Significance of Mitochondrial Reactive Oxygen Species Production in Male Infertility

Adam John Koppers
BSc (Biotechnology) (Honours)

A thesis submitted to the Discipline of Biological Sciences, University of Newcastle for the degree of Doctor of Philosophy

Submitted August, 2010
Declaration

Statement of Originality

This thesis contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. I give consent to this copy of my thesis, when deposited in the University Library, being made available for loan and photocopying subject to the provisions of the Copyright Act 1968.

*Signature:* .................................................. *Date:* ..........................................................
Acknowledgements

I am very grateful for the supervision of Laureate Professor John Aitken. I would not have learnt so much and gained all I have without his great guidance, advice, help and support. Special thanks also to Associate Professor Eileen McLaughlin, Dr Brett Nixon and Dr Geoff De Iuliis for their help whenever it was needed.

To every member of the reproductive science group, thank you for making the lab such a great and enjoyable place to work. You have all made it a wonderful experience.

To my fellow PhD students, Jono, Lisa, Yun, Tegs and Dan, thanks for all your help but most importantly your friendship over the years.

Thank you to my family for all their support throughout all my time at University, I know you are as glad as I am to finally finish. To Lauren, thank you so much for all happiness and fun we have together and the support you have given me.

The greatest gratefulness goes to Jodie Powell, for without her endless dedication and effort this work would not have been possible.
# Table of Contents

Declaration........................................................................................................................................ i  
Acknowledgements....................................................................................................................... ii  
Table of Contents........................................................................................................................ iii  
List of Abbreviations ................................................................................................................... vi  
Abstract......................................................................................................................................... ix  

## Chapter 1: Introduction

1.1 Male Infertility .................................................................................................................. 1  
1.2 Y-Chromosome Deletions .............................................................................................. 6  
1.3 Reactive Oxygen Species and Antioxidants ................................................................. 8  
1.4 Oxidative Stress and Spermatozoa.................................................................................. 14  
1.5 Antioxidants Treatment of Oxidative Stress in Human Spermatozoa ...................... 20  
1.6 Sources of Reactive Oxygen Species in Spermatozoa .............................................. 23  
1.7 Conclusions and Research Aims ..................................................................................... 29  

## Chapter 2: Materials and Methods

2.1 Reagents and Solutions .................................................................................................. 30  
2.2 Human Semen Donation .............................................................................................. 30  
2.3 Preparation of Human Spermatozoa ............................................................................ 30  
2.4 Detection of Reactive Oxygen Species Production by Chemiluminescence ....... 32  
2.5 Flow Cytometric Analysis.............................................................................................. 33  
2.6 JC-1 Assay: Mitochondrial Membrane Potential Indicator ..................................... 34  
2.7 MitoSOX Red Assay: Mitochondrial Superoxide Indicator ........................................ 35  
2.8 Dihydroethidium Assay: Superoxide Indicator............................................................ 36  
2.9 O₂ Consumption............................................................................................................. 36
<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.10 BODIPY (581/591) C&lt;sub&gt;11&lt;/sub&gt;: Lipid peroxidation Probe</td>
<td>36</td>
</tr>
<tr>
<td>2.11 Motility</td>
<td>37</td>
</tr>
<tr>
<td>2.12 Measurement of Fatty Acids in Spermatozoa Using Gas Chromatography</td>
<td>38</td>
</tr>
<tr>
<td>2.13 Immunocytochemistry localization of proteins on fixed spermatozoa</td>
<td>39</td>
</tr>
<tr>
<td>2.14 Protein Extraction and Quantitation</td>
<td>40</td>
</tr>
<tr>
<td>2.15 One dimensional SDS-polyacrylamide gel electrophoresis (1D SDS-PAGE)</td>
<td>41</td>
</tr>
<tr>
<td>2.16 Immunoblotting</td>
<td>43</td>
</tr>
<tr>
<td>2.17 Enhanced chemiluminescence (ECL)</td>
<td>44</td>
</tr>
<tr>
<td>2.18 8-hydroxy-2'-deoxyguanosine (8OHdG) Assay</td>
<td>44</td>
</tr>
<tr>
<td>2.19 Terminal deoxynucleotidyl transferase mediated dUTP Nick End Labeling (TUNEL) Assay</td>
<td>45</td>
</tr>
<tr>
<td>2.20 Measurement of Activated Caspase</td>
<td>46</td>
</tr>
<tr>
<td>2.21 Annexin-V Externalisation</td>
<td>46</td>
</tr>
<tr>
<td>2.22 Ultrastructure</td>
<td>47</td>
</tr>
<tr>
<td>2.23 Statistics</td>
<td>48</td>
</tr>
</tbody>
</table>

**Chapter 3: Significance of Mitochondria in Reactive Oxygen Species in Generation of Oxidative Stress in Human Spermatozoa**

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.1 Introduction</td>
<td>49</td>
</tr>
<tr>
<td>3.2 Experimental Design</td>
<td>53</td>
</tr>
<tr>
<td>3.3 Results</td>
<td>54</td>
</tr>
<tr>
<td>3.4 Discussion</td>
<td>68</td>
</tr>
</tbody>
</table>

**Chapter 4: Stimulation of Mitochondrial Reactive Oxygen Species by Unsaturated Fatty Acids in Defective Human Spermatozoa**

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.1 Introduction</td>
<td>71</td>
</tr>
<tr>
<td>4.2 Experimental Design</td>
<td>74</td>
</tr>
<tr>
<td>4.3 Results</td>
<td>75</td>
</tr>
<tr>
<td>4.4 Discussion</td>
<td>90</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>8OHdG</td>
<td>8-hydroxy-deoxyguanosine</td>
</tr>
<tr>
<td>AA</td>
<td>Arachidonic acid, C20:4 n-6</td>
</tr>
<tr>
<td>AIF</td>
<td>Apoptosis inducing factor</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>ART</td>
<td>Assisted reproductive technology</td>
</tr>
<tr>
<td>AZF</td>
<td>Azoospermia factor</td>
</tr>
<tr>
<td>BAD</td>
<td>Bcl-2-associated death promoter</td>
</tr>
<tr>
<td>BCA</td>
<td>Bicinchoninic acid</td>
</tr>
<tr>
<td>BWW</td>
<td>Biggers, Whitten and Whittingham</td>
</tr>
<tr>
<td>CASA</td>
<td>Computed assisted sperm analysis</td>
</tr>
<tr>
<td>CCCP</td>
<td>Carbonyl cyanide m-chlorophenyl hydrazone</td>
</tr>
<tr>
<td>CoQ10</td>
<td>Coenzyme Q10</td>
</tr>
<tr>
<td>CoQ⁻</td>
<td>Coenzyme ubisemiquinone</td>
</tr>
<tr>
<td>CoQH₂</td>
<td>Reduced form of coenzyme Q10</td>
</tr>
<tr>
<td>DABCO</td>
<td>1,4-diazobicyclo-[2.2.2]-octane</td>
</tr>
<tr>
<td>DDA</td>
<td>Docosadienoic acid, C22:6 n-3</td>
</tr>
<tr>
<td>Δψ</td>
<td>Mitochondrial membrane potential</td>
</tr>
<tr>
<td>DGLA</td>
<td>Dihomo-γ-linoleic acid, C20:3 n-6</td>
</tr>
<tr>
<td>DHA</td>
<td>Docosahexaenoic acid, C22:6 n-3</td>
</tr>
<tr>
<td>DHE</td>
<td>Dihydroethidium</td>
</tr>
<tr>
<td>ECL</td>
<td>Enhanced chemiluminescence</td>
</tr>
<tr>
<td>EDA</td>
<td>Eicosadienoic acid, C20:2 n-6</td>
</tr>
<tr>
<td>ETC</td>
<td>Electron transport chain</td>
</tr>
<tr>
<td>EPA</td>
<td>Eicosapentanoic acid, C20:5 n-3</td>
</tr>
<tr>
<td>FA</td>
<td>Fatty acid</td>
</tr>
<tr>
<td>FAD</td>
<td>Flavin adenine dinucleotide</td>
</tr>
<tr>
<td>Acronym</td>
<td>Definition</td>
</tr>
<tr>
<td>---------</td>
<td>------------</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
</tr>
<tr>
<td>FMN</td>
<td>Flavin mononucleotide</td>
</tr>
<tr>
<td>GC</td>
<td>Gas chromatography</td>
</tr>
<tr>
<td>GLA</td>
<td>γ-linoleic acid, C18:3 n-6</td>
</tr>
<tr>
<td>GPx</td>
<td>Glutathione peroxidase</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>ICSI</td>
<td>Intracytoplasmic sperm injection</td>
</tr>
<tr>
<td>IVF</td>
<td>In vitro fertilization</td>
</tr>
<tr>
<td>JC-1</td>
<td>5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethyl benzimidazolylcarbocyanine iodide</td>
</tr>
<tr>
<td>L•</td>
<td>Carbon-centred radical</td>
</tr>
<tr>
<td>LA</td>
<td>Linoleic acid, C18:2 n-6</td>
</tr>
<tr>
<td>LH</td>
<td>Polyunsaturated FA (Diagramatic)</td>
</tr>
<tr>
<td>LOO•</td>
<td>Lipid peroxyl radical</td>
</tr>
<tr>
<td>LOOH</td>
<td>Lipid hydroperoxide</td>
</tr>
<tr>
<td>Luminol</td>
<td>3-Aminophthalhydrazide, 5-Amino-2,3-dihydro-1,4-phthalazinedione</td>
</tr>
<tr>
<td>MA</td>
<td>Myristic acid, C14:0</td>
</tr>
<tr>
<td>MACS</td>
<td>Magnetic activated cell sorting</td>
</tr>
<tr>
<td>MDA</td>
<td>Malondialdehyde</td>
</tr>
<tr>
<td>MUFA</td>
<td>Monounsaturated fatty acid</td>
</tr>
<tr>
<td>NADH</td>
<td>Nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>NADPH</td>
<td>Nicotinamide adenine dinucleotide phosphate</td>
</tr>
<tr>
<td>OLA</td>
<td>Oleic acid, C18:1 n-9</td>
</tr>
<tr>
<td>OZA</td>
<td>Zymosan opsonized</td>
</tr>
<tr>
<td>PA</td>
<td>Palmitic acid, C16:0</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PI3 Kinase</td>
<td>Phosphoinositide 3 Kinase</td>
</tr>
<tr>
<td>PLA</td>
<td>Palmitoleic acid, C16:1 n-7</td>
</tr>
<tr>
<td>PS</td>
<td>Phosphatidylserine</td>
</tr>
<tr>
<td>PUFA</td>
<td>Polyunsaturated fatty acid</td>
</tr>
<tr>
<td>Acronym</td>
<td>Definition</td>
</tr>
<tr>
<td>---------</td>
<td>------------</td>
</tr>
<tr>
<td>Q</td>
<td>Ubiquinone</td>
</tr>
<tr>
<td>QH$_2$</td>
<td>Ubiquinol</td>
</tr>
<tr>
<td>Q.-</td>
<td>Ubisemiquinone</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
</tr>
<tr>
<td>SA</td>
<td>Stearic acid, C18:0</td>
</tr>
<tr>
<td>SDH</td>
<td>Succinate dehydrogenase</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>SDS-polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SFA</td>
<td>Saturated fatty acid</td>
</tr>
<tr>
<td>SMAC</td>
<td>Second mitochondrial derived activator of caspases</td>
</tr>
<tr>
<td>SOD</td>
<td>Superoxide dismutase</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris buffered saline</td>
</tr>
<tr>
<td>TBST</td>
<td>TBS containing 0.01% Tween-20</td>
</tr>
<tr>
<td>TdT</td>
<td>Terminal deoxynucleotidyl transferase</td>
</tr>
<tr>
<td>TUNEL</td>
<td>Terminal deoxynucleotidyl transferase mediated dUTP Nick End Labeling</td>
</tr>
<tr>
<td>Tween-20</td>
<td>0.1% polyoxyethylenesorbitan monolaurate</td>
</tr>
<tr>
<td>VA</td>
<td>Vaccenic acid, C18:1 n-7</td>
</tr>
<tr>
<td>VDAC</td>
<td>Voltage dependent anion channel</td>
</tr>
</tbody>
</table>
Abstract

Male infertility is a relatively common condition affecting 1 in 20 men of reproductive age. The aetiology of this condition is thought to involve the excessive generation of reactive oxygen species (ROS) by human spermatozoa; however the cause and sub-cellular origins of this aberrant activity is unknown. The objective of this doctoral thesis was to determine the importance of sperm mitochondria in creating oxidative stress associated with defective sperm function and subsequently to investigate causes of increased mitochondrial ROS generation.

In the first study, intracellular measurement of mitochondrial ROS generation and lipid peroxidation was performed using the fluorescent probes MitoSOX red and BODIPY C11 in conjunction with flow cytometry. Effects on sperm movement were measured by computer-assisted sperm analysis. Defective human spermatozoa spontaneously generated mitochondrial ROS in a manner that was negatively correlated with motility. The induction of mitochondria ROS resulted in peroxidative damage to the midpiece and a loss of sperm movement that could be prevented by the concomitant presence of α-tocopherol. Concluding that the sperm mitochondria make a significant contribution to the oxidative stress exhibited by defective human spermatozoa.

In order to investigate causes of irregular mitochondrial ROS production, we demonstrated that defective human sperm populations are characterized by high cellular contents of both total and free fatty acids using gas chromatography. The free unsaturated fatty acid content of these cells was positively correlated with the induction of mitochondrial superoxide generation. This relationship was causal and mediated by the range of unsaturated fatty acids that are present in human spermatozoa. Direct exposure of these cells to free unsaturated fatty acids stimulated mitochondrial superoxide generation and precipitated a loss of motility and an increase in oxidative DNA damage, two key attributes of male infertility.
We followed with examination of the ability of human spermatozoa to undergo apoptosis and generate mitochondrial ROS. We report that these cells are prevented from entering the apoptotic pathway as long as the PI3kinase/AKT complex is phosphorylated. If PI3 kinase phosphorylation is inhibited with wortmannin then these cells default to an intrinsic apoptotic cascade characterized by caspase activation in the cytosol, annexin V binding to the cell surface, mitochondrial ROS generation, cytoplasmic vacuolization, oxidative DNA damage and motility loss. However the physical architecture of the cell subsequently prevents endonucleases released from the mitochondria or activated in the cytosol from penetrating the sperm nucleus and, as a result DNA fragmentation does not occur although oxidative DNA adducts can clearly be detected.

These results for the first time highlight the importance of mitochondrial ROS generation in human spermatozoa in the aetiology of male infertility. Subsequent studies revealed that higher intracellular concentrations of unsaturated fatty acids and the stimulation of apoptosis are both mechanisms of mitochondrial ROS generation which can result in increased lipid peroxidation, loss of sperm motility and oxidative DNA damage. These data and further investigation will aid in the diagnosis, prevention and treatment of male infertility.
Chapter 1:

Introduction
Chapter 1: Introduction

This doctoral thesis describes research studying the role of oxidative stress in male fertility, specifically its causes and sources. For that reason, the following chapter reviews male infertility as a phenomenon and its relationship to oxidative stress, and DNA damage. Finally, current theories and data relating to the origins of oxidative stress in spermatozoa will be discussed.

1.1 Male Infertility

1.1.1 Introduction to Male Infertility

Male infertility is a common condition that is known to affect 1 in 20 males in Australia (McLachlan & de Kretser, 2001). However, the cause of this condition is largely unknown. In recent years, we have only seen two significant advances in our understanding of the causes of male infertility – Y-chromosome deletions and oxidative stress.

Currently, there is no single diagnostic test that can accurately ascertain semen quality or fertilising capacity. The World Health Organisation (WHO, 1999) has published arbitrary cut-off values to classify men into groups for their ‘Potential for natural conception’ based on semen volume, concentration, motility and morphology (Table 1.1). However the conventional semen profile is not a sensitive tool for determining fertility potential unless it is seriously compromised (Aitken, 2006).
Table 1.1 Potential for natural conception based on values from standard semen classification (WHO, 1999).

1.1.2 Trends in Semen Quality

A landmark investigation by Carlsen et al (1992) generated circumstantial data on secular trends in semen quality that has led to a growing concern over the state of male fertility. A review of publications reporting sperm counts in men lacking any known fertility issues found a significant decrease in mean sperm concentrations from \(113 \times 10^6\) to \(66 \times 10^6\) in men between 1940 and 1990. This was further compounded by a reduction in mean seminal volume over the same period from 3.4 to 2.75 ml. Subsequent studies have generated information on temporal changes in sperm concentration in different countries. A recent study by Shine et al (2008) showed the average sperm concentration in New Zealand had more than halved over the 20-year period between 1987 and 2007; from \(110 \times 10^6 / ml\) to \(50 \times 10^6 / ml\) (Fig 1.1). The change in sperm concentration was comparable to data reported by Auger et al., (1995) from France, in which average concentration dropped from \(89 \times 10^6 / ml\) to \(61 \times 10^6 / ml\) between 1973 and 1992. Again similar findings have been described in studies by Irvine et al (1996) in Scotland. These authors emphasized that this was a birth cohort effect of men born before 1970 possessing an average sperm concentration of \(98 \times 10^6 / ml\) compared to \(78 \times 10^6 / ml\) for those born after this date. The study by Auger et al (1995) reported decreases in the percentage of motile and morphologically normal sperm of 0.6% and 0.5% per year respectively between 1973 and 1992.
1.1.3 Increasing Use of ART

Although no direct evidence of declining fertility has been reported, over the same period of declining sperm counts and sperm motility there has also been a subsequent and rapid rise (Fig 1.2) in the use of in vitro fertilisation (IVF) and assisted reproductive technologies (ART), so much so that approximately 1 in every 35 babies given birth in Australia are conceived using this technology (ABS, 1995). The use of intra-cytoplasmic sperm injection (ICSI) since its inception in 1992 has been widely debated with regards to the health of the offspring due to the technique’s ability to by-pass natural selection barriers to fertilisation (Plachot, 1996; Lambert, 2002; Lewis S & Klonoff-Cohen, 2005; Feng et al., 2008; Alukal & Lamb, 2008).
Introduction

Reports have now published data highlighting increased rates of major birth defects in children conceived through ART in comparison to natural spontaneous pregnancy. One such publication by Hansen et al (2002) published in The New England Journal of Medicine, compared the number of children with reported birth defects at the age of one in three different groups; IVF, ICSI and natural pregnancies. The study found that major birth defects were present in 8.6% and 9.0% of babies from the ICSI and IVF cohorts respectively. This was a marked two-fold increase compared with the rate of 4.2% found in the naturally conceived group. When sub-divided into separate congenital defect groups (musculoskeletal, chromosomal, cardiovascular and urogenital), the same increase could be

**Figure 1.2** Cycles of ART started each year has continually risen in Australia with a 3-fold increase between 1992 and 2005 (from AIHW, 2002).
found between all groups. Conversely since the introduction of ICSI, research into the
diagnosis and management of male infertility has declined (McLachlan & De Kretser,
2001); nowadays the sole aim of the clinicians and their patients is to achieve pregnancy
rather than address the underlying causative mechanisms, despite the inherent risks of ART.
Therefore, there is even greater need to further understand causes of male infertility.

<table>
<thead>
<tr>
<th>Causes of Male Infertility</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oxidative stress</td>
</tr>
<tr>
<td>Genetic defects incl. Y-chromosome deletions</td>
</tr>
<tr>
<td>Infection</td>
</tr>
<tr>
<td>Testicular torsion</td>
</tr>
<tr>
<td>Varicocele</td>
</tr>
<tr>
<td>Hormonal Abnormalities</td>
</tr>
<tr>
<td>Environmental factors - radiation and chemical injury</td>
</tr>
<tr>
<td>Sperm transport blockage</td>
</tr>
<tr>
<td>Production of spermatozoa antibodies</td>
</tr>
</tbody>
</table>

**Table 1.2** List of known causes of male infertility.

Although they have varying degrees of prevalence, there are some known causes of male infertility that are listed in table 1.2. This literature review will now briefly discuss Y-chromosome deletions as a well-defined cause yet male infertility, before a detailed analysis of oxidative stress as a cause of male infertility.
1.2 Y-chromosome Deletions

Whilst oxidative stress mediated male infertility is the focus of this thesis, the other major known cause of male infertility are Y-chromosome deletions.

The association between microdeletions present in the long arm of the Y-chromosome in men resulting in impaired fertility due to azoospermia or severe oligospermia, caused by aberrant spermatogenesis was first discovered by Tiepolo and Zuffardi in 1976. This led to the naming of the azoospermia factor (AZF), for which now three distinct loci have been identified (AZFa, AZFb and AZFc; Fig 1.3) and their respective roles in spermatogenesis defined (Vogtet et al., 1996). Each locus is related to different stages of spermatogenesis, as such microdeletions in each region result in different phenotypes. AZFa causes Sertoli Cell Only Syndrome due to the lack of germ cells within the testes; AZFb causes arrest of spermatogenesis; AZFc however has a greater amount of variability with phenotypes varying from no germ cells to oligospermia (Aitken and Krausz, 2001).

