The Effect of Paternal Obesity on Sperm Function, Embryo Development and Subsequent Pregnancy Outcomes

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Thesis abstract

Obesity and its health consequences are an increasing health burden for Australian society, with more than seven million adults in Australia being overweight or obese. According to the Burden of Disease and Injury in Australia (BoD) study, high body mass was responsible for 7.5% of the total burden of disease and injury and is increasing. It is now clear that maternal obesity reduces fertility, in part through actions on the egg, which affect the health of the resultant pregnancy. However, the potential role of male obesity in infertility has been essentially ignored. This is surprising, as the male gamete contributes half of the genetic material of the embryo. Furthermore, sperm function constitutes the single most common cause of infertility. Therefore, factors influencing the health of the sperm and the underlying mechanisms behind any pathology are paramount.

The overall aim of this thesis was to determine the relationship between paternal obesity and male fertility. A novel and unique animal model was developed to elucidate the effects of paternal obesity on embryo development and pregnancy. Results showed that paternal obesity has negative effects on both sperm quality as well as embryo development and quality.

To confirm the results seen in the mouse, the pregnancy and live birth outcomes of a large cohort of patients undergoing assisted reproductive technology were assessed in relation to male obesity. Confirming the results observed in the mouse, it was clearly demonstrated that both sperm function parameters as well as embryo development were significantly reduced where the male partner is obese. This observation was evident after controlling for important factors such as maternal and paternal age as well as maternal body mass index (BMI). Furthermore, clinical pregnancy rates were reduced, miscarriage rates increased and live birth rates were reduced.

Moreover, analysis revealed that as paternal BMI increased, both seminal plasma glucose as well as insulin levels also increased, implicating the role of glucose, in particular, as detrimental to sperm function.

To further elucidate the metabolic markers involved, additional in-vitro studies were conducted whereby factors such as elevated glucose were shown to negatively affect sperm quality in vitro with elevated reactive oxygen species levels and DNA damage observed. These results are consistent with the effects seen on obese men and mice.

In summary, the current studies have shed some light on some longstanding questions regarding the effects of paternal obesity on fertility. Additional questions have been raised as to further understanding the mechanisms behind these effects (and their potential reversibility). This thesis has shed some much-needed light on a major knowledge gap in men's heath and will undoubtedly stimulate further interest in this very important area of applied science and medicine.

Declaration

This work contains no material that 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.

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Hassan W. Bakos

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1 Literature review

1.1 Introduction

Obesity and its health consequences are an increasing health burden in Australian society, with more than 7 million adults in Australia being overweight or obese. According to the Burden of Disease and Injury in Australia (BoD) study, high body mass was responsible for 7.5% of the total burden of disease and injury and is on the increase (Begg, *et al* 2008). Obesity is a condition in which excess body fat is accumulated. It has been associated with many other conditions such as diabetes, cardiovascular disease and certain types of cancer (Proietto and Baur 2004, Visscher and Seidell 2001). Obesity and its associated co-morbidities present significant health concerns throughout the world, and, indeed, obesity is increasingly prevalent in the population of people trying to conceive. In Australia, 62% of the population aged 18 years and over are obese or overweight (National Health Survey 2007–2008), a figure reflective of many countries, including the United States of America (Health, United States 2008) and England (Health Survey for England 2007). More specifically, the prevalence of obesity in young reproductive age men has tripled since the early 1970s (National Center for Health Statistics 2008).

Further, the incidence of male infertility (at least in some geographical areas) is on the rise coincident with a worldwide reduction in sperm quality, which to date is unexplained (Andersson, *et al* 2008) and which appears to be suggestive for humans (Setchell 1997). With an increase in the prevalence of obesity it is necessary to broaden our understanding of the clinical significance of a male partner being overweight or obese. In general, male health is an under-researched area compared with female health, and there are broad knowledge gaps in our understanding of how the health of the male influences the health of the sperm and subsequent pregnancy outcomes.

Several examples of how paternal health around the time of conception affects the health of the offspring are now available. The best characterised is the effects of paternal smoking, which

has been associated with an increased prevalence of asthma, respiratory tract infection, diabetes and obesity in offspring (Chang 2009, Sorahan*, et al* 1997, Toschke*, et al* 2007). Furthermore, a known causal association exists between paternal age and significant medical conditions in the offspring, such as autism (Sartorius and Nieschlag 2010). Further evidence that male health and molecular function of sperm are important to offspring health is the association between paternal occupation where there are exposures to organic solvents during preconception and increases in infant mortality and deaths due to congenital malformation (Lin*, et al* 2008, Sung*, et al* 2009). Research also shows an association with increased paternal body mass index (BMI) around the time of conception with an increase in the BMI of the offspring (Danielzik*, et al* 2002, Li*, et al* 2009). However, while it is clear that there are long-term effects of paternal health around the time of conception on offspring health, there is little information from direct studies on the mechanism involved in paternal obesity at either the sperm or embryo level.

