed light on the importance of sperm DNA quality to the development of the next generation. Finally, as it has already been shown that sperm DNA damage can produce miscarriage and possibly affect the health of the next generation, I would like to correlate sperm DNA quality at the time of IVF insemination with pregnancy/child health outcomes.

If sperm DNA damage or hypomethylation are conclusively linked with adverse health outcomes in children, it would be interesting to study if antioxidant supplementation can prevent these adverse outcomes. The likely sample size required for such a study is several thousand cycles of IVF, a formidable administrative task. However, as the health of the next generation is of utmost importance, I hope that such studies can be organised in the future.
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