Figure 1.3 Y-chromosome with 3 distinct loci of the azoospermia factor regions; AZFa, AZFb, AZFc.
A number of genes from the AZF region have been identified including USPY, RMMY1, DAZ, DBY and HSFY (Foresta et al., 2000; Sun et al., 1999; Elliot et al., 1997; Rei jo et al., 1995; Vinci et al., 2005), it has been hypothesised that some redundancy exists as no single gene deletion found is capable of causing the azoospermia found in men (Krausz et al., 1999). Therefore it is likely to be caused by the removal of multiple adjacent genes. The incidence of microdeletions in each region varies with 60% occurring in AZFa, 15% in AZFb and 5% in AZFc with 20% of cases containing microdeletions across multiple loci (Foresta et al., 2001).

Whilst little can be done with AZFa,b microdeletions as this results in azoospermia, AZFc will commonly cause oligospermia, allowing IVF/ICSI as an option for conceiving offspring (Page et al., 1999). However, further consideration must be placed on the education of men with these Y-chromosome deletions, in order for them to make an informed choice regarding not only the effect on themselves, but also the long term affect on a son who would inherit the same Y-chromosome deletion, and thus their infertility.
1.3 Reactive Oxygen Species and Antioxidants

In order to discuss oxidative stress related male infertility, ROS and antioxidants will be briefly introduced. The term reactive oxygen species (ROS) covers a broad range of oxygen containing molecules, including oxygen free radicals, oxidising agents and carbon based oxygen radicals.

1.3.1 Oxygen Free Radicals

Superoxide anion (O$_2^-$) is the product of the one-electron reduction of molecular oxygen (O$_2$), creating a negatively charged free-radical (Equation I). Superoxide anion is produced in a number of different biological systems including the mitochondria (Boveris et al., 1973) and NADPH oxidase (Cheson et al., 1977). In low concentrations, it also known to be a signaling molecule for different pathways including mitogen-activated protein kinase and c-Jun amino-terminal kinase (Sundaresan et al., 1995; Stevenson et al., 1994).

\[
\text{(Equation I)} \quad O_2 + e^- \rightarrow O_2^-
\]

Unlike superoxide anion, the hydroxyl radical (OH•) is a neutral ROS, however it is considered significantly more reactive and consequently has a much shorter half-life. It is this high reactivity that also makes the OH• more difficult to routinely measure. The hydroxyl radical is formed from hydrogen peroxide and superoxide anion using iron (II) as a catalyst (Gutteridge 1984) (Equation II).

\[
\text{(Equation II)} \quad Fe^{2+} + O_2^- + H_2O_2 \rightarrow Fe^{3+} + OH^+ + OH^- + O_2
\]
1.3.2 Oxidising Agents

Although not a free radical, hydrogen peroxide (H$_2$O$_2$) is still a highly reactive oxidising agent. It is usually formed through the spontaneous dismutation of superoxide anion molecules, a reaction that can also be catalysed by the enzyme superoxide dismutase (Gutteridge, 1984).

1.3.3 Organic Peroxides

The most common organic peroxides are lipids that have undergone peroxidation. The process of lipid peroxidation is known as a chain reaction due to its self propagating nature and as such is characterized by three distinct phases: initiation, propagation and termination (Fig 1.4; Ernster, 1993).

The initiation phase of a lipid peroxidation chain reaction is the step where a carbon-centred radical is produced. Most commonly, the initiation is caused by a ROS, usually OH•, which reacts with a hydrogen atom on an unsaturated fatty acid to make water and a fatty acid radical (Fig 1.4). Although less reactive, H$_2$O$_2$ in the presence of transition metals such as copper, iron and manganese can cause OH• formation via the Fenton reaction (Gutteridge, 1986).

The second phase of lipid peroxidation in propagation; whereby the recently formed and unstable fatty acid radical readily reacts with the abundant molecular oxygen, thereby creating a lipid-peroxyl radical (LOO•). However, the lipid-peroxyl radical is also an unstable compound that will react with another nearby unsaturated fatty acid producing another fatty acid radical and a lipid hydroperoxide (LOOH). This creates a continual cycle of lipid peroxidation is constantly generating lipid hydroperoxides (Fig 1.4)
The final phase of the chain reaction is termination, the lipid peroxidative chain ends when two radicals combine to form a non-radical, however, this usually occurs at high concentrations of lipid peroxyl radicals. Antioxidants such as α-tocopherol can cause termination by reacting with the radical instead of another lipid, thus ending the chain. The resultant α-tocopheroxyl radical is then recycled by other antioxidants such as ascorbate (Johnson, 1979). If the lipid chain reaction is not terminated, irreversible cellular damage to the membranes can occur causing cell lysis (Benedetti et al., 1977). Also, the peroxidation chain produces highly reactive lipid hydroperoxides such as malondialdehyde (MDA). MDA is a known carcinogen by forming adducts with deoxyguanosine of DNA (Esterbauer et al., 1991).

Figure 1.4 Lipid peroxidation chain containing three phases, initiation, propagation and termination. LH, polyunsaturated FA; L•, carbon-centred radical; LOO•, lipid peroxyl radical; LOOH, lipid hydroperoxide.
1.3.4 Major Antioxidants

ROS are a common by-product of cellular metabolism and as a result most cells contain an abundance of antioxidants in order to prevent or minimise the oxidation of cellular constituents by ROS. Cellular antioxidants are a combination of both non-enzymatic and enzymatic systems. However, if the levels of ROS exceed antioxidant levels within the cell, oxidative stress occurs which can result is significant oxidative damage.

There are two forms of superoxide dismutase (SOD), the cytosolic Cu/ZnSOD and the mitochondrial Mn/FeSOD. Superoxide anion is the substrate for SOD which readily converts the free radical into another ROS, hydrogen peroxide $H_2O_2$. Whilst this may seem counter productive it does provide some level of protection in the mitochondria from the highly abundant superoxide anion, which readily attacks the iron/sulphur containing enzymes present in the mitochondrial electron transport chain (Gardner et al., 1995). Heterozygous MnSOD knockout mice accumulate greater levels of DNA damage as well as higher rates of cancer compared to control mice (Li et al., 1995). Whilst SOD removes superoxide anion, the subsequent $H_2O_2$ has to be removed by other antioxidants, particularly glutathione peroxidases and catalase. Considering the importance of SOD as an antioxidant, the testes also contains a unique extracellular SOD that is secreted by both Sertoli and germ cells (Mruk et al., 2002).

Glutathione is a tripeptide, consisting of a single glutamate, cysteine, and glycine. Its antioxidant ability comes from the cysteine residue, which contains a free sulphhydryl group that scavenges free radicals (Meister & Anderson, 1983). Although not an antioxidant on its own, glutathione reductase is critical through its role in the regeneration of oxidised glutathione by reducing it with electrons from nicotinamide adenine dinucleotide phosphate (NADPH) (Fig 1.5) (Meister, 1988). Catalase is equally important in the removal of hydrogen peroxide, and is present within seminal fluid (Jeulin et al., 1989).
Figure 1.5  Antioxidant pathways involved in ROS neutralisation. The formation of superoxide anion by the one-electron reduction of oxygen, which is subsequently converted to hydrogen peroxide by superoxide dismutase. Hydrogen peroxide is then converted to water and oxygen by either catalase or the glutathione peroxidase system. GSH, glutathione; GSSG, glutathione disulfide; NADP(H), nicotinamide adenine dinucleotide phosphate.
Vitamin E is the collective term for all 8 tocopherol molecules, of which α-tocopherol is the major compound. As discussed, lipid peroxidation is a self-propagating cycle that causes major damage to unsaturated fatty acids. α-tocopherol breaks the lipid peroxidation chain by reacting with lipid peroxyl radicals, therefore preventing reaction with other unsaturated fatty acids, thereby ending the chain and stopping further oxidation of lipids (Ernster, 1993).

Ascorbic acid (vitamin C) is a monosaccharide with an attached carboxyl group that is unable to be produced by humans and therefore must be consumed through our diet (Smirnoff, 2001). It is a highly important antioxidant due to its ability to prevent oxidative damage by a variety of ROS including hydroxyl radical, superoxide anion and H₂O₂. It also has a role in preventing the lipid peroxidation of membranes through its ability to regenerate α-tocopherol (Johnson, 1979), as such it is the most abundant antioxidant in seminal plasma (Lewis et al., 1997). Uric acid is a common antioxidant found in seminal plasma (Lewis et al., 1997), and is also a strong reducing agent that has a role in the regeneration of α-tocopherol.

Unlike somatic cells, spermatozoa are unique in that they are no longer capable of gene transcription (Dadoune, 1995). This, and the lack of cytoplasmic space to house antioxidant enzymes, leaves the spermatozoon in a precarious position. However the male reproductive tract compensates for the lack of internal antioxidant capacity by bathing these cells in a complex mixture of ROS scavenging enzymes and small molecular mass organics both when they are travelling down the epididymis, a process that may take 12 days (Amann et al., 1976) and when these cells are ejaculated into seminal plasma. A significant increase in ROS generation as a consequence of, for example, infection, may over time exceed the levels of antioxidant protection provided in these fluids and cause a state of oxidative stress.
1.4 Oxidative Stress and Spermatozoa

Male infertility affects approximately 1 in 20 males in Australia, being the major factor in 40% of couples unable to conceive (McLachlan & de Kreter, 2001). A large portion of the male population is unable to achieve conception despite producing sufficient numbers of spermatozoa. Since the early observation by Macleod (1943) that spermatozoa in high oxygen media lose motility at an accelerated rate, but could be rescued by the antioxidant catalase, oxidative stress has become an area of significant research into male infertility. Tosic and Walton (1946) followed with the discovery that bovine spermatozoa produce hydrogen peroxide, resulting in decreased sperm metabolism. Subsequent studies found ROS generation by spermatozoa in a number of species including rabbit (Holland & Storey, 1981), mouse (Alvarez & Storey, 1984), rat (Lewis & Aitken, 2001) and horse (Ball et al., 2001). Defective sperm function has now been characterised as the most common cause of male infertility (Hull et al., 1985). The importance of ROS generation in this phenomenon is highlighted by reports suggesting that oxidative stress is involved in between 30-80% of all cases of male infertility (Shekarriz et al., 1995; Agarwal et al., 2008).

Assessing the effects of oxidative stress on human spermatozoa is not a simple process due to the large number of molecules that are substrates for free radical attack (particularly lipids, proteins and DNA) and the results of these interactions in terms of sperm function. In the following sections, I review some of the major consequences of free radical attack on spermatozoa with emphasis on the induction of lipid peroxidation, the loss of sperm motility and the creation of DNA damage.

1.4.1 Lipid Peroxidation and Sperm Motility

Lipid peroxidation involves the oxidation of unsaturated fatty acids, and as discussed earlier, results in an extremely harmful self-propagating chain of oxidative damage. Unsaturated fatty acids are more susceptible to attack due to the weakening of the hydrogen
bonds between their double bonds adjoining methylene groups. As such sperm plasma membranes are extremely vulnerable to peroxidation due to their significantly higher content of unsaturated fatty acids in comparison to other cell types (Jones et al., 1979; Ollero et al., 2001). On the other hand, it is believed that the presence of unsaturated fatty acids is essential for the membrane fusion events involved in fertilisation in particular the acrosome reaction and sperm / oolemma fusion.

Following on from the study by MacLeod (1943) the next major breakthrough was achieved by Jones et al (1979), who used a ferrous iron promoter to induce oxidative stress in vitro and as a result measured significant increases in lipid peroxidation and decreased sperm motility. This finding that has been validated in a number of subsequent studies although it has also now been shown that lipid peroxidation affects other aspects of sperm function in addition to motility including sperm-oocyte fusion (Aitken et al., 1989; Twigg et al., 1998; Kemal Duru et al., 2000). Thus, a common product of lipid peroxidation is MDA, which has been accurately measured in human spermatozoa. Using such assays, correlations have been found showing a relationship between increased levels of malondialdehyde (MDA) and decreases in both sperm motility (Fig 1.6; Gomez et al., 1998) and sperm-egg fusion (Aitken et al., 1993).

Increased ROS generation in human spermatozoa, both spontaneous and induced, has been consistently correlated with decreased motility, a major marker of defective spermatozoa and male infertility (Iwasai & Gagnon, 1992; Agarwal et al., 1994; Aitken et al., 1995). There are a number a number of hypotheses on how increased ROS generation may affect sperm motility, one of the strongest is the role of lipid peroxidation. A loss of motility is correlated with increased lipid peroxidation. Additionally the ability of α-tocopherol to both preserve motility and suppress lipid peroxidation indicates that there is a causal relationship between these two factors (Suleiman et al., 1996). Another hypothesis for the relationship between motility loss and oxidative stress involves a direct effect of ROS on axonemal proteins that essential for motility (de Lamirande and Gagnon, 1995).
1.4.2 Oxidative Stress and DNA Damage

During spermatogenesis, once in the spermatid stage, the cell no longer possesses the normal DNA repair mechanisms, greatly increasing the significance of any DNA damage on the spermatozoon. On the other hand the remodelling of sperm DNA, as histones are replaced by protamines, provides extremely tight packaging of the chromatin and therefore greater protection from oxidative attack. It must be taken into consideration that
approximately 15% of DNA in human spermatozoa remains histone bound, and it is even higher in cases of male infertility due to errors in chromatin remodelling during spermiogenesis (Sakkas et al., 1998); this is highest in sub- or infertile males, suggesting the presence of possible histone rich ‘hot-spots’ for DNA damage that are more susceptible to attack.

Oxidative stress is considered the major cause of DNA damage in human spermatozoa (Aitken & De Iuliis, 2007). ROS can directly damage DNA in a number of ways such as modification (i.e. oxidation) of bases, creation of base free sites, deletions and cross-linking (Bailly et al., 1989). Oxidative DNA damage is also known to cause both single and double stranded DNA fragmentation. This is most likely caused by oxidation of the bases (in particular guanine) and the subsequent weakening of the DNA backbone between the base and sugar resulting in depurination, leaving a weakened abasic site which can subsequently generate DNA strand breaks (Wiseman & Halliwell, 1996). This is further supported by recent evidence that shows a strong correlation between poor chromatin packaging and the formation of 8-hydroxy-deoxyguanosine (8OHdG) adducts and DNA strand breaks, as well as supporting the hypothesis that the majority of DNA damage in human spermatozoa is oxidative (Fig 1.7; De Iuliis et al., 2009).

Higher levels of DNA damage in spermatozoa are correlated with male infertility. The role of oxidative stress in DNA damage was first discovered in studies conducted by Kodama et al (1997) which found that in vivo antioxidant therapy significantly reduced oxidative DNA damage in human spermatozoa. These data were subsequently reinforced by Wang et al (2003) who showed that oxidative stress in seminal fluid is correlated with an ensuing increase in DNA damage. The stimulation of ROS in human sperm in vitro has been shown to induce significant amounts of DNA damage (Aitken et al., 1998; De Iuliis et al., 2009). Finally it has been shown that treatment with antioxidants in vivo can subsequently reduce levels of DNA damage observed in infertile men (Comhaire et al., 2000; Greco et al., 2005).
Figure 1.7 DNA damage measured by 8OHdG (oxidative) and TUNEL (strand breaks) assays both show correlations with poor chromatin packaging measured by the CMA₃ assay. TUNEL and 8OHdG assays also correlate indicating majority of DNA damage is oxidative (from De Iuliis et al., 2009).
Whilst it is important to note that the oocyte is capable of DNA repair post-fertilisation (Jaroudi et al., 2009), it is limited in its ability to repair large amounts DNA damage (Agarwal & Prabakaran, 2005). As such there are major consequences for excessive DNA damage in human spermatozoa including significantly reduced rates of pregnancy by either impaired fertilisation, disrupted pre-implantation embryonic development, low rates of implantation and an increased incidence of miscarriage; but most significantly there are high rates of morbidity in the offspring and diminished fertility in vivo and in vitro (Lewis & Aitken, 2005; Zini et al., 2008).

All these data suggest an effective solution in the form of antioxidant therapy in order to counteract the oxidative DNA damage seen within the male patient population. The use of antioxidants both in vitro and in vivo to prevent oxidative damage to spermatozoa is discussed below.
1.5 Antioxidants Treatment of Oxidative Stress in Human Spermatozoa

Due to the major role that oxidative stress plays in the causation of male infertility, numerous studies have focused on benefits of oral antioxidant treatment and will be reviewed here. While oxidative stress is clearly accepted as a major factor in male infertility, results from clinical trials using oral antioxidants are less clear. Although a number of studies report improved semen quality and pregnancy rates, definitive conclusions cannot be drawn due to inconsistent treatments, dose regimes and measured endpoints. Current results of in vitro and in vivo treatment with antioxidants on lipid peroxidation, sperm motility and DNA damage are discussed below.

1.5.1 Effect of Antioxidants on Lipid Peroxidation and Sperm Motility

The most studied antioxidant in terms of male infertility and oxidative stress in vitamin E. Numerous studies have revealed its ability to prevent lipid peroxidation in vitro (Aitken et al., 1989; Verma & Kanwar, 1998). Other studies have further shown that not only does vitamin E prevent lipid peroxidation but also averts losses in sperm motility (Aitken and Clarkson, 1988; Aitken et al 1989), indicating a relationship between the two factors. This further emphasised by the ability of vitamin E to prevent motility loss following cryopreservation (Askari et al, 1994). More antioxidants have subsequently been demonstrated to prevent in vitro lipid peroxidation including pentoxifyline (McKinney et al., 1996) or prevent losses in sperm motility such as vitamin C (Verma & Kanwar, 1998), glutathione (Parinaud et al, 1997), Coenzyme Q10 (Lewin & Lavon, 1997), hypotaurine and catalase (Baker et al., 1996).

In vivo results have shown that Vitamin E alone (Sulieman et al, 1996) or in combination with selenium (Keskes-Ammar et al, 2003) significantly reduces the MDA levels in spermatozoa in comparison to control groups. Similar results were shown for glutathione, which in vivo reduces lipid peroxidation (Lenzi et al., 1994)
1.5.2 Effect of Antioxidants on DNA Damage

Treatment with antioxidants has also been shown to decrease DNA damage found in spermatozoa. Donnelly et al (1999) demonstrated that vitamin C was capable of preventing DNA damage induced by the addition of H$_2$O$_2$. In vivo studies using a combination of vitamins C and E plus glutathione (Kodama et al., 1997) or just vitamins C and E (Greco et al., 2005) both found significant reductions in the levels of DNA damage measured in spermatozoa.

1.5.3 Effect of Antioxidants on Pregnancy Outcomes

While the majority of studies have focused on sperm quality rather than pregnancy, Sulieman et al (1996) found treatment with Vitamin E for 6 months resulted in a decrease in MDA levels but also an increase in spontaneous pregnancy rates in comparison to placebo controls. Whilst a similar study involving a combination of Vitamins E and C found no improvement, the study however was for only 2 months in comparison to 6 months (Rolf et al., 1999).

Menevit (Bayer Healthcare) is a commercially available antioxidant treatment specifically aimed at treating oxidative stress-induced male infertility with a combination of antioxidants. Unusually this preparation contains garlic in order to reduce the inflammation associated with increased levels of leukocytic infiltration that is thought to frequently accompany male infertility (Fraczek & Kurpisz, 2007). This formulation also contains zinc, selenium and folate aimed at enhancing protamine packaging (Tremellen, 2008). In vivo trials of Menevit have shown significant improvement in pregnancy rates of 38.5%, compared to 16% observed in the control group (Tremellen et al., 2007).
Current antioxidant therapy research is critical for both understanding the effects of oxidative stress in male infertility and also for refining the immediate clinical treatment of patients. However, fully elucidating the causes and sources of excessive ROS generation is essential for understanding the fundamental aetiology of male infertility. Current theories and data on sources of oxidative stress in the testes and spermatozoa are discussed below.
1.6 Sources of Reactive Oxygen Species in Spermatozoa

Since the importance of oxidative stress in male infertility has been confirmed by many independent laboratories, the next obvious step in the research is to identify the sources of aberrant ROS generation. Extrinsic, intrinsic and other external sources are discussed below.

1.6.1 Extrinsic Sources of ROS

The most studied extrinsic source of ROS generation is the presence of leukocytes in seminal plasma. Whilst the presence and ROS producing capabilities of leukocytes (approximately 1000 times greater than spermatozoa; Plante et al., 1994) are not in question, whether they have a role in male infertility is.

A number of studies have reported no differences between the number of leukocytes present in the seminal fluid of both fertile and infertile men (Christiansen et al., 1991; Tomlinson et al., 1993; Aitken and Baker, 1995; Rodin et al., 2003). A study by Aitken et al (1995) found no relationship between seminal leukocyte concentration and basic semen parameters (Fig 1.8). Conversely a similar number of studies have found correlations between seminal leukocyte concentration and ROS generation (Aitken et al., 1994; Whittington et al., 1999). Interestingly, in one study by Henkel et al (2005) when the ROS production in seminal fluid was measured in spermatozoa (measured using DHE assay via microscopy) – be more explicit and leukocyte (measured using chemiluminescence on total semen as ROS from spermatozoa was deemed negligible in comparison to leukocytes; Plante et al., 1994) groups, both were shown to exhibit a correlation between ROS production and DNA damage observed in human spermatozoa; however the intrinsic relationship was stronger.
The development of a technique combining density-gradient centrifugation and magnetic Dynabead removal of leukocytes using CD45 antibodies has allowed better analysis of leukocyte free populations of human spermatozoa (Aitken et al., 1996). Subsequent investigation still found a strong relationship between ROS generated by spermatozoa and defective sperm function. This indicates that in relation to male infertility the most relevant source of ROS in seminal fluid is the spermatozoa themselves.