It is now clear that maternal obesity reduces fertility, through actions on the oocyte, which affects the health of the resultant pregnancy (Sorahan*, et al* 1997). Studies have also shown that overweight women are more likely to have reduced fertility (Bolumar*, et al* 2000, Gesink Law*, et al* 2007, Pasquali*, et al* 2006, Rich-Edwards*, et al* 2002). In contrast, research has largely ignored the relationship between obesity and male infertility. This is surprising, as the male gamete contributes to half of the genetic material of the embryo and now more than 50% of reproductive age males are overweight (Begg*, et al* 2008). The increasing incidence of obesity in this population of people is a cause for concern. Given the extensive literature highlighting the increased infertility with elevated maternal BMI, evidence is now required to elucidate the impact of male obesity on fertility.

1.2   Hormonal regulation of spermatogenesis

The production of androgens and spermatozoa are interdependent. Therefore at puberty, androgen levels increase and spermatogenesis is initiated.

In a normal adult male, the hypothalamus secretes gonadotropin-releasing hormone (GnRH) in a pulsatile manner. GnRH interacts with cell surface receptors on the plasma membrane of pituitary gonadotrophs stimulating the release of luteinising hormone (LH) and follicle-stimulating hormone (FSH) (Figure 1.1). LH binds to the LH receptor on the plasma membrane of Leydig cells in the testes resulting in the synthesis of the enzymes of testosterone biosynthesis (from acetate and cholesterol). LH secretion is under negative feedback control by gonadal steroids, both testosterone and oestradiol, at the level of the hypothalamus with the androgens effecting a slowing of the hypothalamic pulse generator and consequently a decrease in the frequency of the LH pulsatile release (Griffin 2003) (Figure 1.1).

FSH acts to control spermatogenesis following its binding to the FSH receptor of the plasma membrane of sertoli cells. FSH secretion is modulated by activin and inhibin. Activin binds to activin receptor type II [ACT RII] in the gonadotroph cells in the pituitary and stimulates the secretion of FSH. Increases in FSH secretion are also thought to be via activin stimulation of GnRH in the hypothalamus (Kumanov, *et al* 2005). Inhibin B is mostly produced in the sertoli cells, and it shows a diurnal rhythm parallel to that of testosterone. FSH stimulates inhibin's production and, in turn, inhibits the secretion of FSH via a negative feedback mechanism, reviewed by (Mah and Wittert 2010). Inhibin has also been shown to bind to ACT RII, reducing activin binding to the receptor and therefore activin's stimulation of FSH secretion (de Kretser et al. 2004; Meachem et al. 2001). FSH levels increase with the loss of germinal elements in the testis (Griffin 2003) and FSH has been used as a marker of spermatogenesis. Inhibin B used in

combination with FSH is a more sensitive marker of spermatogenesis when correlated with testicular volume and sperm counts (Mah and Wittert 2010).

NOTE:
This figure is included on page 5
of the print copy of the thesis held in
the University of Adelaide Library.

Figure 1.1: Hormonal regulation of spermatogenesis: Gonadotropin-releasing hormone, which is secreted by the hypothalamus, controls the secretion of the hormones produced by the pituitary (FSH and LH). FSH is secreted by the pituitary which stimulates sertoli cells. LH is also produced by the pituitary and acts on leydig cells to produce testosterone. Testosterone, together with FSH, acts on sertoli cells to develop sperm. Image courtesy of Andrology Australia.

1.3   Stages of spermatogenesis

Spermatogenesis is a highly specialised process. Broadly it consists of three stages: (1) mitotic proliferation; (2) meiotic division; and (3) cyto-differentiation (spermiogenesis) (Everitt 2003).

The first phase begins at puberty when a distant group of cells called A1 spermatagonia emerge from spermatogonial stem cells. Each of these cells undergoes a certain number of

mitotic divisions thus increasing the pool of cells. Following several divisions (five in the human), intermediate spermatagonia give rise to type B spermatagonia, which then give rise to pre-leptotene primary spermatocytes. A distinct feature of this step of spermatogenesis is that while nuclear division is complete, cytoplasmic division remains incomplete until the late stages of the process prior to mature spermatozoa being released.

The second proliferative phase of spermatogenesis is marked where pre-leptotene spermatocytes duplicate their DNA as they enter their first meiotic prophase. During prophase, the paired homologous chromosomes come together. During this step, the chromatids break and exchange genetic material and then rejoin. The first meiotic division ends with the separation of homologous chromosomes, leading up to the separation of each primary spermatocyte into two secondary spermatocytes. Each secondary spermatocyte contains one set of chromosomes. Each chromosome consists of two chromatids. These chromatids then also separate and each secondary spermatocyte yields two round spermatids. It should be mentioned that during prolonged meiotic prophase, spermatocytes are especially sensitive to damage.

The third phase of spermatogenesis involves dramatic changes to the structure and function of the cell, a process known as spermiogenesis. The major change during this step involves the remodelling of the cytoplasm of each round spermatid. This process involves the elongation of the nucleus, shedding of the majority of the cell's cytoplasm and the formation of the acrosome, which contains important molecules for the penetration of the oocyte. During this phase, the mid-piece is also formed which contains the mitochondria as well as the centriole which connects the head of the spermatozoa with the mid-piece. Condensation of the chromatin also occurs during this phase. Condensation of the chromatin occurs when histones are replaced with transitional proteins and then smaller, highly basic arginine and (in eutherians) cysteine-rich protamines (Braun 2001, Dadoune 1995, Kierszenbaum 2001, Miller, *et al*). All of these tight packaging

processes occur in order to deliver DNA that can be expressed correctly by the developing embryo (Sakkas, *et al* 1999, Schulte, *et al* 2009).

The process of spermatogenesis is also vulnerable to disruption. Environmental factors, such as high temperature, pro-inflammatory factors, lifestyle, medications and illicit drugs, radiation therapy and xenobiotics can all disturb the biochemical events that occur during spermatogenesis. This disruption can lead to abnormal chromatin structure, which is incompatible with fertility as well as having a negative impact on testicular function. Clearly, gene mutations and chromosomal abnormalities can also have a direct impact on the chromatin of the sperm (Alvarez 2003, Varghese, *et al* 2008).
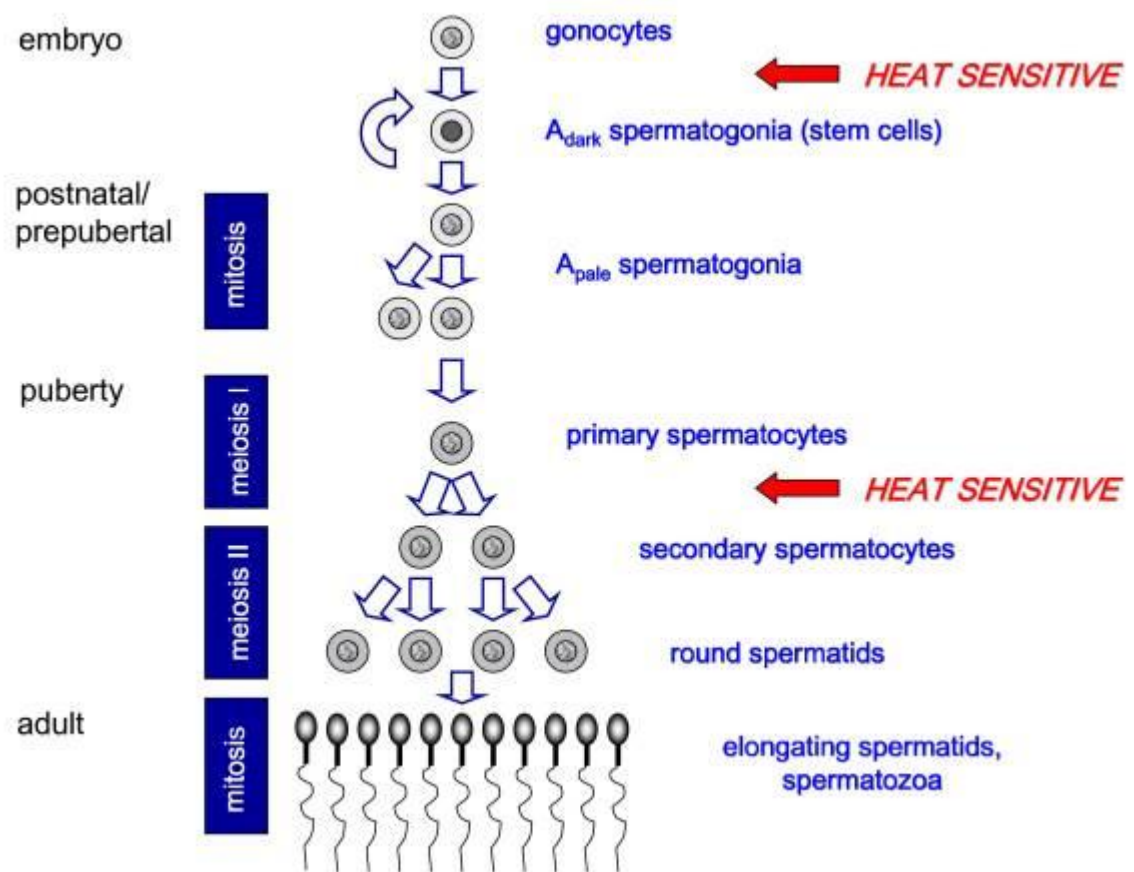
Figure 1.2: Diagram to illustrate human spermatogenesis. Diagram taken from (Ivell 2007).