*Figure 1.8* No relationship exists between leukocyte concentration in semen and basic sperm parameters including motility, concentration, total count and morphology (from Aitken et al., 1995).
1.6.2 Intrinsic Sources of ROS

For some time the presence of NADPH oxidase in spermatozoa was inconclusive, due to data indicating that the addition of NADPH was found to elicit a ROS response when measured by chemiluminescence (Aitken et al., 1997). However Richer & Ford (2001) were unable to confirm NADP –dependent ROS generation using electron paramagnetic resonance spectroscopy. Since then a number of studies have shown the presence of a calcium-dependant NADPH oxidase, NOX 5, to be present within sperm (Banfi et al., 2001; Armstrong et al., 2002; Sabeur and Ball, 2007). The identified NOX 5 appears to be a unique form, as it is not controlled by protein kinase C as occurs in the leukocyte form of NADPH oxidase (Armstrong et al., 2002). Subsequently, evidence for a NOX 2 was reported by Shukla et al (2005) within the head region of mouse spermatozoa. Since these findings were made no data have been presented showing that any NADPH oxidase is over-expressed or stimulated in defective sperm or cases of male infertility.

One of the first reports of mitochondrial ROS production came from Boveris and colleagues (1973), who used antimycin A, an inhibitor of the mitochondrial ETC complex III, to induce superoxide anion and H\textsubscript{2}O\textsubscript{2} generation. Since then it has not only become evident that mitochondria are capable of low levels of ROS production during normal physiology (Inoue et al., 2003; Turrens, 2003), but also ROS have been implicated in the signalling pathways involved in apoptosis, also known as programmed cell death (Brookes et al., 2002).

The main source of mitochondrial ROS is at complex III of the electron transport chain (ETC). Electron transport ubiquinone at site Q forms an ubisemiquinone radical, although during normal respiration the flow of electrons would continue along the chain, occasionally electrons are released to oxygen with the resultant formation of superoxide anion. The use of antimycin A blocks electron flow after the formation of ubisemiquinone, therefore electrons cannot flow to complex III and as a consequence more electrons are
released to oxygen and form superoxide anion (Turrens et al., 1997; St-Pierre et al., 2002; Muller et al., 2003). The other major source of ROS production is via complex I, however the mechanism has yet to be elucidated, although it is probable that a similar ‘leak’ of electrons occurs at complex III (Turrens et al., 1997; St-Pierre et al., 2002). Although the primary ROS generated by the mitochondria is superoxide anion it is readily converted to H$_2$O$_2$ due to the high concentration of superoxide dismutase present in the intermembrane space (Okado-Matsumoto and Fridovich, 2001).

Whilst the mitochondrial electron transport chain is capable of ROS production under normal physiological conditions, mitochondrial dysfunction can result in an increase in ROS production and has been implicated in numerous pathological conditions including Alzheimer’s disease (Beal, 1995) and ischemia (Stone et al., 1992). Although no data exist for humans, rat and rabbit spermatozoa have been shown to generate ROS from the mitochondria via the electron transport chain (Holland & Storey, 1981; Vernet et al., 2001; Agarwal et al., 2003).

1.4.3 External Sources of ROS

A number of external influences have been implicated as a cause of ROS generation. These include diabetes, alcohol consumption, phthalates, heavy metals and varicocele, which are discussed below.

Diabetes was first suggested to have a role in oxidative stress in the testes by Zhao et al. (2004). A recent study by Shrilatha and Muralidhara (2007) confirmed this hypothesis by showing that the streptozotocin-induced diabetic rat model was found to exhibit a major increase in testicular oxidative stress within 6 weeks of initiation of the diabetic state, with significant increases in both lipid peroxidation and ROS generation after just two weeks. Similar results were subsequently found using the same model in pre-pubertal rats (Chandrashekar & Muralidhara, 2009). Agbaje and colleagues have also found that the spermatozoa of diabetic men contain higher rates of DNA fragmentation (Agbaje et al.,
2007) as well as oxidative DNA damage (Agbaje et al., 2008), indicating a role of oxidative stress in this aetiology. Although the cause is unknown, these studies highlight a relationship between men with diabetes and oxidative stress.

Studies in mice have shown that chronic alcohol (ethanol) consumption can lead to male infertility. Anderson et al (1983) showed that chronic alcohol consumption resulted in decreased sperm production as well as an increase in morphological defects. A clinical study into the effects of alcoholism on human fertility was undertaken by Muthusami & Chinnaswamy (2005), who found that all major sperm parameters; semen volume, sperm count, motility, and number of morphologically normal sperm were all significantly decreased. Whilst none of these studies investigated the effect of alcohol on ROS generation, high rates of alcohol consumption have been associated with oxidative stress (Koch et al., 2004).

The generation of ROS can be exacerbated by a multitude of environmental factors. The increasing presence of chemicals and by-products of manufacturing in the environment have been suggested to pose a serious threat towards the reproductive health of humans around the world (Aitken et al., 2006). Phthalates are present in plastic food packaging, and can be released into foods upon contact, increasing human exposure levels to these compounds (Rock et al, 1986). Phthalate exposure in rats has shown to induce significant levels of DNA damage in spermatozoa as well as impairing normal spermatogenesis (Agarwal et al., 1985). Additional studies have shown that exposure of germ cells to phthalates in vitro results in oxidative stress, mitochondrial dysfunction, cytochrome c release, and apoptosis (Richburg and Boekelheide, 1996; Kasahara et al, 2002).

Other major products of industrial waste are heavy metals, such as cadmium, manganese and lead. Exposure to lead has been shown to increase testicular oxidative stress (Acharya et al., 2003). Unpublished observations by De Iuliis et al, has shown that various heavy metals are capable of inducing lipid peroxidation and oxidative DNA damage. Providing
further evidence for the detrimental effect of exposure to environmental toxins on male reproductive health.

Varicocele is a relatively common condition affecting 15-25% of the male population, with its occurrence rising to 40% in men suffering infertility (Pryor & Howards, 1987; Meacham et al., 1994). The condition, which occurs predominately on the left side, is caused by increased dilation in the veins draining the testes (known as pampiniform plexus).

Despite significant research in the area, no single-defined cause of varicocele has been identified, which suggests a hypothesis leading towards a multi-factorial cause such as genetic predisposition and other external factors. Phenotypically, impaired spermatogenesis is the major observation; however varicocele is also extremely varied in terms of its effects on sperm production and function, which provides further difficulties in research. In fact, an exact cause of varicocele has not been identified despite a number of possible causes including increased testicular temperature, hypoxia, lower testicular testosterone and oxidative stress (Agarwal et al., 2009).

Although evidence of pregnancies from men with varicocele has been reported (Richardson et al., 2008), other data have shown numerous defects in sperm function (WHO, 1999). These alterations in sperm function include decreased sperm motility, morphology, long-term survival as well as an inability to capacitate, bind to the zona pellucida or acrosome react (WHO, 1999). These changes in sperm function somewhat mirror those seen during cases of oxidative stress in human spermatozoa. In fact there is some research suggesting that ROS mediated oxidative stress is a major factor in varicocele (Barbieri et al., 1999). However, the research is currently not definitive as there are also reports indicating there is no difference in ROS levels between men with and without varicocele (Cocuzza et al., 2008).
1.7 Conclusions and Research Aims

Elucidating sources of reactive oxygen species in spermatozoa will ultimately aid in identifying causes of oxidative stress. As discussed, most of the research has been valuable in ascertaining the importance and cellular consequences of oxidative stress and the therapeutic role of antioxidants on male infertility, however now is the time to further the research into sources and causes. Therefore the aims of the research were as follows.

1. To investigate possible sources of aberrant ROS generation in human spermatozoa and therefore causes of male infertility (i.e. mitochondria).

2. To investigate causes of the aberrant ROS generation in spermatozoa by the source(s) identified.

3. To investigate mechanisms of oxidative cellular damage in spermatozoa.
Chapter 2:

Materials and Methods
Chapter 2: Materials and Methods

2.1 Reagents and Solutions

Unless stated otherwise all chemicals and reagents used throughout this research were purchased from Sigma (Sigma Aldrich, USA). A complete compilation of all the solutions used is contained in the Appendix.

2.2 Human Semen Donation

Human semen samples were obtained from an unselected panel of volunteer donors of unknown fertility status assembled for the Reproductive Sciences Group of the University of Newcastle. Semen samples were obtained via masturbation after a minimum period of 48 hours abstinence into sterile sample containers and delivered to research staff within 45 min of ejaculation. Scientific use of the samples was approved by the University of Newcastle Human ethics Committee and the State Minister of Health.

2.3 Preparation of Human Spermatozoa

2.3.1 Semen Analysis

Prior to semen analysis, all samples were kept at 37°C for 15 min to allow them to liquefy. The parameters used to analyse the quality of each sample include liquefaction, debris, consistency and volume were determined macroscopically. Other parameters such as cell density, motility and vitality were determined using microscopic methods, according to the criteria laid down by the World Health Organization (WHO; 1999).
The concentration of spermatozoa in each semen sample was measured by diluting the sample 1:20 with sperm diluting fluid (Appendix). A 10 µl aliquot of the diluted sample was then placed on a Neubauer haemocytometer. The spermatozoa present in five major squares were then counted (four corners and the centre square); the final sperm concentration was expressed as: total number of cells counted x 10^6 cell /ml.

Motility was assessed by placing 10 µl of each sample on a slide and 100 spermatozoa were scored as either motile or non-motile. Sperm motility was then expressed as a percentage (%). Cell vitality was measured using an eosin exclusion staining technique. A mixture of 5 µl of semen and 5 µl of eosin (Appendix) was placed on a slide. Eosin is membrane permeable to dead cells only resulting in a pink staining, whilst live cells remain unstained. A total of 100 cells were counted as either dead or alive and expressed as a percentage (%).

2.3.2 Purification of Human Spermatozoa Using a Percoll Gradient

All human semen samples were purified using Percoll density gradient centrifugation. Each gradient consisted of 3 ml of 90% Percoll (Appendix) overlaid by 3 ml of 45% Percoll (Appendix) in a 15 ml conical bottomed tube. A single semen sample was carefully layered on top of the gradient. The samples were then centrifuged for 30 min at 600 × g. This created three different fractions: the seminal plasma and debris which remained on the top, in the middle was the low density (45% Percoll) fraction, which consisted of lower-motility / dysfunctional spermatozoa and the high density (90%) Percoll fraction pelleted at the bottom which consists of viable / motile spermatozoa (Aitken & West, 1990).

The seminal plasma was discarded, the low density fraction was transferred to a new 15 ml tube and the remaining supernatant removed. Both high and low density fractions were re-suspended in 5 ml of Biggers, Whitten and Whittingham (BWW; Appendix) and centrifuged at 600 × g for 15 minutes in order to wash the cells. The supernatant was then discarded and the pellets were re-suspended in 1 ml of BWW.
2.3.3 Determination of Sperm Concentration

Sperm concentration of each fraction was finally determined using methods stated in section 2.3.1. The sperm concentration was the adjusted to the appropriate concentration depending on the experiment.

2.4 Detection of Reactive Oxygen Species Production by Chemiluminescence

2.4.1 Removal of Contaminating Leukocytes

Leukocytes are known produces of significantly high levels of reactive oxygen species and have also been identified to be present in human semen samples (Aitken and West, 1990). This contamination by leukocytes removes the validity of all chemiluminescence analyses of reactive oxygen species production by spermatozoa, unless addressed.

Before any chemiluminometry experiments were performed all contaminating leukocytes were removed through the use of magnetic Dynabeads (Dynal Biotech., Australia). The Dynabeads are magnetic polystyrene beads coated with a monoclonal mouse antibody specific for the epitope common to all isoforms of CD45. CD45 is a leukocyte cell-surface marker, not present on human spermatozoa and therefore allows the removal of leukocytes via magnetic separation. The presence of leukocytes was determined after the human sperm purification using the determination of cell concentration method described in section 2.3.2. Prior to use the Dynabeads were washed once in BWW to remove chemical preservatives. Then four times the number of leukocytes in Dynabeads was added to the sperm populations, for example if $2 \times 10^6$ leukocytes/ml were detected, $8 \times 10^6$ Dynabeads/ml was added to ensure saturation. The samples were then slowly rotated for 30 min. After this the magnetic Dyna-beads were removed through the use of a magnetic tube holder. To ensure
the removal all contaminating leukocytes, zymosan opsonized with autologous pooled serum, a glucan made susceptible to leukocytes, was used as a specific stimulator of leukocyte reactive oxygen species production, was added to each reaction and the chemiluminescence response monitored using luminol and peroxidase using methods as discussed later in section 2.4.2. Only samples that exhibited no response to OZA stimulation were included in data analysis.

2.4.2 Luminol Peroxidase-Dependent Chemiluminescence

Luminol (3-Aminophthalhydrazide, 5-Amino-2,3-dihydro-1,4-phthalazinedione) is a common chemiluminescent probe used for the detection of reactive oxygen species production (Aitken et al., 2003; Wymann et al., 1987). Luminol stock solutions were prepared at 25 mM in DMSO. The final concentration of spermatozoa in each reaction was 125 x 10⁶ cells/ml, cell free controls were also performed to ensure that any responses were dependent on the presence of spermatozoa. The final concentration of the probes was 250 µM, with the luminol reactions also supplemented with 8 µl of horseradish peroxidase prepared at 2 mg/ml in BWW to provide a final activity of 11.5 U/ml. The final volume for all reactions was 400 µl.

All chemiluminescence reactions were recorded using a Berthold 953 luminometer (Berthold, Germany). The results were recorded as both continuous traces as well as the average integration of photon counts every 1.25 minutes.

2.5 Flow Cytometric Analysis

Flow cytometric analysis was performed on human spermatozoa following the incubation with a variety of fluorescent products as described below. Sample analysis was performed using a FACScalibur (BD Biosciences, USA) fitted with an argon laser operating with excitation wavelength of 488 nm coupled with the following emission filters; 530/30 band
pass (FL-1 / green), 585/42 band pass (FL-2 / red) and >620 nm long pass filter (FL-3 / far red). Data was collected using CellQuest Software (BD Biosciences, USA). A minimum of 10,000 events were measured for each sample.

### 2.6 JC-1 Assay: Mitochondrial Membrane Potential Indicator

One of the most common fluorescent probes used for assessing the mitochondrial membrane potential is 5,5’,6,6’-tetrachloro-1,1’,3,3’-tetraethyl benzimidazolylcarbocyanine iodide (JC-1; Molecular probes, USA). In mitochondria with a low membrane potential JC-1 forms monomers within mitochondria emitting green fluorescence (Ex. 488 nm, Em. 536 nm). However, in mitochondria with a high membrane potential JC-1 forms multi-mers known as J-aggregates resulting in an orange/red fluorescence (Ex. 488 m, em. 590 nm; Jelley, 1937; Reers et al., 1991).

JC-1 stock solutions were prepared in DMSO at 7.5 mM, as specified by the manufacturers’ instructions. A 1:375 dilution of JC-1 was made up in BWW. A 20 µl aliquot of JC-1 was then added to a 180 µl sample, containing 2 x 10^6 cells/ml, to give a final concentration of 2 µM. The samples were then incubated for 15 minutes at 37°C. This was followed by centrifugation for 5 min at 600 × g, removal of the supernatant and re-suspension in 1 ml of BWW.

Just prior to flow cytometric analysis, the dead cell indicator, propidium iodide (stored in phosphate buffered saline; PBS; Appendix) was added to the samples to give a final concentration of 0.01 mg/ml. Flow cytometric analysis was performed as described in section 2.5; propidium iodide positive cells were measured FL-3 and excluded from JC-1 analysis, assessed using FL-1/2.
2.7 MitoSOX Red Assay: Mitochondrial Superoxide Indicator

2.7.1 MitoSOX Red Staining of Spermatozoa

Intracellular generation of mitochondrial superoxide was estimated using MitoSOX Red (Molecular Probes, USA), a novel fluorescent probe selectively targeted to the mitochondria, that upon oxidation by superoxide anion exhibits red fluorescence upon binding to nucleic acids.

For this assay, MitoSOX Red stock solutions (5 mM in DMSO) and SYTOX Green (dead cell indicator) stock solutions (125 μM in DMSO) were diluted 1:1:250 in BWW. A 20 μl aliquot of MitoSOX Red and SYTOX Green mixture was then added to a 180 μl sample, containing 2 x 10^6 cells/ml, to give a final concentration of 2 μM and 0.05 μM respectively, and incubated for 15 min at 37°C. This was followed by centrifugation for 5 min at 600 x g and the pellet re-suspended in 1 ml of BWW. Flow cytometric analysis was performed as described in section 2.5, SYTOX Green and MitoSOX red were measured on the FL-1 and FL-2 filters respectively.

2.7.2 MitoSOX Red Imaging

Prior to imaging the localization of these probes, live cells were fixed to glass slides using Cell-Tak™ (BD Biosciences, USA). Images were then collected on a Zeiss LSM510 confocal microscope (Carl Zeiss GmbH, Germany) using an argon laser excitation (488 nm) with emission collection at 500–530 nm (green) and helium neon laser excitation (543 nm) with emission collection at more than 560 nm (red).
2.8 Dihydroethidium Assay: Superoxide Indicator

Dihydroethidium (DHE) exhibits a weak blue fluorescence; however, once this probe is oxidised by superoxide anion, it intercalates within DNA, staining the cell nucleus or mitochondria with a red fluorescence. For this assay, DHE stock solutions (5 mM in DMSO) and SYTOX Green stock solutions (125 µM in DMSO) were diluted 1:1:250 in BWW. A 20 µl aliquot of DHE and SYTOX Green mixture was then added to a 180 µl sample, containing 2 x 10^6 cells/ml, to give a final concentration of 2 µM and 0.05 µM respectively, and incubated for 15 min at 37°C. This was followed by centrifugation for 5 min at 600 × g and re-suspension in 1 ml of BWW. Flow cytometric analysis was performed as described in section 2.5, SYTOX Green and DHE were measured on the FL-1 and FL-2 filters respectively.

2.9 O$_2$ Consumption

Oxygen consumption was measured using an Apollo 4000 free radical analyser (WPI, USA) in a sealed 0.8 ml reaction chamber maintained at 37°C. Spermatozoa were incubated for 120 min in the presence or absence of mitochondrial inhibitors. Respiratory activity was defined as nmoles of oxygen consumed/min/10^6 cells, calculated using the ideal gas equation. Cell concentration used was 100 × 10^6 cells/ml.

2.10 BODIPY (581/591) C$_{11}$: Lipid peroxidation Probe

2.10.1 BODIPY (581/591) C$_{11}$ Staining of Spermatozoa

Lipid peroxidation was assessed using BODIPY (581/591) C$_{11}$ as the probe (Molecular Probes, USA). This probe incorporates into membranes where it undergoes a fluorescence emission shift upon peroxidation by lipid radicals from red to green (Aitken et al., 2007).
BODIPY C\textsubscript{11} stock solution (2 mM in ethanol) was diluted in BWW to give a final concentration of 5 $\mu$M, and a cell concentration of $10 \times 10^6$ cells/ml and incubated for 30 min at 37°C. Followed by washing twice in BWW ($650 \times g$ for 5 min), spermatozoa were divided into separate aliquots before the addition of any treatments. Just prior to flow cytometric analysis, the dead cell indicator, propidium iodide (stored in PBS) was added to the samples to give a final concentration of 0.01 mg/ml.

Flow cytometric analysis was performed as described in section 2.5, propidium iodide positive cells were measured using FL-3 and excluded from BODIPY (581/591) C\textsubscript{11} analysis assessed via FL-1/2

\subsection*{2.10.2 BODIPY (581/591) C\textsubscript{11} Imaging}

Prior to imaging the localization of these probes, spermatozoa were adhered to glass slides using Cell-Tak™ (BD Biosciences, USA). Images were then collected on a Zeiss LSM510 laser scanning confocal microscope (Carl Zeiss GmbH, Germany) using an argon laser excitation (488 nm) with emission collection at 500–530 nm (green) and helium neon laser excitation (543 nm) with emission collection at more than 560 nm (red).

\subsection*{2.11 Motility}

Evaluation of sperm motility parameters was conducted using computer-assisted sperm analysis (CASA) using a Hamilton Thorne Version 12 IVOS (Hamilton Thorne Biosciences, USA). For each measurement, a 2.5 $\mu$l aliquot of spermatozoa was loaded onto a standard 4 chamber slide (Leja, NL). A total of at least 200 or more spermatozoa were examined for each sample using standard settings (30 frames acquired at a frame rate of 60 Hz and a temperature of 37°C in 20 $\mu$m deep chambers). Samples were analyzed for percent motility as well as progressive motility (average path velocity of more than 25 $\mu$M/sec).
2.12 Measurement of Fatty Acids in Spermatozoa Using Gas Chromatography (GC)

2.12.1 Preparation of Samples for GC

Measurements of fatty acids were performed on populations of spermatozoa, free of contaminating leukocytes using methods in 2.4.1. Prior to GC preparation samples were stored at -80°C.

Total fatty acid (FA) concentration was analysed via direct trans-esterification of lipids and gas chromatography. A 2 ml aliquot of Methanol:toluene (4:1 v/v) containing 20 µg/ml of C19:0, as an internal standard, was added to the sample (200µl). Acetyl chloride (200µl) was added while vortexing and then heated for 1 hr at 100°C. The tubes were cooled in water for 5 min and 5 ml of 6% K₂CO₃ was added and centrifuged at 3000 x g for 5 min with a temperature of 4°C. The upper toluene phase was collected and stored in a gas chromatograph (GC) vial at -20°C for GC analysis.