1.4   Basic diagnosis of male subfertility

Male infertility constitutes the cause of approximately 50% of all infertility cases (de Kretser 1997), with 1 in 20 men in Australia considered infertile, and defective sperm function being the most commonly defined cause of infertility (McLachlan and de Kretser 2001). The sperm cell with its polyunsaturated fatty acid cell membrane, failure of protamination and the absence of anti-oxidants in its reduced cytoplasm make it a highly susceptible cell to environmental effects. Although the capacity of assisted reproductive technology (ART) to bypass male factor infertility has improved dramatically, male infertility remains an issue and may also be on the rise (Sharpe and Irvine 2004).

Some studies suggest that sperm numbers in males are declining at a rate of 1% a year (Carlsen, *et al* 1992, Swan, *et al* 1997). Although this decline seems to be variable based on geographical locations, it appears to be more suggestive for humans rather than animals (Setchell 1997); and also more pronounced in the developed world where there is a coincident rise in the prevalence of obesity and type II diabetes. Despite these statistics male infertility has received little research and public health attention in comparison to female fertility.

The basic diagnosis of male fertility is best described by the World Health Organisation (WHO) laboratory manual for the examination of human semen and sperm-cervical mucus interaction (WHO 1999). The first edition of this manual was published in 1980 in response to a growing need for the standardisation in the ways in which semen analysis and processing were conducted. For the purposes of this thesis, the 4th edition of the manual will be referred to and used. Although a more recent edition of this manual has now been published, references to this manual will be limited as it was published after the work described in this thesis had been conducted.

Decades of research were invested on the development of functional assays to determine sperm function. These include, but are not limited to assessments of zona-free hamster oocyte binding, human zona pellucida binding potential, acrosome reaction and antibody-coating of spermatozoa (WHO 1999). However, according to the 4th edition of the WHO manual, there are three major criteria to evaluate the fertility of a man: the number of sperm per millilitre of semen; the percentage of progressively motile sperm within an ejaculate; and an assessment of sperm morphology (head, mid-piece and tail structural features). Strict criteria stipulate that a normal sperm must contain a 30–70% acrosome, a mid-piece containing very little or no cytoplasmic droplets that is inserted at 90° into the head, , and a tail that contains no sharp bends. For detailed reference values, see Table 1.1.

Table 1.1: Basic diagnostic reference values of male subfertility according to the 4th edition World Health Organisation.

Volume	≥2.0 ml
pH	≥ 7.2
Sperm concentration	≥ 20 X l 0^6 spermatozoa/ml
Total sperm number	≥ 40 X 10^6 spermatozoa per ejaculate
Motility	≥ 50% motile (grades a + b) or ≥ 25% progressive (grade a) within 60 minutes of ejaculation
Normal morphology	≥ 15%
Vitality	≥ 50% live

Although this WHO standard is considered to be the gold standard for the diagnosis of male subfertility, it has been argued that the parameters measured according to this manual are