Spermatozoon non-esterified (free) fatty acid concentration was analysed by a single step esterification of free FA and gas chromatography. A 2.5 ml aliquot of Methanol:acetyl chloride (50:1, v/v) containing 20 µg/ml of C19:0; as an internal standard was added to the sample (200µl) in a glass culture tube. The sample was then incubated (45 min at 25°C) and vortexed for 5 min. Following, 1.25 ml of 6% K₂CO₃ was added and then hexane (75µl), and subsequently vortexed for 2 min. The samples were centrifuged at 1500 x g for 10 min, vortexed for 2 min and centrifuged again at 1500 x g for 10 min. The hexane supernatant was transferred to a GC vial containing a 200µl insert and stored at -20°C for GC analysis.
2.12.2 GC Analysis of Total and Free Fatty Acids

Methylated total and free FA samples were analysed by GC using a fixed carbon-silica column 30m x 0.25mm (DB-225) (J & W Scientific, USA). The GC was equipped with a flame ionization detector, autosampler and autodetector. Injector and detector ports were set at 250°C. Oven temperature was 170°C for 2 min, increased 10°C/min up to 190°C where it remained stationary for one minute. Temperature then increased 3°C/min up to 220°C, which was maintained for a total run time of 30 minutes per sample. A split ratio of 10:1 and an injection volume of 3µl were used. A known fatty acid mixture was used to compare with analysed samples to identify peaks according to retention time and their concentration was determined using a Hewlett Packard 6890 Series GC with Chemstations Version A. 04.02.

2.13 Immunocytochemistry localization of proteins on fixed spermatozoa

Spermatozoa were fixed in 4% paraformaldehyde, and washed three times with PBS and plated onto poly-L-lysine coated cover slips, and air-dried. All the following incubations were performed in a humid chamber at 37°C. The cells were permeabilized with 0.2% Triton X-100 for 15 min, rinsed with PBS and blocked with 1% serum / 3% BSA for 1 hr. Cover slips were washed three times with PBS for 5 min and incubated in an appropriate dilution (Appendix) of primary antibody at 4°C overnight. Cover slips were then subjected to 3 x 5 min washes with PBS and incubated in a 1:100 dilution of fluorescein isothiocyanate (FITC)-conjugated secondary antibody for 2 hours at 37°C. Following incubation cover slips were again washed, and mounted in 10% mowiol 4-88 (Calbiochem, USA) with 30% glycerol in 0.2 M Tris (pH 8.5) with 2.5% 1,4-diazobicyclo-[2.2.2]-octane (DABCO) onto glass slides. Cells were examined using a LSM510 laser scanning confocal microscope (Carl Zeiss GmbH, Germany) using an argon laser excitation (488 nm) with emission collection at 500–530 nm.
2.14 Protein Extraction and Quantitation

2.14.1 Total cellular protein extraction

For total sperm protein extraction, the cells were lysed in an anionic sodium dodecyl sulfate (SDS) detergent. Spermatozoa were centrifuged at 600 x g for 5 min; pelleted cells were resuspended in 100 µl SDS Extraction Buffer (Appendix). Followed by vortexing for 1 min, boiling for 5 minutes in order to solubilize the protein, the samples were then centrifuged at 16 000 g for 15 min. The supernatant containing the extracted protein was removed and placed in a clean tube for further quantitation. All protein samples were stored at -20°C.

2.14.2 Total Cellular and Quantitation

The concentration of extracted proteins samples was determined using a bicinchoninic acid (BCA) Protein Assay Kit (Pierce, USA) according to manufacturer’s instructions. This assay involves colorimetrically estimating the concentration of protein in aqueous solution. The assay combines the reduction of Cu²⁺ to Cu¹⁺ by protein in an alkaline medium with the colorimetric detection of the cuprous cations utilizing the bicinchoninic acid-containing reagent. To obtain accurate protein estimation a dilution series of protein standards (0-2 mg/ml) were prepared using the supplied BSA stock solution. Triplicate aliquots of each standard (5 µl), an appropriate diluent blank, and the unknown samples were added to a 96 well microplate. Each well was then mixed with 200 µl of working reagent (BCA Reagent A: BCA Reagent B = 50:1). The microplate was then incubated at 37°C for 1 hr, after which the absorbance was measured at 550 nm using an Fluostar Optima Fluorescence Microplate Reader (BMG Labtech, Germany).
2.15 One dimensional SDS-polyacrylamide gel electrophoresis (1D SDS-PAGE)

2.15.1 Gel Apparatus Preparation

Extracted human sperm proteins were separated using one dimensional gel electrophoresis. The polyacrylamide gels were made in a commercial, small format gel chamber (0.75 mm x 6 cm x 10 cm; Bio-Rad), consisting of a separating (resolving) gel layer, topped by a stacking gel and secured in an electrophoresis apparatus. The sodium dodecyl sulfate – polyacrylamide gel electrophoresis (SDS-PAGE) gels used in this study consisted of a 10% polyacrylamide resolving gel (Appendix) and a 5% polyacrylamide stacking gel (Appendix). The resolving layer was poured into the gel chamber between the glass plates using a transfer pipette and overlaid with a thin layer of water-saturated isobutyl alcohol and allowed to polymerize. The water-saturated isobutyl alcohol was poured off and the gel rinsed with Milli-Q H₂O, following which the stacking gel was poured over the resolving gel to the top of the gel chamber. A 10 well comb was then inserted into the gel which was allowed to polymerize. Once set, the gels were assembled in an electrophoresis tank and immersed in SDS-PAGE Running Buffer (Appendix).

2.15.2 Gel Electrophoresis

Prior to electrophoresis the protein samples were thawed and either 5 or 10 µg aliquoted into clean Eppendorf tubes, along with 5 µl of SDS-PAGE loading buffer (Appendix). The samples were then denatured via heating at 100°C for 5 min and subsequently loaded into the appropriate wells of the stacking gel. A pre-stained marker, comprising proteins of known molecular mass was also loaded into a separate well of the gel to allow correct estimation of resolved proteins following enhanced chemiluminescence (section 2.17). The marker used throughout the following study was Prestained Protein Ladder (Fermentas,
The gel electrophoresis was performed under a constant voltage of 80 V for 15 min to all the proteins to migrate through the stacking gel, and from there on the voltage was increased to 140 V for the remainder of the separation. When the dye front had reached the bottom of the resolving gel, the electrophoresis was stopped and the gels removed from the tank for transfer on to nitrocellulose membrane.

2.15.3 Protein Transfer

In order to transfer proteins from the electrophoresis gel to the membrane, a protein transfer cassette was constructed (Fig 2.1). All components were soaked Western Blot Transfer Buffer (Appendix) for 5 minutes to allow for equilibration. In order to construct the cassette the various components were layered in the following order into the black side of an open ‘transfer; a scourer, 3 pieces of filter paper, the electrophoresis gel, a nitrocellulose membrane, 3 additional pieces of filter paper and finally another scourer. The cassette was closed and the set up was assembled into a transfer tank in a vertical orientation (Mini Trans-Blot Cell; Bio-Rad) according to manufacturer’s instructions and immersed in Western Blot Buffer. A constant current of 300 mA was then applied for a total of one hr. After the completion of the protein transfer the membrane was removed from the cassette and immunoblotting.
Materials and Methods

2.16 Immunoblotting

Once the protein transfer from the gel to nitrocellulose membrane was completed, non-specific binding sites on the membrane were blocked by incubation in 3% BSA (Research Organics, USA) / 0.1% polystyreneesorbitan monolaurate (Tween-20) in Tris Buffered Saline (TBS; Appendix) overnight at 4°C on a solid-state rotator. On conclusion of this incubation, the membrane was quickly rinsed for 20 seconds in TBS containing 0.01% Tween-20 (TBST; Appendix) to remove excess blocking solution. The membrane was then incubated in primary antibody at an appropriate concentration (Appendix), for 1.5 hours at room temperature on a solid-state rotator. After this incubation the membrane was washed 3 times in TBST for 10 min on an orbital shaker to remove excess and unbound antibody. The membrane was then incubated again for one hr with a horseradish peroxidase (HRP) conjugated secondary antibody at an appropriate concentration (Appendix), at room temperature on a solid-state rotator. Following this incubation, the secondary antibody was once gain removed from the membrane washing 3 times for 10 minutes in TBST.
Following immunoblotting nitrocellulose membranes were developed using enhanced chemiluminescence.

### 2.17 Enhanced chemiluminescence (ECL)

This technique enables the secondary antibody containing a HRP tag that remains bound to the membrane (section 2.16) to be visualized with an ECL substrate (GE Healthcare, Sweden). The subsequent emission of light from the bound complex during the reaction can then be captured on film. This was performed by incubating the antibody-probed nitrocellulose membrane for 5 min in the ECL detection reagents (Solution A: Solution B = 1:1) according to manufacturer’s instructions. Excess detection reagent was then removed by blotting paper and the membrane wrapped in cling wrap. The membrane was then transferred to a LAS-4000 Luminescent Imaging System (Fujifilm, Japan) for developing.

### 2.18 8-hydroxy-2'-deoxyguanosine (8OHdG) Assay

#### 2.18.1 Fluorescent Labelling with 8OHdG Antibody

The formation of the 8OHdG base lesion, which is a strong biomarker for oxidative stress, was measured using a FITC-conjugated antibody (Biotrin OxyDNA Test Kit, Biotrin International Ltd, Ireland). Spermatozoa were pelleted after treatment by centrifugation at $600 \times g$ for 5 min. For the positive control, spermatozoa were incubated for 1 hr at room temperature with $\text{H}_2\text{O}_2$ (2 mM) and $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (1 mM) in a final volume of 200 µl BWW. All cells were then washed twice in BWW, resuspended in 100 µl of 2 mM dithiothreitol in BWW, in order to decondense the highly compacted sperm chromatin (Mitchell et al., unpublished), and incubated for 45 min at 37°C. After centrifugation at $600 \times g$ for 5 min, the cells were then fixed by resuspending the pellet in 2% paraformaldehyde and incubated at 4°C for 15 min. The cells were then washed in PBS and stored in 200 µl of 0.1 M glycine at 4°C. After storage, fixed cells were washed and resuspended in 100 µl of
0.2% Triton-X. They were then incubated at room temperature for 15 min, centrifuged (600 \( \times \) g for 5 min) and washed once in Wash Solution (Biotrin OxyDNA Test Kit); 50 \( \mu l \) blocking solution (Biotrin OxyDNA Test Kit) was then added before incubation at 37°C for 1 hr. This was followed by centrifugation (600 \( \times \) g for 5 min) and washed once in Wash Solution. The anti-8OHdG antibody (1:50) was then added and incubated for 1 hr and finally, the cells were washed twice, resuspended in 1 ml PBS and transferred to 5 ml FACS tubes for flow cytometric analysis. Flow cytometric analysis was performed as described in section 2.5, 8OHdG conjugated fluorescence was measured using FL-1.

2.19 Terminal deoxynucleotidyl transferase mediated dUTP Nick End Labeling (TUNEL) Assay

The hallmark of apoptosis is DNA degradation, however this may also be present due to oxidative DNA damage. The DNA cleavage may yield double-stranded and single-stranded DNA breaks. Both types of breaks can be detected by labelling the free 3'-OH terminal with fluorescent nucleotides (i.e. fluorescein-dUTP) in an enzymatic reaction. The enzyme terminal deoxynucleotidyl transferase (TdT) catalyzes the polymerization of deoxyribonucleotides to the 3'-end of single- and double-stranded DNA.

Following treatment, spermatozoa were centrifuged (600 \( \times \) g for 5 min) and re-suspended in 2 mM dithiothreitol in BWW for 45 min. After centrifugation at 600 \( \times \) g for 5 min, the cells were then fixed by resuspending the pellet in 2% paraformaldehyde and incubated at 4°C for 15 min. The cells were then washed in PBS and stored in 200 \( \mu l \) of 0.1 M glycine at 4°C. For analysis, spermatozoa were centrifuged (600 \( \times \) g for 5 min) before resuspending the pellet in 100 \( \mu l \) of permeabilization solution (Appendix) and incubating for 2 min at 4°C. The cells were then centrifuged (600 \( \times \) g for 5 min) and the pellets resuspended in PBS. The positive control was treated with 100 \( \mu l \) of DNase I (1 mg/ml) and 10 \( \mu l \) MgSO\(_4\) (10 mM) for 30 min at 37°C.
TUNEL labelling was achieved with the In Situ Cell Death Detection Kit (Roche Applied Science, USA) according to the manufacturer’s instructions. Cells were then washed twice in PBS, diluted to a final volume of 500 µl in PBS and kept in the dark for analysis using flow cytometry. Flow cytometric analysis was performed as described in section 2.5, TUNEL fluorescence was measured using FL-1.

2.20 Measurement of Activated Caspase

The FLICA Poly-Caspases Detection Kit (Immunochemistry Technologies, USA) was used to measure active caspases in whole cells. The kit relies on a cell permeable inhibitor sequence of caspases conjugated to a carboxyfluorescein. In the presence an active caspase enzyme inside the cell, it will bind and form a covalent bond with FLICA.

The FLICA dye was stored as a 150x stock solution in DMSO, according to the manufacturers instructions. The stock solution was then diluted in BWW and added to spermatozoa to give a final concentration of 1x FLICA dye. Spermatozoa were incubated for 1 hr with the dye, in the last 15 min Sytox Green was added at a final concentration of 0.05 µM. This was followed by centrifugation for 5 min at 600 × g and re-suspension in 1 ml of BWW. Flow cytometric analysis was performed as described in section 2.5, SYTOX Green and FLICA were measured on the FL-1 and FL-2 filters respectively.

2.21 Annexin-V Externalisation

The externalisation of phosphatidylserine from the inner to outer leaflet of the plasma membrane is a feature of apoptosis. The Annexin-V-FITC Apoptosis Detection Kit (Sigma-Aldrich, USA) allows the detection of phosphatidylserine on the cell surface through FITC conjugated Annexin-V. The addition of the dead cell indicator, propidium iodide, allows the differentiation between apoptotic cells (annexin-V positive, PI negative), necrotic cells (annexin V positive, PI positive), and viable cells (annexin-V negative, PI negative).
After treatment, spermatozoa were centrifuged (600 \times g for 5 min), and the pellet was resuspended in binding buffer containing FITC-conjugated Annexin-V according the manufacturers instructions. Spermatozoa were incubated with the antibody for 1 hr, prior to centrifugation and re-suspension in 1 ml of BWW.

Just prior to flow cytometric analysis, the dead cell indicator, propidium iodide (stored in PBS) was added to the samples to give a final concentration of 0.01 mg/ml. Flow cytometric analysis was performed as described in section 2.5, FITC conjugated Annexin-V and propidium iodide fluorescence was measured using FL-1 and FL-3 respectively.

2.22 Ultrastructure

Spermatozoa were centrifuged at 1000 x g for 10 min, then resuspended and fixed in 500 μl of 2% formaldehyde / 2.5% glutaraldehyde in 0.1 M PBS (pH 7.3) for 2 hr at 4°C. To remove the fixative, spermatozoa were washed 3 times by centrifugation at 1000 x g for 5 min and resuspended in 0.1 M PBS. After the 3rd centrifugation, spermatozoa were submitted to successive dehydration steps. Cells were immersed progressively in increasingly concentrated ethanol solutions (v/v) at 50%, 75%, 95% for 5 min each, then in 3 changes of 100% ethanol for 5 min each and in 2 changes of 100% acetone for 5 min. After that, successive Spurr’s resin (Appendix) filtration steps began by immersing the dehydrated samples in more concentrate resin solution in acetone (v/v), at first 33% and 50% for 1 hr each at room temperature, then 66% and 100% each over night at room temperature, and finally transferred to filtrate in fresh 100% resin and polymerized for 24h at 60°C in a laboratory oven.

Sections (70nm) of the embedded tissue were cut on an Ultracut E ultramicrotome (Reicher-Jung, Australia), floated out on milli-Q water and transferred onto 100-mesh copper grids. Grids were then stained with 0.5% uranyl acetate in 30% ethanol for 10 min, then rinsed with distilled H2O, further staining was achieved with lead citrate for 10 min. Lead precipitates on sections grids were removed by rinsing in 0.05 M NaOH before
further rinsing in distilled H₂O. Grids were left at room temperature to dry and the stained sections were examined using a JEOL-1200EX electron microscope (JEOL, Japan), operating at 80kV.

2.23 Statistics

All experiments presented in this study were replicated a minimum of three times. All graphical data are represented as the mean ± SEM. Statistical differences between group means were determined using an analysis of variance (ANOVA) or unpaired T-test; post hoc comparison of group means was by Fisher’s Protected Least Significant Difference test All P values < 0.05 were considered statistically significant.

The following statistical significance abbreviations were used:

*    = P < 0.05
**   = P < 0.01
***  = P < 0.001
a    = P < 0.05
b    = P < 0.01
c    = P < 0.001
Chapter 3:

Role of Mitochondria in Reactive Oxygen Species Production in Human Spermatozoa
Chapter 3: Role of Mitochondria in Reactive Oxygen Species Production in Human Spermatozoa

3.1 Introduction

3.1.1 Mitochondria as a Source of Reactive Oxygen Species

A large body of evidence exists identifying the role that oxidative stress plays in male infertility, owing to vast amounts of research since MacLeod et al (1943) showed that incubation of human spermatozoa in high oxygen tension medium resulted in significant decreases in motility. However, despite the fact that numerous candidates have been researched including leukocytes and NADPH oxidase (reviewed in Aitken & De Iuliis, 2007; Agarwal et al., 2003), no definitive sources of reactive oxygen species production has been elucidated.

The major source of ROS generation in somatic cells is thought to involve electron leakage from the mitochondrial electron transport chain (ETC) during cellular respiration, with an estimated 2% of consumed oxygen being converted to superoxide anion via this route (Boveris & Chance, 1973). Usually, this activity is balanced by antioxidants such as SOD, catalase, a-tocopherol, cytochrome c, glutathione and glutathion reductase within the intermembrane space and mitochondrial matrix (Andreyev et al., 2005). However, mitochondrial ROS production may overwhelm these defence mechanisms, creating a source of oxidative stress that has been implicated in numerous pathological conditions including both Alzheimer’s (Hirai et al., 2001) and Parkinson’s disease (Betarbet et al., 2000). Currently, evidence exists for the generation of ROS by rabbit and rat sperm mitochondria, but no equivalent data is available for human gametes (Holland & Storey, 1981; Chapman et al., 1985; Vernet et al., 2001).
Figure 3.1 Schematic diagram showing the main pathways of electron flux through the mitochondrial electron transport chain. Sites of action for the mitochondrial inhibitors used in this study are also indicated. VDAC, Voltage Dependent Anion Channel; SDH, succinate dehydrogenase; Q, ubiquinone; QH$_2$, ubiquinol; , Q$^\cdot$, ubisemiquinone; SOD, superoxide dismutase; GPx, glutathione peroxidase; FMN, flavin mononucleotide; NADH, nicotinamide adenine dinucleotide; FAD, flavin adenine dinucleotide.
The mitochondrial electron transport chain is a complex series of electron donor and transport proteins and molecules. As such the generation of reactive oxygen species can be as equally complex. The site of electron blockage or leakage has a profound effect on the topology of ROS generation. It has been recognized that the two major sites of electron leakage arise from either complexes I or III, resulting in ROS generation on either side of the inner mitochondrial membrane (St-Pierre et al., 2002).

Unlike somatic cells, sperm mitochondria are not dispersed throughout the cytoplasm. Instead they are located at the proximal end of the tail, beneath the head. Positioned in gyre structures around the axoneme, the mitochondria are physically attached to the sheath. Due to this unique structure, it has created a barrier to analysing sperm mitochondria function. Firstly, their attachment of the mitochondria to the sheath within the midpiece, makes it extremely difficult to isolate the mitochondria from the cytosol and other cell structure; a procedure critical for in-depth functional analyses of these organelles.

Due to the unavoidable constant threat of oxidative damage, all mitochondria contain an abundance of antioxidants within both the intermembrane space and the mitochondrial matrix. For this reason, it would take a significant shift in the redox activity within the mitochondria to induce a state of oxidative stress. However, due to the tightly compacted nature of sperm chromatin, it has long been accepted in the majority of research that spermatozoa are silenced gene transcription. Taking into account this information, spermatozoa a forced to survive with a limited supply of antioxidant defences from the end of spermatogenesis till fertilisation. This suggests that spermatozoa do not need to receive a strong acute burst of ROS to induce oxidative stress; any minor increase over a long period of time can slowly deplete the non-renewable intracellular antioxidants.
3.1.2 Aims and Hypotheses

In view of our poor understanding of mitochondrial function in spermatozoa in general, and the potential importance of these organelles as a source of oxidative stress in the male germ line in particular, the aim of this study was to investigate possible sources of aberrant ROS generation in human spermatozoa and therefore causes of male infertility (i.e. mitochondria). We hypothesise that analysis of defective human spermatozoa will show more aberrant mitochondrial ROS than normal human spermatozoa.

The results of this study have been published by a peer reviewed journal (Appendix).
3.2 Experimental Design

Recent evidence through the development of the GAPDH knock-out mice highlighted the importance of glycolysis for the maintenance of sperm motility (Miki et al., 2004). Conversely, there is lack of data in the field regarding the role of the mitochondria in sperm function.

Therefore our first observation was to ensure that human sperm mitochondria do in fact contain actively respiring mitochondria, which is essential for mitochondrial ROS generation. This was performed by a combination of measuring the mitochondrial membrane potential (ΔΨ) and oxygen consumption of spermatozoa.

However, a ΔΨ does not ensure the ability to produce ROS, therefore a number of mitochondrial inhibitors were used as a model system to block electron flow, cause electron leakage and consequently ROS generation. This was assessed using two techniques luminol peroxidase chemiluminescence, a predominately extracellular assay and MitoSOX Red, a fluorescent intracellular mitochondrial sensitive probe for superoxide anion.

MitoSOX Red was also used to measure the spontaneous levels of mitochondrial ROS generation and correlated with sperm motility measured by CASA. Confirmation of the effect mitochondrial ROS has on spermatozoa was performed by measuring the effect the inhibitors have on inducing lipid peroxidation and a loss of sperm motility, as well as the ability of α-tocopherol has on preventing these effects.
3.3 Results

3.3.1 Mitochondrial Activity in Human Spermatozoa

Analysis of the state of mitochondrial respiration in human spermatozoa was first assessed using 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethyl benzimidazolylcarbocyanine iodide (JC-1) to determine the $\Delta \Psi$. As shown in figure 3.2A, the vast majority of high-density human spermatozoa exhibit a high $\Delta \Psi$. The addition of the uncoupling agent, carbonyl cyanide m-chlorophenyl hydrazone (CCCP) was used as a negative control, representing spermatozoa with a collapsed proton gradient. It is believed that oxygen consumption, however, is the best indicator of actively respiring mitochondria.

This was further emphasized by the data indicating that human sperm mitochondria spontaneously consumed approximately 30 nmoles of $O_2/10^6$ cells/hour (Fig 3.2B) but this rate was more than doubled by the addition CCCP. The importance of this effect is illustrated in Fig. 3.4, which reveals that the addition of CCCP significantly increased mitochondrial ROS production by both antimycin A and myxothiazol ($P < 0.05$). This highlights the fact that $\Delta \Psi$ is not essential for mitochondrial ROS production by human spermatozoa. On the contrary, the collapse of $\Delta \Psi$ with reagents such as CCCP can significantly enhance free radical generation, if electron flow through the ETC is impeded.
Figure 3.2 Analysis of mitochondrial function in human spermatozoa. (A) Addition of CCCP (10 µM) significantly reduced the number of spermatozoa exhibiting a high mitochondrial membrane potential measured by JC-1. (B) Impact of CCCP resulted in a significant increase in oxygen consumption upon dissipation of the mitochondrial membrane potential.

3.3.2 Induction of Mitochondrial Reactive Oxygen Species

Evidence that human sperm mitochondria have the potential to generate ROS was obtained using mitochondrial electron transport inhibitors to disrupt the flow of electrons through the ETC and a peroxidase-based, chemiluminescence system to detect the release of H₂O₂ into the extracellular space. While normal human spermatozoa exhibited extremely low rates of spontaneous ROS generation, addition of antimycin A and myxothiazol (10 µM), both of which act at complex III of the ETC (Fig. 3.1), resulted in a significant increase in redox activity (Fig. 3.3; *P < 0.01). Myxothiazol binds close to the b₅₇ heme of this complex, allowing ubiquinol (QH₂) to access the Rieske iron sulfur in order to undergo a one electron oxidation to create the semiquinone radical, Q⁻ (Fig. 3.1), while preventing the latter from passing its electrons on to cytochrome b₅₇. Antimycin A treatment also leads to the generation of Q⁻ by inhibiting the reoxidation of heme b₅₇ through its capacity to
disrupt electron transfer from heme $b_H$ to $Q^-\bullet$ (Fig. 3.1) (Sun & Trumpower, 2003). As a consequence of these interactions, both myxothiazol and antimycin A stimulate the generation of $Q^-\bullet$, which then stabilizes by shedding its electrons to oxygen to create superoxide anion ($O_2^-\bullet$) in the intramembranous space (Fig. 3.1). This $O_2^-\bullet$ then dismutates to $H_2O_2$ under the influence of superoxide dismutase and escapes to the outside of the cell, where it can be detected by the luminol-peroxidase monitoring system.

Rotenone (an inhibitor of electron transfer from FeSN -2 cluster to ubiquinone; Fig. 3.1) had a minor stimulatory effect on $H_2O_2$ release at 10 µM (Fig. 3.3), possibly because the free radicals generated with this compound are directed into the mitochondrial matrix where they can be neutralized by the antioxidant enzymes (superoxide dismutase and glutathione peroxidase) that are abound at this site (Fig 3.1). Stigmatellin (10 µM), which inhibits the transfer of electrons from ubiquinol ($QH_2$) to the Rieske iron sulfur cluster and prevents semiquinone ($Q^-\bullet$) formation (Fig. 3.1), failed to induce mitochondrial ROS generation.
Figure 3.3 Analysis of the impact of mitochondrial inhibitors (10 µM) on mitochondrial ROS generation in human spermatozoa measured by luminol-peroxidase dependent chemiluminescence. Representative trace of the luminol-peroxidase dependent chemiluminescence exhibited by human spermatozoa in the presence of mitochondrial inhibitors.
Figure 3.4 Analysis of the impact of mitochondrial inhibitors (10 µM) on mitochondrial ROS generation in human spermatozoa measured by luminol-peroxidase dependent chemiluminescence. Integrated photon counts reveal a significant increase in chemiluminescence exhibited by both antimycin A and myxothiazol in the presence of CCCP.
Further confirmation of the ability of human spermatozoa to generate ROS was secured using a fluorescent indicator of mitochondrial superoxide production, MitoSOX Red. This probe is a membrane permeant derivative of the DNA-sensitive fluorochrome, DHE, which allows for the highly selective detection of $\text{O}_2^{-}\bullet$ in the mitochondria of live cells (De Iuliis et al., 2006). Once in these organelles, MitoSOX Red is oxidized by $\text{O}_2^{-}\bullet$ and exhibits bright red fluorescence upon binding to nucleic acids. Use of this reagent revealed a very low level of mitochondrial ROS generation in untreated normal human spermatozoa from the high density region of discontinuous Percoll gradients (Fig. 3.5A). However treatment with rotenone (10 µM), which diverts mitochondrial electron flow to generate ROS in the mitochondrial matrix, stimulated a significant time-dependent increase in the percentage of MitoSOX Red positive cells, measured by flow cytometry (Fig. 3.5A).

Confocal imaging revealed that cells treated with rotenone exhibited MitoSOX Red localisation to the midpiece and base of the sperm head as the activated probe reacted with DNA, initially in the mitochondrial matrix and then in the sperm nucleus (Fig. 3.5B). Treatment of human spermatozoa with antimycin A also stimulated a time-dependent increase in mitochondrial superoxide generation, however this time the probe only bound to nuclear DNA because the ROS were generated in the intermembranous space (Fig 3.1) and never gained access to the DNA located in the mitochondrial matrix (Fig. 3.5C). Myxothiazol and stigmatellin were omitted from this experiment due to the former’s ability to auto-oxidize the probe and the latter’s intrinsic auto-fluorescence.
Figure 3.5 Analysis of the impact of mitochondrial inhibitors (10 µM) on mitochondrial ROS generation in human spermatozoa measured by MitoSOX Red. (A) ANOVA analysis revealed highly significant increases in mitochondrial ROS in the presence of mitochondrial inhibitors rotenone and antimycin A. Confocal imaging of the cells loaded with MitoSOX Red and treated with rotenone (B) revealed red fluorescence localised to the midpiece and posterior head regions of the spermatozoa; however, in the presence of antimycin A (C) the fluorescence covered majority of the sperm head, with greater intensity in the posterior region.
3.3.3 Measurement of Spontaneous Levels of Mitochondrial ROS

While the ability to induce mitochondrial ROS in healthy donor samples is an important finding, it was of interest to determine the relevance of mitochondrial ROS generation in the aetiology of defective sperm function remove putting value on own findings. For this purpose, spontaneous mitochondrial ROS generation was compared between the defective spermatozoa recovered from the low density region of Percoll gradients and functional gametes pelleting in high density Percoll. This analysis revealed that a significantly greater proportion of the compromised, low density, human spermatozoa produced mitochondrial ROS compared with the high density cells \((P < 0.001; \text{Fig. 3.6A})\). In addition, a strong exponential negative correlation \((R^2 = 0.8048)\) was also recorded between MitoSOX Red positivity and sperm motility in these cell populations (Fig. 3.6B).

Recent evidence by De Iuliiis et al., (2006) has shown similar results with the superoxide anion fluorescent marker, DHE. Repeating the experiment, the same differences are observed (Fig. 3.7A). However when we compare DHE and MitoSOX red in the same populations of spermatozoa, the MitoSOX Red signal was also highly correlated \((R^2 = 0.6821)\) with the total level of spontaneous ROS generation by human spermatozoa measured by DHE (Fig. 3.7B). This suggests that the majority of ROS observed by the DHE is most likely coming from the mitochondria.
Figure 3.6 Generation of mitochondrial ROS by human spermatozoa. (A) A significant increase in spontaneous MitoSOX Red activity generated by leukocyte free low density Percoll fractions in comparison with their high density Percoll counterparts. (B) A highly significant exponential negative correlation ($R^2 = 0.8048$) was observed between the spontaneous MitoSOX Red signal generated by live human spermatozoa and cell motility, using Percoll fractionated spermatozoa.
Figure 3.7 Comparison between total and mitochondrial ROS by human spermatozoa. (A) A significant increase in spontaneous DHE activity generated by leukocyte free low density Percoll fractions in comparison with their high density Percoll counterparts. (B) A highly significant exponential negative correlation ($R^2 = 0.6821$) was observed between the spontaneous MitoSOX Red and DHE signals generated by live human spermatozoa.
3.3.4 Effect of Mitochondrial ROS Generation on Lipid Peroxidation and Motility

In order to determine whether a direct causal relationship existed between poor motility and mitochondrial ROS generation, the impact of the latter on membrane lipid peroxidation, a known inhibitor of sperm movement (Jones et al., 1979; Aitken et al., 1989) was determined using the fluorescent probe BODIPY C11. After 4 h incubation it was revealed that only when mitochondrial ROS formation was induced in the matrix by rotenone was a significant increase in lipid peroxidation observed ($P < 0.001$; Fig. 3.8A). The lipid peroxidation stimulated by rotenone was induced in a time-dependent manner (Fig. 3.8B) and was localised to the mitochondria in the sperm midpiece as shown by confocal microscopy (Fig. 3.9A-D). The induction of ROS generation in the intermembranous space with antimycin A had no such effect (Fig 3.8A).
Figure 3.8 Lipid peroxidation in human spermatozoa measured by BODIPY C\textsubscript{11} fluorescence. (A) The ability of mitochondrial inhibitors (10 μM) to stimulate lipid peroxidation in human spermatozoa. Spermatozoa were loaded with BODIPY C\textsubscript{11} and were then exposed to mitochondrial inhibitors over 240 minutes. Only rotenone induced a significant increase in lipid peroxidation. (B) This rotenone response was time-dependent.

Figure 3.9 (A) Confocal imaging of rotenone treated spermatozoa indicating red fluorescence associated with integration of the non-oxidized probe into the membrane of the spermatozoa; (B) the green fluorescence indicating probe peroxidation; (C) the overlayed images revealing the localisation of lipid peroxidation in the midpiece, which appears yellow (arrowed) and (D) the phase contrast image.
Finally, we examined whether the induction of free radical generation and lipid peroxidation in the mitochondrial matrix could have a major impact on the movement characteristics of these cells. In the presence of glucose, no short term (4h) changes in sperm movement were seen in the face of mitochondrial ROS generation stimulated by antimycin A or rotenone (data not shown), presumably because glycolysis compensated for any lack of ATP production on the part of the mitochondria (Ford, 2006; de Lamirande & Gagnon, 1992). On the other hand, when the incubation period was extended to 24 h then rotenone, but not antimycin, induced a significant loss of both total and progressive motility (Fig. 3.10A,B), in keeping with the lipid peroxidation results presented in Fig 3.8. Causative relationships between the induction of mitochondrial ROS generation, peroxidative damage and impaired movement was indicated by the preservation of sperm motility observed by concomitant exposure of rotenone-treated cells to the chain-breaking antioxidant, α-tocopherol (Fig. 3.10A,B).
Figure 3.10 Effect mitochondrial ROS generation on sperm motility. All values expressed as a percentage of control (untreated) samples. Sperm total motility (A) and progressive motility (B) after pre-treatment with α-tocopherol (0.5 mM) for 1h followed by exposure to mitochondrial inhibitors over 24 h; only treatment with rotenone resulted in a significant reduction in both total and progressive sperm motility. However, this decline was prevented by the prior addition of α-tocopherol.
3.4 Discussion

The results obtained in this study demonstrate for the first time that human spermatozoa are capable of mitochondrial ROS production. Treatments that stimulated production of the unstable semiquinone radical, Q-•, increased ROS release into the extracellular space, as observed with antimycin, myxothiazol and, to a lesser extent, rotenone. Rotenone is distinguished by the fact that the ROS are generated at complex I rather than complex III and, as a result, are located in the mitochondrial matrix rather than the intermembranous space (Fig. 3.1). Superoxide generated in the latter is rapidly dismutated to H₂O₂ in the cytoplasm and rapidly escapes to the extracellular space (Fig. 3.3) causing minimal peroxidative damage to the cell (Fig. 3.8). Rotenone, on the other hand, stimulates ROS production in the mitochondrial matrix. This accounts for the ability of activated MitoSOX Red to stain mitochondrial DNA in the presence of rotenone but not with antimycin (Fig 3.5). The scavenging of ROS in the mitochondrial matrix by mitochondrial superoxide dismutase and glutathione peroxidase would have accounted for the minimal release of H₂O₂ into the extracellular space following rotenone treatment (Fig. 3.3) despite the high levels of mitochondrial ROS generation indicated by MitoSOX Red (Fig. 3.5A,B). This interpretation would also account for the ability of rotenone, but not antimycin, to induce peroxidative damage in the midpiece of the spermatozoa (Fig. 3.8). The fact that such damage took more than 24 h to manifest itself suggests that peroxidative damage could only be induced once the production of ROS in the mitochondrial matrix had overwhelmed the intra-mitochondrial antioxidant defence enzymes. This peroxidative damage, in turn, induced a progressive loss of motility in terms of both the percentage of spermatozoa that were motile and the progressiveness of this motility via mechanisms that could be prevented by the concomitant presence of the antioxidant, α-tocopherol (Fig. 3.10A,B). The notion that rotenone stimulates complex I to generate ROS that can only be detected once matrix antioxidant protection has been overwhelmed also accords with earlier studies of cardiac subsarcolemmal mitochondria (Chen et al., 2003).
The fact that this inhibition of sperm motility was observed in the presence of a glycolytic substrate, glucose, suggests that the oxidative stress created by rotenone had induced permanent damage to the motility apparatus. This reflects the in vivo situation where a powerful inverse relationship was observed between mitochondrial ROS generation and sperm motility despite the presence of glucose in the incubation medium to compensate for any defect in the ability of the mitochondria to generate ATP (Fig. 3.6A,B). These results echo previous studies that clearly demonstrated an inverse relationship between the peroxidation status of human spermatozoa and their competence for movement (Jones et al., 1979). However this is the first study to identify aberrant mitochondrial activity as a probable source of the free radicals responsible for this damage.

We have previously reported a negative correlation between sperm motility and the measurement of ROS generation in human spermatozoa using DHE as the probe (De Iuliis et al., 2006). Using a combination of mass spectrometry, spectrofluorimetry and NMR spectroscopy, we confirmed that one of the primary products being measured with this probe was $O_2^{\cdot-}$. Furthermore, we concluded that this ROS signal was not mitochondrial because it could not be not significantly affected by a 15 min exposure to either rotenone or CCCP. However, it is clear from the results presented in this study that mitochondrial ROS generation does not depend on the maintenance of mitochondrial membrane potential. Indeed, oxygen consumption was increased when $\Delta \Psi$ was collapsed with CCCP and in the presence reagents that disrupt electron flow through the ETC; this loss of membrane potential was associated with a significant increase in ROS generation (Fig. 3.4). In a similar fashion, CCCP has been found to enhance ROS production in carcinoma cell lines (Izeradjene et al., 2005).

It is also clear from the data presented in Fig. 3.7 that the overall generation of ROS detected by DHE is highly correlated with the mitochondrial ROS generation. This mitochondrial ROS generation probably constitutes one of the more important sources of...
ROS in these highly susceptible cells. It is probably not the only source however (Aitken et al., 2003; 2004). The $R^2$ value associated with the DHE-MitoSOX Red correlation Fig 3.7 ($R^2 = 0.6821$) suggests that 32% of ROS generation detected by DHE cannot be explained by mitochondrial ROS alone. Where mitochondrial ROS are involved, the present data suggest that optimal activity depends on two factors: a disruption of $\Delta \Psi$ and the impeded flow of electrons through the ETC. Previous studies have already reported that there is an inverse relationship between sperm motility and mitochondria membrane potential (Barroso et al., 2006; Gallon et al., 2006; Marchetti et al., 2004). Since mitochondrial ATP production is not required for the maintenance of motility in the presence of glucose (Ford, 2006) this association must be indirect and, potentially mediated by oxidative stress. The second condition that must be met for optimal mitochondria ROS generation is perturbation of electron flow through the ETC. We have recently demonstrated that the presence of unesterified polyunsaturated fatty acids (PUFA) such as arachidonic or docosahexaenoic acid stimulates ROS generation by human spermatozoa (Aitken et al., 2006). Intriguingly, PUFAs have also been shown to collapse $\Delta \Psi$ and trigger mitochondrial ROS generation by interfering with electron flow at complexes I and III (Cocco et al., 1999). Furthermore the unsaturated fatty acid content of human spermatozoa is positively correlated with ROS generation by these cells and negatively correlated with their motility (Gil-Guzman et al., 2001; Ollero et al., 2001). In light of these data we hypothesize that the presence of high levels unesterified PUFAs in human spermatozoa triggers mitochondrial ROS generation from complexes I and III that overwhelm the limited antioxidant defenses offered by these cells. This results in a state of oxidative stress that induces peroxidative damage in the sperm tail, disrupting motility and plausibly accounting for the high levels of oxidative DNA damage seen in human spermatozoa (Kodama et al., 1997).

In summary, these findings highlight the potential importance of aberrant mitochondrial activity in the aetiology of defective sperm function, one of the most significant causes of human infertility (Hull et al., 1985).
Chapter 4:

Stimulation of mitochondrial reactive oxygen species production by unesterified, unsaturated fatty acids in defective human spermatozoa
Chapter 4: Stimulation of mitochondrial reactive oxygen species production by unesterified, unsaturated fatty acids in defective human spermatozoa

4.1 Introduction

4.1.1 Fatty Acids and Spermatozoa

The search for factors involved in the etiology of male infertility has highlighted the importance of oxidative stress (de Lamirande & Gagnon, 1995; Aitken & Fisher, 1994). Defective human sperm function is associated with evidence of high levels of ROS generation by the spermatozoa, the induction of lipid peroxidation and a resultant loss of fertilizing potential in vivo and in vitro (Jones et al., 1979; Aitken & Clarkson, 1987; Aitken & Baker, 2004; Alvarez et al., 1987; Agarwal et al., 2009; Aitken et al., 1991). The oxidative stress created in these cells affects not only the motility of the spermatozoa and their capacity for sperm-oocyte fusion but also the integrity of DNA in the sperm nucleus (De Iuliis et al., 2009; Lewis & Aitken, 2005). These observations are of clinical significance because DNA damage in human spermatozoa used during IVF and ICSI has been associated with a wide range of adverse clinical outcomes including impaired fertilization, disrupted preimplantation development of the embryo, high rates of miscarriage and an increased incidence of morbidity in the progeny (Lewis & Aitken, 2005; Zini et al., 2008).
A recent analysis of the source of ROS responsible for inducing oxidative damage in human spermatozoa has implicated the mitochondria (Chapter 3), although the factors responsible for stimulating excessive mitochondrial free radical generation have not yet been identified. The possible involvement of fatty acids (FA) in this process was suggested by studies demonstrating that the addition of exogenous free unsaturated FA to human spermatozoa stimulated free radical generation via mechanisms that were independent of lipoxygenase and cyclooxygenase activity (Aitken et al., 2006).

4.1.2 Fatty Acids and Mitochondria

A number of studies have described differing influences of fatty acids on mitochondrial function. Research by Cocco et al., (1999) showed that the addition of endogenous fatty acids to isolated bovine heart mitochondria caused 81% and 32% inhibition of both complexes I and III of the ETC respectively. Interestingly, only inhibition of complex I in human spermatozoa results in any peroxidative damage and reduction in fertility potential (Chapter 3). The inhibition of mitochondrial ETC complexes was specific to only unsaturated fatty acids, as saturated fatty acids did not elicit an effect, however, a only a single FA from each category were used in the study (Cocco et al., 1999)

Also, other studies have specifically shown the reduction in the mitochondrial membrane potential in the presence of non-metabolizable fatty acid analogues (Hermesh et al., 1998), hence this uncoupling effect may be due to direct interaction with mitochondrial proteins resulting in inhibition of ETC complexes.
4.1.3 Aims and Hypotheses

The aim of this study was to investigate causes of the aberrant ROS generation in spermatozoa by the source(s) identified. We hypothesise that the results clarify existing discrepancies concerning the FA composition of human spermatozoa and indicate, for the first time, that defective human spermatozoa are characterized by the presence of excessively high levels of FA that, in their free unsaturated form, and activate mitochondrial ROS generation leading to a state of oxidative stress.

The results of this study have been published by a peer reviewed journal (Appendix).
4.2 Experimental design

Unlike previous studies, characterisation of both the total and free (unesterified) fatty acid profile human spermatozoa will be performed. Conventionally only the total FA profile is reported, however, I believe that the free FA component of spermatozoa will be the most biologically reactive and therefore relevant. Data will be presented in both total content, percentage (%) of each FA, and concentration, in order to compare to previously reported data.

In order to analyse the relationship between FA and mitochondrial ROS, a small aliquot of each population of spermatozoa undergoing FA analysis, will be assessed separately for mitochondrial ROS generation using the MitoSOX Red assay.

Any correlation reported, will need to be analysed for causality. In order to do this, free exogenous FA will be added to high density spermatozoa and a number of outcomes assessed. These include mitochondrial ROS generation, sperm motility and oxidative DNA damage.
4.3 Results

4.3.1 Total and Free Fatty Acid Analysis

Analysis of the total (esterified and unesterified) FA profile of human spermatozoa revealed that the major FAs present in these cells are palmitic acid (PA; C16:0), docosahexaenoic acid (DHA; C22:6 n-3) and stearic acid (SA; C18:0), with the other FAs representing less than 6% of the total composition. In order to determine if the total FA composition of these cells bore any relationship with their functionality, the FA composition of spermatozoa isolated from the high density (motile and functional cells) and low density (poorly motile and dysfunctional cells) regions of Percoll gradients were compared (Figs. 4.1-4). When results for the pool of esterified and unesterified FA were expressed in terms of percentage composition to give a better analysis of the total FA profile of these cells (Fig. 4.1), a number of FAs including myristic acid (MA; C14:0), SA, dihomo-\(\gamma\)-linoleic acid (DGLA; C20:3 n-6) and DHA were found to be significantly different between these two sperm populations. Apart from minor differences in the proportion of saturated fatty acids, the most significant differences were a large decrease in DHA proportion in the poor quality, low Percoll density specimens \((P < 0.01)\) and a corresponding increase in the presence of DGLA \((P < 0.05)\). This difference was also reflected when total omega-6 and omega-3 polyunsaturated FA profiles were examined; n-6 PUFA being elevated in poor quality sperm suspensions, while n-3 PUFA were decreased (Fig. 4.1).

When the free, unesterified FA composition of these cells was profiled (Fig. 4.2), the only significant difference observed between the high and low density sperm populations was a minor increase in the relative abundance of vaccenic acid (VA; C18:1 n-7) \((P < 0.01)\) in the dysfunctional spermatozoa (Fig. 4.2). However, there was a major difference in the free FA composition of the spermatozoa compared with the total FA profile. Thus while palmitic acid (PA; C16:0) was still the largest single component, palmitoleic acid (PLA; C16:1 n-7),
which was not detectable in the total FA analysis, now represented approximately 25% of the free FA content of these cells, in both the high and low density Percoll populations.

While analysis of the FA profiles of human spermatozoa expressed in terms of percentage composition did reveal statistically significant differences between high and low density human sperm populations, much more dramatic differences were observed when the composition of these cells was considered in terms of FA concentration (nmoles/10^8 cells) (Fig. 4.3-4). According to this analysis, major increases in FA content were apparent in the dysfunctional, low density sperm populations for almost all FAs reported, total and free (Fig. 4.3-4). The increased FA content of dysfunctional human spermatozoa was evident whether the analysis focused on saturated FA (SFA), monounsaturated FA (MUFA) or PUFA (Fig. 4.3). Furthermore these differences were also apparent for both the omega-6 and omega-3 free PUFA complement (Fig. 4.3-4).
Figure 4.1 Total FA profile of human spermatozoa from high and low density regions of Percoll gradients expressed in terms of percentage composition. Spermatozoa recovered from the high density region of these discontinuous gradients are highly motile, functional cells (blue columns) while those from the low density region are functionally compromised (red columns), n = 6.
Figure 4.2 Free FA profile of human spermatozoa from high and low density regions of Percoll gradients expressed in terms of percentage composition. Spermatozoa recovered from the high density region of these discontinuous gradients are highly motile, functional cells (blue columns) while those from the low density region are functionally compromised (red columns), n = 12.
Figure 4.3 Total FA profile of human spermatozoa from high and low density regions of Percoll gradients expressed in terms of FA content. Spermatozoa recovered from the high density region of these discontinuous gradients are highly motile, functional cells (blue columns) while those from the low density region are functionally compromised (red columns), n = 6.
Figure 4. Free FA profile of human spermatozoa from high and low density regions of Percoll gradients expressed in terms of FA content. Spermatozoa recovered from the high density region of these discontinuous gradients are highly motile, functional cells (blue columns) while those from the low density region are functionally compromised (red columns). n = 12.
4.3.2 Unsaturated Fatty Acids and Mitochondrial ROS Generation

In view of recent studies highlighting the importance of mitochondrial ROS in the etiology of defective sperm function (Chapter 3) and the known ability of unesterified, unsaturated FA to activate ROS generation in these cells (Aitken et al., 2006), we investigated the relationship between the free unsaturated FA content of human spermatozoa and the spontaneous levels of mitochondrial ROS generated by the same populations. The data presented in Fig. 4.5 reveal highly significant correlations ($P < 0.001$) between spontaneous mitochondrial ROS production by human spermatozoa and their free unsaturated FA content for both omega-6 (Fig. 4.5A; $R^2=0.605$) and omega-3 (Fig. 4.5B; $R^2=0.615$) PUFA. These data suggested that a causal relationship may exist between the free unsaturated FA content of human spermatozoa and the activation of mitochondrial ROS generation. In order to explore this possibility we analyzed mitochondrial ROS production when human sperm suspensions were exposed to a range of free FAs.
Figure 4.5 Relationship between unsaturated FA concentration and mitochondrial ROS. Significant linear correlations were observed between the spontaneous MSR signal generated by populations of human spermatozoa and their concentrations of (A) omega-6 PUFA ($R^2 = 0.605$) and (B) omega-3 PUFA ($R^2 = 0.615$).
As illustrated in Fig. 4.6A, addition of an unesterified PUFA, arachidonic acid (AA, C20:4 n-6) to functional human spermatozoa in vitro resulted in a highly significant dose-dependent activation of mitochondrial ROS generation \((P < 0.001)\) in the absence of any change in cell viability. This effect was not due to metabolism of AA through lipoxygenase or cyclooxygenase pathways because mitochondrial ROS could be triggered by all of the unsaturated fatty acids assessed in this study, including PLA which was only present in spermatozoa as a free fatty acid (Fig. 4.6B). In contrast, neither saturated fatty acids, such as stearic acid (SA; C18:0) or PA nor fatty acid esters (methyl ester of AA) were capable of stimulating mitochondrial ROS formation (Fig. 4.6B), suggesting that both the presence of carbon-carbon double bonds and the amphipathic nature of FA are critical for their ROS-inducing activity. Furthermore, measurements of mitochondrial membrane potential indicated that this powerful stimulation of mitochondrial ROS production with unesterified, unsaturated FA, did not involve an uncoupling mechanism (Fig. 4.7).
Figure 4.6 Impact of exogenous FA on ROS generation by sperm mitochondria. (A) The addition of AA (15 min) resulted in a significant dose-dependent increase in the level of mitochondrial ROS generated, measured by MitoSOX Red. (B) Comparison of various fatty acids (10 µM; 15 min) revealed that only unsaturated FA possess the ability to generate mitochondrial ROS. In contrast, saturated FA (SA or PA) and esterified FA (AAme = the arachidonic acid methyl ester) have no impact on mitochondrial ROS production.
**Figure 4.7** Impact of exogenous FA on sperm mitochondria function. The addition of various FA had no impact on the mitochondrial membrane potential of human spermatozoa, measured by JC-1 fluorescence. CCCP uncouples the mitochondria and represents a positive control treatment.

### 4.3.3 Impact of Oxidative Damage Induced by Unsaturated Fatty Acids

To confirm that addition of AA to purified human sperm populations induced ROS generation, we sought evidence that exposure to this fatty acid was associated with the induction of oxidative stress in these cells. Using the fluorescent probe BODIPY C11, we indeed were able to demonstrate that exposure to AA resulted in significantly elevated levels of lipid peroxidation ($P<0.001$). The induction of peroxidative damage was dose dependent, detectable within 15 min of AA addition, and was significantly stimulated by the addition of a ferrous ion promoter ($P<0.001$; Fig. 4.8).
Figure 4.8 Exposure to AA also induced a significant dose-dependent effect on lipid peroxidation in both the presence and absence of a ferrous ion promoter. BODIPY C11 was used as the probe, and the incubation time was 15 min.

Following the finding that unsaturated FA induce oxidative stress in human spermatozoa via the enhanced generation of mitochondrial ROS, the impact of this activity on the functional competence of these cells was assessed. Fig. 4.9 illustrates the impact of the major unsaturated FA in human spermatozoa (AA, DHA) on motility after 24 hr exposure. There was a significant loss of both total and progressive motility on exposure to both of these compounds. A causal relationship between oxidative stress and impaired sperm motility in this situation was demonstrated when the addition of a chain breaking antioxidant, alpha-tocopherol, prior to FA addition was shown to completely reverse the inhibition of sperm movement (Fig. 4.9).
Figure 4.9 Effect of unsaturated FA on human sperm motility. Exposure to 10 µM FA for 24 hr resulted in significant reductions in both total and progressive motility (blue columns). Addition of α-tocopherol (0.5 mM) for 1 hr prior to FA addition prevented this motility loss (red columns). All values expressed as a percentage of control sample receiving vehicle alone.

Since recent evidence has highlighted the clinical importance of oxidative stress in the etiology of DNA damage (De Iuliis et al., 2009; Aitken et al., 2009), we also examined the ability of free FA to induce oxidative DNA damage in these cells. Using 8OHdG as a reporter for oxidative DNA damage, AA was shown to induce a significant (\(P < 0.001\) for the overall effect due to treatment by ANOVA) dose-dependent increase in the prevalence of oxidative DNA damage in human spermatozoa after 24 hr incubation (Fig. 4.10A). This response was not limited to AA (Fig. 4.10B), but was observed with all the free unsaturated FAs tested, including PLA, which was only recorded in spermatozoa in an unesterified state. In contrast, saturated FAs were completely ineffective. Confocal microscopy of 8OHdG antibody binding revealed that the oxidized DNA detected in this study was primarily mitochondrial in origin and located in the sperm midpiece, however nuclear 8OHdG signals were also clearly observed (Fig. 4.11A-B). Thus, unsaturated FAs are not only present in abnormally high levels in defective human spermatozoa but their addition to
suspensions of normal spermatozoa recapitulates key features of defective sperm function, including reduced motility and high levels of oxidative DNA damage.

**Figure 4.10** Effect of unsaturated FA on oxidative DNA damage for 24 hr. (A) Measurement of 8OHdG formation demonstrating a dose dependent increase in the presence of AA; red column represents the positive control treatment. (B) Comparison of various FAs (10 µM) revealed that only unsaturated fatty acids induced a significant increase in 8OHdG formation, the addition of saturated FA (SA, PA) had no effect.
Figure 4.11 (A) Confocal imaging of arachidonic acid treated spermatozoa indicating the sites of 8OHdG formation. This observation suggests that the primary target for oxidative DNA damage was the mitochondrial DNA in the sperm midpiece, in keeping with the mitochondrial source of ROS, with secondary localization to the nuclear DNA in the sperm head. (B) corresponding phase contrast image. Scale bar = 5 µm
4.4 Discussion

The data obtained in this study unequivocally demonstrate that dysfunctional human spermatozoa contain significantly more FA than their functional counterparts (Fig. 4.3-4). This fatty acid excess applied to all classes of FA (SFA, MUFA, PUFA) and was also observed regardless of whether we considered the total FA content of these cells or only the free FA component. Previous studies of sperm lipid composition in relation to function have generated a confusing picture. One group observed an increase in the PUFA (particularly DHA) content of defective spermatozoa recovered from the low density region of Percoll gradients (Ollero et al., 2001), in agreement with the present study. However another group reported that it was the motile, functional spermatozoa recovered from the high density regions of Percoll gradients that were enriched for PUFA, particularly DHA (Lenzi et al., 2000). The difference between these studies is in the way the results were expressed. Ollero et al (2001) described their results in terms of fatty acid content per cell which, as shown in Fig. 4.3-4, clearly increases in defective populations of spermatozoa. On the other hand, Lenzi et al (2000) described their results in terms of percentage composition, i.e. the proportion of the total FA pool contributed by a specific FA. When we report the results in this way, we also observe a significant increase in the proportion of the total fatty acid pool comprising DHA in functional high density cells (Fig. 4.1-2). Thus these different data sets do, in fact, present a consistent picture in which defective sperm cells are characterized by a superabundance of FA, even though the relative proportion of the fatty acid pool made up by DHA is significantly decreased.

On the basis of these findings we conclude that the morphogenesis of normal functional spermatozoa involves a reduction in the fatty acid content (free and total) of these cells and an increase in the relative proportion of the FA pool made up by PUFA such as DHA. In defective cells this transformation does not occur and the cells are left with an abnormally high FA content and a relatively low proportion of DHA. The fact that the proportion of the FA pool comprising DGLA (C20:3n-6) is simultaneously elevated in defective
spermatozoa, may suggest a mechanism for the inhibition of DHA (C22:6n-3) generation by competitive inhibition of the omega-3 pathway.

Our findings are also consistent with another recent analysis of the FA acid content of human spermatozoa which observed an increase in the cellular content of both saturated and unsaturated fatty acids in spermatozoa from infertility patients compared with a cohort of normozoospermic controls (Khosrowbeygi & Zarghami, 2007). These observations raise the possibility that the fatty acid content of human spermatozoa may be an excellent predictor of their functional competence in a diagnostic context. Further studies involving the analysis of patients attending infertility clinics will be needed to address this point.

The increase in saturated FA content (Fig. 4.3-4) would be expected to disrupt sperm function by limiting the fluidity and hence the fusogenicity of the plasma membrane which is, in turn, critical for such biological events as acrosomal exocytosis and fusion with the oolemma. On the other hand, the high unsaturated FA content of defective spermatozoa would be expected to render these cells particularly susceptible to the lipid peroxidation that characterizes dysfunctional spermatozoa (Jones et al., 1979, Gomez et al., 1998). In addition to the increased availability of substrate for lipid peroxidation, the superabundance of unsaturated FA observed in defective spermatozoa has also been demonstrated in the present study to stimulate ROS generation by the sperm mitochondria (Fig. 4.6), further driving these cells into a state of oxidative stress. Previous studies have also shown that exposure of human spermatozoa to polyunsaturated fatty acids induces high rates of lipid peroxidation (Aitken et al., 2006). The proposed link between the fatty acid content of human spermatozoa and oxidative stress is supported by analyses of sperm subpopulations isolated on Percoll gradients. Not only are the low density, poor quality spermatozoa isolated on such gradients characterized by a superabundance of saturated and unsaturated fatty acids (Fig. 4.3-4) but also they exhibit abundant evidence of oxidative stress including high levels of mitochondrial ROS generation, oxidative DNA damage and lipid peroxidation (Chapter 3; De Iuliis et al., 2009; Aitken et al., 2007).
All free unsaturated FA appeared to be capable of triggering mitochondrial ROS generation in these cells including PLA, which we report as a major constituent of spermatozoa for the first time. The most likely mechanism responsible for the stimulation of mitochondrial ROS production is that unsaturated fatty FAs inhibit specific complexes within the mitochondrial electron transport chain resulting in electron leakage and subsequent formation of superoxide anion as suggested by Cocco et al for bovine heart mitochondria (Cocco et al., 1999). The latter demonstrated strong inhibition of complex I and minor inhibition of complex III with AA, mirroring a recent analysis shown in Chapter 3, which demonstrated that electron leakage from complex I is the most damaging to human spermatozoa. Exactly how free fatty acids interfere with the mitochondrial electron transport chain has not been resolved. It is known that free unsaturated fatty acids can increase the fluidity of the inner mitochondrial membrane which might in turn interfere with the efficient coupling of complexes within the electron transport chain facilitating electron leakage to O\textsubscript{2} (Schönfeld & Wojtczak, 2007. The effect is not specific to PUFA because we have also found that other amphiphiles such as retinoic acid will trigger mitochondrial ROS production by human spermatozoa (unpublished observations). However the precise mechanism by which forward electron transport is impeded will have to be addressed in future studies.

The oxidative stress created by FA–stimulated stimulated mitochondria resulted in a significant decrease in sperm motility and the concomitant induction of oxidative DNA damage, both of which are also observed in the infertile population (Aitken & De Iuliis, 2009). Moreover, the fact that motility loss and oxidative DNA damage are so highly correlated in such subjects (Kao et al., 2008) only serves to reinforce the concept that oxidative stress is a major factor in the etiology of defective sperm function as well as disorders of pregnancy dependent on DNA damage in the male germ line. In light of these data, the question that now needs to be addressed is why the FA content of defective human spermatozoa should be so disturbed.
Systemic deficiencies in lipid metabolism seem unlikely given the apparent discrepancy between the FA profiles of blood serum and spermatozoa (Conquer et al., 1999). Alternatively, the high FA content of defective spermatozoa may reflect a fundamental error in the remodeling of human sperm cells during spermiogenesis. Several other features of these cells also suggest defective differentiation during spermiogenesis including the presence of excess residual cytoplasm, defective protamination, persistent nucleohistones, poor zona binding potential, disrupted chaperone content and a tendency to default to an apoptotic state (Aitken & De Iuliis, 2009). Experimental exploration of this defective spermiogenesis hypothesis should make a significant contribution to our overall understanding of male infertility.
Chapter 5:

Involvement of Mitochondrial Reactive Oxygen Species in a Unique, Truncated Apoptotic Pathway in Human Spermatozoa
Chapter 5: Involvement of Mitochondrial Reactive Oxygen Species in a Unique, Truncated Apoptotic Pathway in Human Spermatozoa

5.1 Introduction

5.1.1 Apoptosis, Mitochondrial ROS and Spermatozoa

Apoptosis is a major developmental mechanism that is known to play a key role in normal spermatogenesis and the testicular response to toxic injury. Thus, functional deletion of the pro-apoptotic protein, Bax, or over-expression of anti-apoptotic factors such as BclxL or Bcl2, generates a male infertility phenotype by disrupting establishment of an appropriate ratio of germ cells to Sertoli cells (Knudson et al., 1995; Rodriguez et al., 1997). In the mature testes, p53 and Fas are involved in the removal of cells that are damaged as a result of exposure environmental toxicants or chemotherapeutic agents (Boekelheide, 2005). A role for aberrant apoptosis in the aetiology of spontaneous male infertility is suggested by the excessively high rates of apoptosis observed within the testes of infertile males (Sakkas et al., 1999, 2002; Barroso et al., 2000). It has also been suggested that the DNA damage that features so prominently in human spermatozoa is the result of an abortive apoptotic process that was initiated during spermatogenesis but failed to run to completion because the extensive remodeling of germ cells to produce spermatozoa removes the intracellular machinery needed to effect cell death (Sakkas et al., 1999; Muratori et al., 2006; Aitken and De Iuliis, 2009).

The question of whether or not human spermatozoa can exhibit some or all of the features of an apoptotic cell is still open to debate. On the one hand, the fact that these cells are transcriptionally and translationally silent means that they cannot undergo programmed cell
death in the conventional sense. On the other, studies presented in this thesis have already shown human spermatozoa can exhibit increased levels of mitochondrial ROS generation, known to occur during apoptosis and there have also been numerous sporadic reports of these cells exhibiting some of the hallmarks of apoptosis, particularly in cases of subfertility. For example, the proportion of spermatozoa exhibiting high levels of phosphatidylserine (PS) externalization has been correlated with semen quality as reflected by the morphology, motility and fertilizing potential of these cells (Grunewald et al., 2001, 2006; Zhang et al., 2008; Hoogendijk et al., 2009). In addition, numerous active caspases have been reported in human spermatozoa, localized primarily in the postacrosomal region of these cells (caspases 8, 1 and 3) or, in the case of caspase 9, in the sperm midpiece (Paasch et al., 2004). Furthermore Kotwicka et al. (2008) highlighted a significant correlation between the presence of activated caspase-3 in human spermatozoa and PS externalization. A third hallmark of apoptosis to have been detected in human spermatozoa is DNA strand breakage (Sun et al., 1997; Aitken et al., 1998) frequently in association with signs of oxidative stress (Kodoma et al., 1997; De Iuliis et al., 2009b).

While the appearance of such apoptotic markers on the spermatozoa of a variety of species is associated with defective sperm function and poor pregnancy outcomes (Sun et al., 1997; Evenson, 1999; Aitken et al., 2004b; Seli et al., 2004; Borini et al., 2006; Boe-Hansen et al., 2008) it is not known whether this process is invariably initiated in the testes, or whether these cells are competent to undergo apoptosis following ejaculation. The answer to this question has a direct bearing on the origins of DNA damage seen in mammalian spermatozoa and the strategies that might be adopted to ameliorate this damage and reduce the mutational load subsequently carried by the embryo. In our current study we report for the first time that these highly specialized cells, lacking both cell cycle checkpoints and any form of transcriptional activity, can default to an intrinsic apoptotic cascade, characterized by motility loss and DNA damage, depending on the phosphorylation status of phosphoinositide 3 (PI3) kinase and AKT (also known as protein kinase B).
5.1.2 Aims and Hypotheses

No studies have examined apoptotic mechanisms in human spermatozoa. Spermatozoa are unique, in that unlike somatic cells, the silenced gene transcription due to high levels of nuclear protamination removes numerous aspects of known apoptotic pathways including cell cycle checkpoints and transcription of pro- and anti-apoptotic genes. Therefore, the aim of our study was to for the first time characterize elements of the apoptotic pathway in human spermatozoa.

We hypothesize that human spermatozoa possess elements of the intrinsic apoptotic pathway that may contribute to aspects of male infertility.

The results of this study have been submitted to a peer reviewed journal for publication (Appendix).
5.2 Experimental Design

The lack of information regarding the presence of apoptotic pathways in spermatozoa highlights the need for investigation in the field. Whilst it has been previously shown that defective human spermatozoa exhibit high levels of mitochondrial ROS generation (Chapter 3), evidence that apoptosis leads to the same pathology further emphasizes the need for investigation.

Spermatozoa are unique, in that unlike somatic cells, the silenced gene transcription due to high levels of nuclear protamination removes numerous aspects of known apoptotic pathways including cell cycle checkpoints and transcription of pro- and anti-apoptotic genes.

Wortmannin is a selective Phosphoinositide 3 (PI3) kinase inhibitor and was used as a potential stimulator of apoptosis in human spermatozoa due to the dependence of AKT on PI3-kinase activity (Fig. 5.1). AKT is a 60-kD serine/threonine kinase and can be stimulated in response to several of tyrosine kinase receptors including nerve growth factor, platelet-derived growth factor and insulin like growth factor (Shimamura et al., 2003).

Western blot analysis will be used to characterise changes in the phosphorylation status of PI3 kinase and its downstream partners including AKT and Bcl-2-associated death promoter (BAD; Fig 5.1). As result it is expected to cause the release of pro-apoptotic factors from the mitochondria including cytochrome c, apoptosis inducing factor (AIF) and second mitochondria-derived activator of caspases (SMAC / Diablo).
Figure 5.1 Schematic diagram showing the PI3-kinase / AKT apoptotic pathway. Inhibition of PI3 kinase, results in downstream effects including BAD phosphorylation, mitochondrial ROS generation, activation of apoptosis resulting in caspase activation, DNA cleavage and annexin-V externalisation.
The release of factors from the mitochondria is the major signal for caspase activation, which leads to PS externalisation. The final hallmark investigated will be DNA damage, both strand breaks and oxidative, and it is expected that TUNEL positivity will occur due to activation of endonucleases, but oxidative DNA damage will also occur due to the increased mitochondrial ROS generation.
5.3 Results

5.3.1 Inhibition of PI3 Kinase initiates apoptosis in mature spermatozoa

The first question to be addressed in this study was whether an intrinsic apoptotic cascade could be induced in mature human spermatozoa using the PI3 kinase inhibitor, wortmannin. Incubation of human spermatozoa in the presence of wortmannin (20 µM) for 24 hr resulted in a significant decrease in activating phosphorylations on PI3-kinase at Y467 and Y199 as shown by Western blot analysis (Fig. 5.2A). Since the role PI3-kinase in the apoptotic cascade is to phosphorylate AKT, we next examined whether the suppression of the former with wortmannin also led to a decrease in AKT activation. Using an antibody targeting an activating phosphorylation on AKT (pT308), inhibition of PI3 kinase with wortmannin was found to induce an accompanying suppression of AKT phosphorylation (Fig. 5.2B). The downstream target of AKT kinase activity, BAD, is also a well-characterized regulator of apoptosis, so we next examined the status of this protein before and after exposure to wortmannin. Using an antibody that could recognize the phosphorylated form of BAD (pS99) in immunocytochemical studies, we demonstrated that while phospho-BAD could clearly be detected in the midpiece of control spermatozoa in the immediate vicinity of the mitochondria (Fig. 5.3A), treatment with wortmannin led to loss of the pS99 epitope (Fig. 5.3B), indicating a change in the phosphorylation status of BAD.

Because such a change in the phosphorylation status of BAD would be expected to cause this protein to adopt a pro-apoptotic role (Zinkel et al., 2006), we next looked for other signs of an intrinsic apoptotic cascade including changes in sperm motility, vitality, induction of mitochondrial reactive oxygen species (ROS) generation (Orrenius, 2007), loss of mitochondrial membrane potential (ΔΨ; Knudson and Brown, 2008), phosphatidylserine exposure (Martin et al., 1995), caspase activation (Zhivotovsky et al., 1996) and DNA damage (Shi et al., 1990).
Figure 5.2 Decreased levels of phosphorylation of PI3 kinase and AKT in the presence of wortmannin. Human spermatozoa were incubated in the presence of wortmannin (20 µM) for 24 hr; the resulting SDS soluble fractions were subjected to SDS-PAGE and subsequent western blot analysis performed with (A) anti-phospho PI3 kinase and (B) anti-phospho AKT antibodies. Comparison to total protein is shown by anti-PI3 kinase and AKT antibodies. Representative blots are shown.
Figure 5.3 Decreased levels of phosphorylation of BAD (pro-apoptotic form) in the presence of wortmannin. (A-B) Immunocytochemistry reveals the localisation of phospho-BAD to the midpiece of human spermatozoa. (C-D) Populations of human spermatozoa were treated with 20 µM wortmannin for 24 hr resulted in a reduction in the phosphorylated form of BAD occurs in the presence of wortmannin, as shown by the disappearance of fluorescence localized to the midpiece of the human spermatozoa. Representative images are shown.

5.3.2 Motility and vitality

One of the most obvious and rapid changes to occur following the induction of apoptosis with wortmannin was a highly significant, dose-dependent loss of overall percentage sperm motility ($P < 0.001$) and progressive motility ($P < 0.001$; Fig. 5.4A,B) within 4 hr in the complete absence of any change in sperm vitality (Fig. 5.4C).
**Figure 5.4** Analysis of the impact of wortmannin (20 μM) treatment for 4h on human sperm motility and vitality. CASA analysis revealed highly significant decreases in (A) motility and (B) progressive (**$P < 0.01$, ***$P < 0.001$). The loss of motility was without any significant loss in vitality, eosin staining measured by (C).
5.3.3 Mitochondrial Changes associated with apoptosis

In order to determine whether this loss of motility observed after disruption of the PI3 kinase/AKT/BAD axis with wortmannin, involved an apoptotic cascade in mature human spermatozoa, the ability of this compound to activate mitochondrial ROS generation was investigated. As illustrated in Fig. 5.5A, the addition of wortmannin to human spermatozoa in vitro resulted in a highly significant dose-dependent increase in mitochondrial ROS after 4 hr as measured by MitoSOX Red ($P < 0.001$). Since a loss of $\Delta\Psi$ is also a common signature of apoptotic cells (Saelens et al., 2004) that has been observed to accompany mitochondrial ROS generation (Brookes, 1998), we also examined the impact of wortmannin on this aspect of mitochondrial activity. Use of the fluorescent marker, JC-1, revealed that wortmannin had no effect on $\Delta\Psi$ (Fig. 5.5B) within 4 hr suggesting that the rapid stimulation of mitochondrial ROS was not due to an uncoupling mechanism, nor is the latter an immediate component of this apoptotic pathway in human spermatozoa.
**Figure 5.5** Effect of wortmannin on mitochondria ROS production measured by MitoSOX Red assay (A) Addition of wortmannin resulted in a dose-dependent increase in mitochondrial ROS generation after 4 hr, assessed by the MitoSOX Red assay (**P < 0.01, ***P < 0.001). (B) The presence of wortmannin did not affect the mitochondrial membrane potential gauged by JC-1 fluorescence; CCCP (10 µM) was used as a negative control.
5.3.4 Characterization of Apoptosis Markers

In order to determine whether the suppression of PI3 kinase/AKT resulted in the expression of additional hallmarks of apoptosis other than mitochondrial ROS generation, these cells were also examined for caspase activation and phosphatidylserine exteriorization. Using the fluorescently labeled inhibitor of caspase assay (FLICA), it was demonstrated that the addition of wortmannin caused a highly significant ($P < 0.001$) dose-dependent increase in caspase activation in human spermatozoa (Fig. 5.6A) over the course of a 4 hr incubation. Interestingly, the addition of other activators of apoptosis in somatic cells including staurosporine, lipopolysaccharide, Kdo (Eley et al., 2005) and genestein (McCabe & Orrenius, 1993) were unable to elicit the activation of caspases in human spermatozoa over the same time period (Fig. 5.6B), emphasizing the dramatic differences that exist between the apoptotic pathway(s) expressed in spermatozoa and other cell types. An exception was H$_2$O$_2$ which could induce motility loss, caspase activation and mitochondrial ROS generation in a dose dependent fashion within 2 hr (data not shown).
Figure 5.6 Pan-caspase activation measured by FLICA in presence of wortmannin (20 μM) after 4 hr treatment. (A) The addition of wortmannin resulted in a dose-dependent increase in pan-caspase activation (*P < 0.05, ***P < 0.001). (B) Presence of common apoptotic stimulants including staurosporine, arachidonic acid, lipopolysaccharide, and genestein were unable to induce caspase activation.
Another well-known marker of apoptosis in somatic cells is Annexin-V, a cellular protein that has a strong binding affinity for phosphatidylserine (PS), which becomes expressed on the cell surface during apoptosis. Since Annexin-V will also bind to PS on the interior of non-viable cells with disrupted plasma membranes, it is critical to monitor cell viability with PI in concert with the analysis of Annexin-V binding. With this safeguard in place, it was clearly demonstrated (Fig. 5.7) that exposure to wortmannin resulted in a highly significant dose-dependent increase in PS externalization on the surface of viable human spermatozoa ($P < 0.001$).

![Figure 5.7](image)

**Figure 5.7** Assessment of phosphatidylserine externalisation using annexin-V binding. Significantly higher levels of phosphatidylserine externalisation (annexin-V positive / PI negative) were observed in the presence of increasing doses wortmannin in human spermatozoa (**$P < 0.01$, ***$P < 0.001$).
The appearance of DNA strand breaks is considered one of the hallmarks of an apoptotic cell. Such apoptosis-associated DNA fragmentation is induced by endonucleases which are either released from the mitochondrial inter-membranous space, (AIF or Endo G), or activated in the cytosol (caspase-activated Dnase; CAD), prior to their translocation to the nucleus. The presence of DNA strand breaks in human spermatozoa has been widely observed in defective sperm populations and is known to be induced by a wide variety of stimuli including various forms of electromagnetic radiation and xenobiotics (Aitken et al., 2004b; Banks et al., 2005; Aitken et al., 2005). However, within the time frame of these studies, the addition of wortmannin was unable to induce DNA strand breaks as measured by the TUNEL assay, (Fig. 5.8A) in direct contrast to apoptosis in somatic cells. Investigation of the localization of 3 common nucleases CAD (Fig. 5.8BC), Endo G (Fig. 5.8DE) and AIF (Fig.5.9), revealed these effectors of apoptosis to be present in the midpiece of human spermatozoa, where the mitochondria and a majority of the sperm cytoplasm is located. Stimulation of apoptosis in these highly compartmentalized cells with wortmannin unable to induce the required translocation of these nucleases from the midpiece to the nucleus (Fig. 5.8 and 5.9). Other effectors of the apoptotic cascade such as cytochrome C (Fig. 5.9 B and C) or SMAC/Diablo (Fig. 5.9 E and F) were also confined to the midpiece of the cell before and after activation of the intrinsic apoptotic cascade with wortmannin.
Figure 5.8 Effect of Wortmannin on DNA damage after 24 hr. (A) Measurement of DNA strand breaks via TUNEL assay demonstrated no increase in the presence of wortmannin. Immunocytochemistry reveals the localisation of CAD (B) and Endo G (D) to the midpiece of untreated human spermatozoa. In the presence of wortmannin (20 µM) for 24 hr no translocation of either CAD (C) or Endo G (E) were observed.
Figure 5.9 Localisation of mitochondrial related factors during apoptosis using immunocytochemistry. In untreated human spermatozoa (A) AIF (B) cytochrome c and (C) SMAC / Diablo are all localised to the midpiece of human spermatozoa. Addition of wortmannin (20 µM) for 24 hr, has no effect of the localisation as shown in B (AIF), D (cytochrome c), F (SMAC/Diablo).
Numerous recent studies have provided evidence highlighting the clinical importance of oxidative stress in the etiology of DNA damage in human spermatozoa (Kodama et al., 1997; De Iuliis et al., 2009b). So, even though the impeded migration of nucleases from the sperm midpiece to the nucleus precluded endonuclease-mediated DNA cleavage during apoptosis, the activation of mitochondrial ROS could have induced oxidative DNA damage that might, in time, generate the expected strand breaks. To examine this possibility we analyzed the formation of 8OHdG, a marker of oxidative DNA damage, following wortmannin exposure. In this study, wortmannin was shown to induce a highly significant ($P < 0.001$) dose-dependent increase in the prevalence of oxidative DNA damage in human spermatozoa after 24 hr incubation (Fig. 5.10).

**Figure 5.10** Effect of wortmannin on oxidative DNA damage after 24 hr. Measurement of 8OHdG formation using a FITC-conjugated antibody, demonstrated a dose dependent increase in the presence of wortmannin ($*P < 0.05$, $***P < 0.001$).
Figure 5.11 Electron microscopy of human spermatozoa treated with wortmannin. (A) Untreated, morphologically normal spermatozoa. (B-C) Treatment with wortmannin (20 \( \mu \text{M} \)) for 24 hr resulted in major morphologically changes including membrane expansion and vacuole formation, as indicated by arrows.
Finally we examined whether the induction of this apoptotic cascade was associated with ultrastructural changes similar to those seen in somatic cells following the induction of this process. Ultrastructural analysis of human spermatozoa under the same conditions that resulted in both caspase activation and PS externalization, revealed several morphological changes compared to control cells (Fig. 5.11). Specifically, activation of the intrinsic apoptotic cascade with wortmannin, resulted in the appearance of large vacuoles and membrane protrusions in the cytoplasm-rich sperm midpiece, although no major changes were recorded in the cytoplasm-poor sperm head or flagellum.
6.4 Discussion

Whilst PI3-kinase/AKT has previously been reported to play a role in sperm motility (Ashizawa et al., 2009) and capacitation (Nauc et al., 2004; O’Flaherty et al., 2006), this is the first study to fully investigate the role of this pathway in the modulation of apoptosis in human spermatozoa. Wortmannin successfully reduced PI3-kinase phosphorylation and thereby disrupted the ability of this kinase to phosphorylate its own downstream target, AKT, on T308 (Fig. 5.2). The subsequent change in AKT activity resulted in BAD altering from a phosphorylated (anti-apoptotic) state to a non-phosphorylated (pro-apoptotic) state (Fig. 5.3). This change in the phosphorylation status of BAD is known to allow Bak/Bax to form pro-apoptotic pores in the outer-mitochondrial membrane (Yang et al., 1995) and to promote its ability to form mitochondrial pores in its own right (Zha et al., 1996; Polzien et al., 2009). Downstream of BAD dephosphorylation we then saw the induction of several changes that were consistent with the intrinsic apoptotic cascade observed in somatic cells including mitochondrial ROS formation, caspase activation and PS exteriorization and cytoplasmic vacuolization (Morey et al., 1993; Lee et al., 1994; Luo and Loison, 2008).

Thus this study clearly indicates that mature functional human spermatozoa can undergo an intrinsic apoptotic cascade. Indeed the latter appears to be a default pathway for these cells. As soon as conditions arise that compromise the phosphorylation status of the PI3-kinase/AKT complex then these cells enter an intrinsic apoptotic pathway associated with the dephosphorylation of BAD and the induction of mitochondrial permeability. Interestingly, most of the stimuli that induce apoptosis in somatic cells are completely without effect in human spermatozoa including staurosporine, lipopolysaccharide, Kdo and genestein. As far as we are aware there are no convincing reports of receptor activated apoptosis in human spermatozoa. The only possible exception could be the apoptosis-inducing properties of progesterone (Lozano et al., 2009); however this steroid probably exerts its pro-apoptotic
effect by mobilizing calcium which then, secondarily, induces ROS formation and oxidative stress (Aitken and Clarkson, 1987; Aitken et al., 1996).

Since we can find no evidence that apoptosis is actively induced via a receptor mediated mechanism in mature human spermatozoa, we propose that these cells revert to this pathway in response to stress, as a form of programmed senescence. Although the range of apoptotic responses that spermatozoa can express is limited by their physical architecture, two responses appear to be critical. The first is the expression of annexin V binding as a consequence of the exteriorization of PS (Fig. 5.7). The significance of this change may be found following insemination when the female reproductive tract contains millions of moribund and senescent spermatozoa that must be phagocytosed by infiltrating leukocytes. Accordingly, there is a massive leukocytic infiltration into the lower female reproductive tract post coitum (Thompson et al., 1992). The phagocytic activity exhibited by these cells must be silent; in other words, the spermatozoa must be efficiently phagocytosed and removed but this activity must not be accompanied by an oxidative burst or the production of pro-inflammatory cytokines (D’Cruz et al., 1992). There are many examples of silent phagocytosis in biology and a common feature of this phenomenon is the expression of apoptotic markers, such as PS, on the surface of the phagocytosed cell. This apoptotic marker is thought to instruct the phagocyte that the target cell should be engulfed in a non-phlogistic manner (Kurosaka et al., 2003). We therefore propose that the activation of this apoptotic cascade in senescent cells is an adaptation that permits the efficient removal of spermatozoa from the female tract by phagocytic leukocytes without provoking an inflammatory response.

In parallel with the surface expression of Annexin V binding sites, apoptotic spermatozoa also exhibit signs of caspase activation (Fig. 5.6) and a loss of motility (Fig. 5.4). Whether there is a causal relationship between caspase activation and motility loss has not yet been established, however these two parameters are highly correlated (Marchetti et al., 2004). Moreover isolation of apoptotic spermatozoa using magnetic beads coated with annexin V, revealed a clear association between annexin V binding, caspase activation and impaired
motility (Said et al., 2006). The motility loss exhibited as part of this apoptotic cascade could again be an adaptive response to ensure that moribund, oxidatively stressed spermatozoa with possible DNA damage, cannot participate in the fertilization process.

In most cell types the activation of the intrinsic apoptotic cascade culminates in extensive DNA damage and cell death. It is at this point in the apoptotic pathway that the highly specialized anatomy of human spermatozoa starts to play a role in limiting the apoptotic phenotype. While in other cell types apoptosis is associated with the movement of endonucleases (AIF, CAD, Endo G) into the nucleus, this does not occur in spermatozoa. Following the induction of apoptosis these proteins, as well as cytochrome C and SMAC/Diablo remain resolutely locked in the midpiece region of the cell (Figs. 5.8 and 5.9). Translocation of the nucleases to the sperm nucleus is presumably impeded by the highly compacted nature of sperm chromatin and the highly compartmentalized architecture of mature spermatozoa, which separates the nucleus in the sperm head from the cytoplasm and mitochondria in the midpiece. The immediate, practical consequence of endonuclease exclusion from the sperm nucleus is that these apoptotic cells do not rapidly exhibit high levels of DNA fragmentation, as measured in the TUNEL assay (Fig. 5.8). However the concomitant generation of mitochondrial ROS does result in the induction of oxidative DNA damage as reflected by 8OHdG formation (Fig. 5.10). Since the spontaneous formation of 8OHdG in human spermatozoa is highly correlated with DNA damage as measured with the TUNEL assay (De Iuliis et al., 2009b), it seems probable that these two events are related and sequential. Thus the formation of oxidative base adducts will affect DNA integrity by labilizing the glycosyl bond that attaches the base to the ribose unit, leading to loss of the affected base and the generation of an abasic site. Abasic sites have a strong destabilising effect on the DNA backbone, which can then result in strand breaks (Wiseman & Halliwell, 1996). We conclude that, ultimately, apoptosis in human spermatozoa does result in DNA fragmentation but it is a prolonged process driven by oxidative stress rather than endonuclease activity.
A similar explanation may apply to the changes in mitochondrial activity observed during apoptosis. When this process is triggered by wortmannin, the mitochondria instantly generate ROS but there is no immediate loss of ΔΨ. Formation of a mitochondrial pore without loss of ΔΨ, although uncommon, has been previously described (Kluck et al., 1997; Yang et al., 1997). The dephosphorylation of BAD leads to a loss of cytochrome c from the mitochondria which then (i) activate caspases by participating in apoptosome formation and (ii) alter the activity of the mitochondrial electron transport chain from the normal 4 electron reduction of $O_2$ to a one electron process that produces superoxide anion rather than water (Cai and Jones, 1998). Reverse electron flow to complex 1 appears to be responsible for the superoxide generation observed under these circumstances (Kushnareva et al., 2002). This is significant because we have previously demonstrated that electron leakage from this site is particularly damaging to spermatozoa, prompting extensive lipid peroxidation and progressive motility loss (Chapter 3). The fact that ΔΨ remains unchanged during the induction of mitochondrial ROS generation indicates that depolarization is not a prerequisite for superoxide production. However the observation that ΔΨ is lost in populations of defective human spermatozoa generating ROS (Espinoza et al., 2009) suggests that eventually, the peroxidative damage sustained by the sperm mitochondria will lead to depolarization and an irreversible loss of mitochondrial function.

In summary, this report provides definitive evidence that mature human spermatozoa can be induced to undergo a limited form of apoptosis characterized by mitochondrial ROS generation, PS externalization, caspase activation, motility loss, cytoplasmic vacuole formation and oxidative DNA damage. The physical architecture of the spermatozoon subsequently prevents endonucleases released from the mitochondria or activated in the cytosol, from translocating to the nucleus. As a consequence, DNA fragmentation, one of the hallmarks of apoptosis in somatic cells, cannot occur immediately but may be precipitated secondarily as a result of oxidative DNA adduct formation. Entry into this apoptotic pathway is actively prevented by the phosphorylation of PI3kinase and AKT which, in turn, maintains BAD in a phosphorylated, anti-apoptotic state. Conversely, dephosphorylation of the PI3 kinase/AKT axis leads to the activation of apoptosis. We
propose that this is an adaptive physiological response to cell senescence associated with oxidative stress, rather than a receptor–mediated event. The purpose of this apoptotic response to oxidative stress may be to facilitate the silent phagocytosis of senescent, moribund spermatozoa following insemination and to prevent oxidatively-damaged spermatozoa from participating in the fertilization process. In light of these findings, we would expect that growth factors/cytokines that stimulate PI3 kinase/AKT would have a powerful pro-survival effect on human spermatozoa while the selection of non-apoptotic cells would be an effective means of removing senescent, DNA-damaged spermatozoa from sperm populations intended for use in assisted conception therapy (Grunewald et al., 2001, 2006; Lewis & Aitken, 2005; Dirican et al., 2008; Zini et al, 2008; Hoodendijk et al., 2009).
Chapter 6:

Final Discussion and Conclusions
Chapter 6: Final Discussion and Conclusions

6.1 Final Discussion and Future Directions

6.1.1 Mitochondrial Targeted Antioxidant Therapy

The research presented in this thesis was aimed at elucidating the sources and causes of increased ROS generation and oxidative stress in human spermatozoa. The results presented in chapter 3 signified for the first time the important role that the mitochondria play in this process. It was shown that mitochondrial ROS generation is negatively correlated with motility. Induced mitochondrial ROS resulted in increased levels of lipid peroxidation resulting in a motility loss that could be prevented by the presence of α-tocopherol.

Clinically, the importance of oxidative stress mediated male infertility has given rise to the use of antioxidant therapy. While a number of research studies have focused on a single antioxidant’s effects (as discussed in Chapter 1), current commercial treatments such as Menevit use a broad range of anti-oxidant compounds in order to address oxidative stress related male infertility. In light of these results it would seem beneficial to develop antioxidant trials and therapies that specifically target mitochondria. From this perspective, the most logical candidate is coenzyme Q10 (CoQ10), a well-known mitochondrial antioxidant component.

Although CoQ10 has a number of physiological roles including redox carrier, activator of uncoupling proteins and in mitochondrial pore formation (Bentinger et al., 2007); CoQ10 in its reduced form (CoQH₂) is a powerful antioxidant through its ability to inhibit destructive lipid peroxidation chains. The effectiveness of CoQH₂ as an inhibitor of lipid peroxidation is through its ability to break the complex chain reactions produced during lipid peroxidation cascades. In this process, CoQ10 prevents the formation of lipid peroxyl...
radicals (LOO•) production during initiation phase of lipid peroxidation (Fig. 6.1). The reduced form of CoQ10 reduces the initiating perferryl radical via the formation of a semiquinone and H2O2. Alternatively, CoQH2 can eliminate LOO• directly. This is achieved again via the formation of a semiquinone, the same mechanism by which α-tocopherol prevents lipid peroxidation (Mukai et al., 1990).

**Figure 6.1** Coenzyme Q10 and α-tocopherol inhibition of lipid peroxidation. LH, polyunsaturated FA; L•, carbon-centred radical; LOO•, lipid peroxyl radical; LOOH, lipid hydroperoxide; CoQ10, coenzyme Q10; CoQH2, reduced coenzyme Q10; CoQH•-, Coenzyme ubisemiquinone.

There are a number of pathologies where oxidative stress is a well-documented contributing factor that also exhibit increases in the synthesis of CoQ10 including Alzheimer’s, prion, other neurodegenerative disease and diabetes, (Turunen et al., 2004). During both ageing and in heart disease there is a significant lowering of CoQ10 content in the target organ.
(Littarru et al., 1996) creating a state of vulnerability to oxidative stress. Since mitochondrial ROS generation is a continual and unavoidable by-product of cellular respiration, conditions associated with a decreased availability of CoQ10 in the male reproductive tract may well be associated with oxidative stress. Given the preliminary evidence for CoQ10 as a major antioxidant in the male reproductive tract (Mancini et al., 1994; 1998; 2005) further studies should be undertaken to examine the potential inclusion of this co-enzyme as a candidate for the antioxidant therapy of male infertility such as the in vivo study by Balercia et al (2009) that showed increased sperm motility in infertile men with asthenozoospermia treated with coenzyme CoQ10 for 6 months.

However, while it is an effective and a viable option for those undergoing ART, antioxidant therapy is an approach that only addresses the symptoms of this condition; it does not go to the underlying causative mechanisms.

6.1.2 Relationship Between High FA Content and Poor Spermiogenesis

The results described in Chapter 4 established a causal link between the levels of unsaturated fatty acids in human spermatozoa, mitochondrial ROS generation and its adverse effect on function. While mechanisms for increasing the cellular FAs content of spermatozoa were not investigated in this study; the data shown in this thesis provide greater understanding of possible causes. An animal model study comparing a diet high in unsaturated FAs compared to a control might yield evidence for an effect on male fertility. On the other hand, almost every FA increased in concentration when high and low density human spermatozoa were compared (for both total and free FA), very little to no change was observed in the overall FA profile (% wt/wt). This gives an indication of a fundamental developmental error in contrast to a specific or acute increase in the uptake of subgroup of FAs.

Recent evidence suggests that systemic deficiencies in lipid metabolism seem an unlikely cause given the apparent discrepancy between the FA profiles of blood serum and
spermatozoa (Conquer et al., 1999). Alternatively, the high FA content of defective spermatozoa is likely reflect a fundamental error in the remodelling of human sperm cells during spermiogenesis, which would be associated with the increased retention of residual cytoplasm and an enhanced cytoplasmic volume. Several other features of these cells also suggest defective differentiation during spermiogenesis including the presence of defective protamination, persistent nucleohistones, poor zona binding potential, disrupted chaperone content and a tendency to default to an apoptotic state (Aitken & De Iuliis, 2009).

Other studies have demonstrated that spermatogenesis is highly dependent on testosterone levels (Sharpe 1987, 1988); as a consequence of low testosterone levels spermiogenesis is not completed properly, culminating in spermatozoa exhibiting increased cytoplasmic volume, a characteristic of the low density spermatozoa used in this study. Thus it is possible that the disruption of normal testosterone production might impair spermatogenesis resulting in the production of immature spermatozoa carrying excess residual cytoplasm as a consequence of which cellular FA content is increased, mitochondrial electron transport is impaired and a state of oxidative stress is induced. This potential chain of cause and effect will need to be investigated in future studies. Clearly one of the issues that would be have to be resolved is whether intratesticular testosterone is ever decreased to the point that the androgen receptors in Sertoli and peritubular myoid cells are not fully saturated.

6.1.3 Importance of Apoptosis Related Research

The clinical importance of apoptosis on human sperm function was not the aim of the study described in chapter 5. This has been previously shown by Grunewald et al., 2001, 2006; Zheng et al., 2008; Hoodendijk et al., 2009. On the other hand, the data presented in this thesis provides greater understanding of the pathways involved, as well as introducing the concept that mitochondrial ROS must be taken into consideration and it can be assumed that other apoptotic events such as PS externalization will occur, therefore causing phagocytosis. The area of relevance lies with assisted reproductive technology and
infertility clinics. Whilst the use of magnetic activated cell sorting (MACS) to eliminate apoptotic spermatozoa has highlighted the benefits in ART with higher cleavage and pregnancy rates observed in one trial (Dirican et al., 2008) it is not currently a routine clinical technique. The significance of this is shown by figure 5.10, indicating high levels of oxidative DNA damage. As mitochondrial ROS has been shown to reduce sperm function (chapter 3) it may in turn lead to IVF use, but the generation of DNA damage within the same populations of spermatozoa has further implications for the health and well being of the immediate offspring as well as of future generations.

As shown throughout this thesis, the generation of mitochondrial ROS will subsequently lead to the formation of DNA oxidative damage. These observations are of great clinical significance because DNA damage in human spermatozoa used during IVF and ICSI has been associated with a wide range of adverse clinical outcomes including impaired fertilization, disrupted preimplantation development of the embryo, high rates of miscarriage and an increased incidence of morbidity in the progeny (Lewis & Aitken, 2005; Zini et al., 2008). The study by Morris et al (2002) found significantly lower rates of cleavage, fertilisation and embryo cell number in patients undergoing IVF/ICSI who had higher levels of DNA damage in the spermatozoa.

Furthermore, in vitro mouse studies have shown that the use of spermatozoa containing higher levels of DNA damage for ICSI have reported reduced fertility, as measured by fertilisation and pregnancy rates (Fernandez-Gonzalez et al., 2008). Of greater concern however is the data indicating that when offspring originating from highly DNA-damaged spermatozoa are born, there are 20% higher mortality rates within the first 5 months, premature ageing and a 70% death rate due to cancer growth. Such results place even greater emphasis on the need to further our understanding of the causes of DNA damage in human spermatozoa.
6.1.4 Other Sources of Mitochondrial ROS generation

While this thesis has covered two causes of mitochondrial ROS generation and DNA damage in human spermatozoa, unsaturated fatty acids and apoptosis, the literature suggests that there are many other potential causes including paternal age and smoking.

The consequences of paternal age on human fertility have come to the fore due to changes in human reproductive patterns, a combination of social changes, prolonged life expectancy and a reliance on ART (Heck et al., 1997). The association between increased paternal age and decreased sperm parameters has been well documented. Jung et al (2002) reported a 27% decrease in progressive motility between younger men (21-25 yrs) and their subjects (>50 yrs). A similar trend was also observed analysing the total sperm count, semen volume and sperm concentration, which all showed approximately a 25% decline between the age groups of 30-35 years and >55 years of age (Levitas et al., 2007). Furthermore recent studies have also shown that ROS production and oxidative stress are increased in human spermatozoa during ageing, suggesting a possible role for the decline in fertility (Cocuzza et al., 2008).

Although the source of ROS in the study by Cocuzza et al (2008) was not investigated, other evidence related to ageing suggests that it may be of mitochondrial origin. A large body of evidence exists implicating the continual generation of ROS by the mitochondria, as it converts 2-3% of all oxygen consumed into superoxide anion, in the aetiology subsequent mitochondrial DNA (mtDNA) damage as well as damage to other macromolecules (James & Murphy, 2002). As well as, mtDNA mutations, oxidative DNA damage and lipid peroxidation, both markers of, or contributors to reduced fertility, are shown to be increased in a variety of tissues during ageing (Lee et al., 1999; Wei et al., 1996; Lu et al., 1999).
While the significance of DNA damage in human spermatozoa has been previously discussed; numerous studies have also investigated the effect of paternal age on sperm DNA damage. Using the sperm chromatin structure assay, one study found a relationship between DNA fragmentation and age (Wyrobeck et al., 2006). This was further confirmed by Singh et al (2003) who found the same association measuring DNA strand breaks using the comet assay. Although not measured in these studies, ROS generation was likely to be a significant contributor to the DNA damage found in older men. Although DNA damage may reduce overall rates of cleavage and pregnancy (Morris et al., 2002), it does not completely exclude the possibility of fathering a child. Furthermore, while the role of the oocyte in repairing DNA is essential, it cannot be assumed to be 100% efficient. In fact, it has been shown that oxidative DNA damage can lead to various genomic defects (Bosco et al., 2005), and therefore increased chance morbidity and mortality in the offspring.

The carcinogenic and toxic effects of cigarette smoke on the health of individuals are well-documented. Smoking has also been shown to result in an increased likelihood of male infertility, with significantly higher levels of seminal ROS generation (Saleh et al. 2002). Previous research has shown a significant elevation in the activity of MnSOD (mitochondrial isozyme) in cigarette smokers (St Clair et al. 1994), indicating that cigarette smoking may cause increased mitochondrial ROS generation, rather than activate a cytosolic or plasma membrane oxidase system. In smokers, the overall ETC function is significantly decreased which also correlates with increased peroxidative damage to lymphocyte membranes (Miro et al., 1999).

An extremely large Danish study in 2007 selected 2542 healthy men, without bias towards reproductive history (Ramlau-Hansen et al., 2007). The investigation discovered that compared with non-smokers, smokers exhibited significantly decreased sperm concentration, semen volume, motility and total sperm count. Although the changes observed were only minor (20-30% decrease), such effects may further compound an already-existing minor reproductive condition or combine with other xenobiotics to further
reduce fertility. Overall, the smokers presented in the study exhibited increased seminal ROS production and decreased antioxidant levels.

A number of independent studies have also shown that smoking results in higher levels DNA damage in the human spermatozoa in comparison to non-smokers (Fraga et al. 1996; Sepaniak et al., 2006; Viloria et al., 2006). Also, a higher rate of smoking observed in men is associated with corresponding increases in the rate of aneuploidy in spermatozoa (Rubes et al., 1998; Shi et al., 2001).

Overall, the results on smoking in men provide further evidence for the role that oxidative stress and DNA damage play in male infertility. Although only suggestive evidence exists, these data also provide a rationale for further investigation into the role of mitochondria in smoking related oxidative stress in the male germ line.
6.1.5 Model for Mitochondrial ROS, Oxidative Stress and Male Infertility

**Figure 6.2** Model of the sources and effects of mitochondrial ROS generation in human spermatozoa.

In summary, the data presented in this thesis and subsequent discussion has developed a model (Figure 6.2) for understanding the detrimental cellular effects of mitochondrial ROS generation as well as possible causes and greater implications. According to this model a single or multiple stimulants may lead to mitochondrial ROS generation, consequently this leads to lipid peroxidation and motility loss in spermatozoa and therefore a reduction in fertility. However, the resultant increase in oxidative DNA damage can also lead to higher rates of disease in the offspring following ART.
6.2 Concluding Remarks

The studies presented in this thesis have for the first time highlighted the significance of the generation of mitochondrial ROS in defective human spermatozoa. Furthermore, they have emphasised its effects on lipid peroxidation, sperm motility and DNA damage.

These data also revealed that higher intracellular concentrations of unsaturated fatty acids and the stimulation of apoptosis are both mechanisms of mitochondrial ROS generation which can result in increased lipid peroxidation, loss of sperm motility and oxidative DNA damage. With this information future studies can investigate causes of higher levels of cellular fatty acids and stimulation or inhibition of apoptosis in human spermatozoa. In turn this will aid in the diagnosis, prevention and treatment of male infertility therefore possibly reduce the current reliance on ART and reducing risk of childhood diseases and cancer.
References


---

**References**


Fraga CG, Motchnik PA, Wyrobek AJ, Rempel DM, Ames BN. Smoking and low antioxidant levels increase oxidative damage to sperm DNA. *Mutat Res.* 351: 199-203.


References


Page DC, Silber S, Brown LG. 1999. Men with infertility caused by AZFc deletion can produce sons by intracytoplasmic sperm injection, but are likely to transmit the deletion and infertility. *Hum Reprod.* 14: 1722-6.


References


Appendix
# Appendix

## A. Buffers and Solutions

<table>
<thead>
<tr>
<th>Solution</th>
<th>Constituents</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>BWW Stock</strong></td>
<td>915 mM NaCl</td>
</tr>
<tr>
<td></td>
<td>4.6 mM KCl</td>
</tr>
<tr>
<td></td>
<td>1.2 mM KH$_2$PO$_4$</td>
</tr>
<tr>
<td></td>
<td>1.2 mM MgSO$_4$.7H$_2$O</td>
</tr>
<tr>
<td></td>
<td>1.7 mM CaCl$_2$.2H$_2$O</td>
</tr>
<tr>
<td><strong>BWW (Working Solution)</strong></td>
<td>915 mM NaHCO$_3$</td>
</tr>
<tr>
<td></td>
<td>(omitted for –HCO$_3^-$ media)</td>
</tr>
<tr>
<td></td>
<td>25 mM D-glucose</td>
</tr>
<tr>
<td></td>
<td>5.6 mM Sodium Pyruvate</td>
</tr>
<tr>
<td></td>
<td>0.27 mM Sodium Lactate</td>
</tr>
<tr>
<td></td>
<td>5 U/ml Penicillin</td>
</tr>
<tr>
<td></td>
<td>5 µg/ml Streptomycin</td>
</tr>
<tr>
<td></td>
<td>20 mM HEPES buffer</td>
</tr>
<tr>
<td></td>
<td>1mg/ml PVA</td>
</tr>
<tr>
<td><strong>Eosin</strong></td>
<td>0.1 g Eosin</td>
</tr>
<tr>
<td></td>
<td>10 ml dH$_2$O</td>
</tr>
<tr>
<td><strong>Percoll 90%</strong></td>
<td>10ml 10xHAMS solution</td>
</tr>
<tr>
<td></td>
<td>90ml Percoll</td>
</tr>
<tr>
<td></td>
<td>1 mg/ml PVA</td>
</tr>
<tr>
<td></td>
<td>0.27mM Sodium Pyruvate</td>
</tr>
<tr>
<td></td>
<td>25 mM NaHCO$_3$</td>
</tr>
<tr>
<td></td>
<td>44 mM Sodium Lactate</td>
</tr>
<tr>
<td><strong>Percoll 45 %</strong></td>
<td>90% Percoll diluted with equal amount of BWW.</td>
</tr>
<tr>
<td><strong>Phosphate Buffered Saline (PBS)</strong></td>
<td>PBS tablets dissolved in Milli-Q according to manufacturer’s instructions (SigmaAldrich)</td>
</tr>
<tr>
<td><strong>Sperm Diluting Fluid</strong></td>
<td>0.6M Sodium hydrogen carbonate</td>
</tr>
<tr>
<td></td>
<td>1% 37% Formaldehyde</td>
</tr>
<tr>
<td>Buffer Type</td>
<td>Composition</td>
</tr>
<tr>
<td>------------------------------------------------</td>
<td>-----------------------------------------------------------------------------</td>
</tr>
<tr>
<td>SDS Extraction Buffer</td>
<td>0.2% w/v SDS, 50% v/v 0.375M Tris, 10% w/v Sucrose, 1 per 10ml Protease Inhibitor Tablet</td>
</tr>
<tr>
<td>SDS-PAGE Loading Buffer</td>
<td>0.2% w/v SDS, 50% v/v 0.375M Tris, 10% w/v Sucrose, 4% v/v 2β-mercaptoethanol, 0.001% Bromophenol Blue</td>
</tr>
<tr>
<td>SDS-PAGE Running Buffer (5x)</td>
<td>1 mM Tris Base, 1M Glycine, 0.1% SDS</td>
</tr>
<tr>
<td>5% Polyacrylamide Stacking Gel</td>
<td>0.6125 ml 40% Acrylamide, 1.5 ml 0.375 M Tris (pH 6.8), 0.05 ml 10 % SDS, 2.7575 ml Milli-Q water, 5 μl NNN-tetra-methylethylenediamine, 0.05 ml 10% AMPS</td>
</tr>
<tr>
<td>10% Polyacrylamide Resolving Gel</td>
<td>1.25 ml 40% Acrylamide, 1.5 ml 0.375 M Tris (pH 6.8), 0.05 ml 10 % SDS, 2.145 ml Milli-Q water, 5 μl NNN-tetra-methylethylenediamine, 0.05 ml 10% AMPS</td>
</tr>
<tr>
<td>Tris Buffered Saline (TBS)</td>
<td>0.1 M Tris, 0.15 M NaCl</td>
</tr>
<tr>
<td>TBST</td>
<td>TBS supplemented with 0.01% Tween-20</td>
</tr>
<tr>
<td>Western Blot Transfer Buffer</td>
<td>0.15 M Tris Base, 0.9 M Glycine, 15% Methanol</td>
</tr>
</tbody>
</table>
B. Primary and Secondary Antibodies

All the antibodies used in this study were all from Abcam (Cambridge, UK) with the exception of the non-phospho AKT antibody which was purchased from Genesearch (QLD, Australia). The final concentrations at which the various antibodies were used were as follows:

**Primary Antibodies (Western Blot)**

- Rabbit anti-phospho PI3 kinase targeting Y467+Y199: 1:500
- Rabbit anti-PI3 kinase: 1:100
- Rabbit anti-phospho AKT targeting T308: 1:500
- Rabbit anti-AKT: 1:100

**Primary Antibodies (Immunocytochemistry)**

- Rabbit anti-AIF: 1:500
- Rabbit anti-cytochrome C: 1:50
- Rabbit anti-SMAC/Diablo: 1:50
- Rabbit anti-Endo G: 1:50
- Rabbit anti-phospho BAD targeting S99: 1:50

**Secondary Antibodies (Western Blot)**

- Goat anti-rabbit IgG: 1:1000

**Secondary Antibodies (Immunocytochemistry)**

- Goat anti-rabbit IgG: 1:100


C. List of Publications