insufficient or in some cases irrelevant, especially following the introduction of sperm intracytoplasmic sperm injection (ICSI) in the mid 1990s. This is because they assess only gross abnormalities such as head and tail defects. Indeed, none of these assessments determine the relative health of the sperm. Consequently, there are calls in the literature for further assessment such as the use of DNA damage as an additional useful test to the standard semen analysis for the diagnosis of male subfertility (Agarwal and Allamaneni 2005, Lewis, *et al* 2008, Varghese, *et al* 2008). Indeed, some groups have also called for a detailed lifestyle history to be taken by the clinician to determine ways in which abnormal results can be explained and acted upon (Varghese, *et al* 2008) (Figure 1.3). However, to date most testing remains limited to the standard count, motility and morphology assessment.
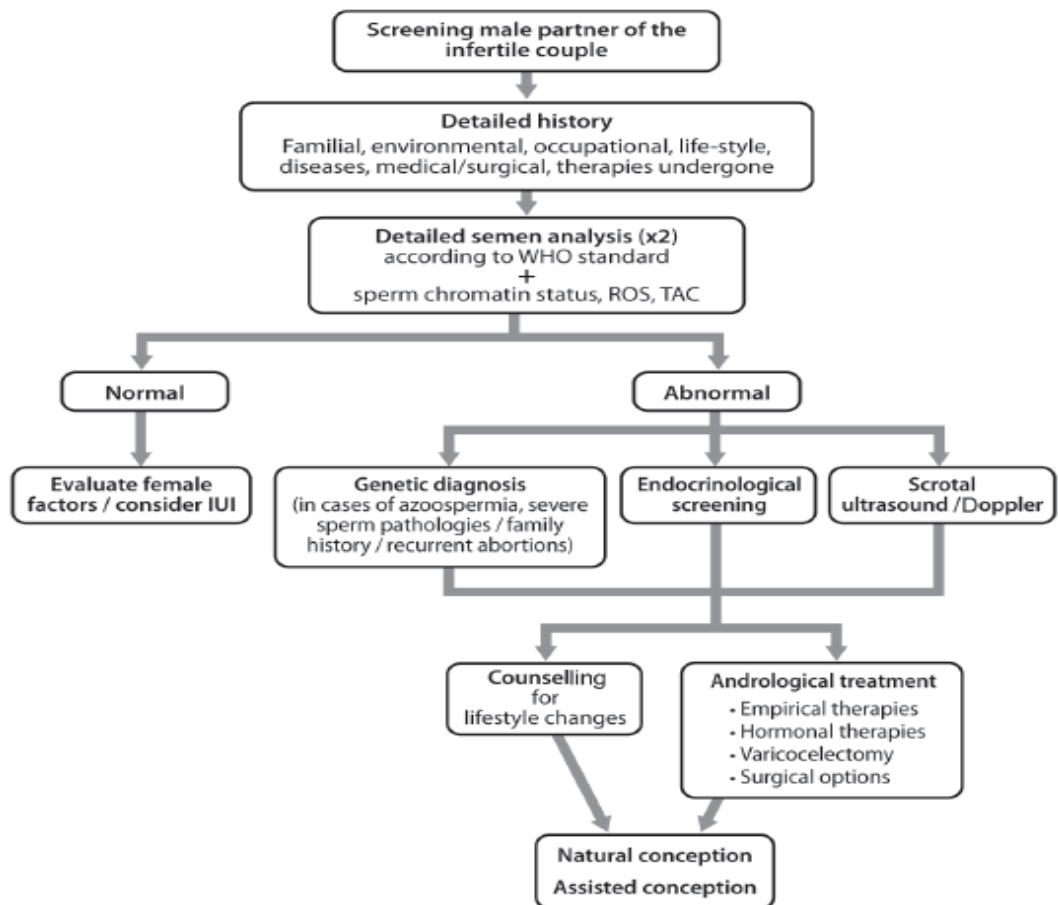
Figure 1.3: Proposed diagnosis and management approach to male infertility. Taken from (Varghese, *et al* 2008).

1.5 Sperm DNA damage

There are several different levels of sperm chromatin abnormalities that are important to consider:

- damage to the actual DNA in the form of single-stranded and/or double-stranded DNA strand breaks
- nuclear protein defects that may interfere with histone to protamine conversion and therefore DNA compaction
- chromatin structural abnormalities causing altered tertiary chromatin configuration (Evenson, *et al* 2002, Schulte, *et al* 2009).

Sperm DNA damage is now measured predominantly by three different procedures:

1.5.1 Sperm chromatin structure assay (SCSA)

DNA damage in spermatozoa was first measured using the Sperm Chromatin Structure Assay (SCSA) in what has become a highly cited publication (Evenson, *et al* 1980). SCSA is an assessment of sperm chromatin integrity by measuring the susceptibility of DNA to acid or heat-induced denaturation (Darzynkiewicz, *et al* 1975, Rigler, *et al* 1969). Therefore, it is an assay which measures DNA damage indirectly by quantifying a metachromatic shift of Acridine Orange (AO) from green to red using flow cytometry after acid treatment. The extent of DNA denaturation is determined and is usually expressed as the DNA fragmentation index (DFI) (Darzynkiewicz, *et al* 1975, Evenson, *et al* 1980).

1.5.2 Terminal deoxynucleotidyl transferase-mediated (TdT) deoxyuridine triphosphate (dUTP) nick end labelling assay (TUNEL)

Although this assay has been shown to be complicated by the fact that terminal transferases are unable to penetrate into the densely compacted chromatin (Mitchell, *et al*), TUNEL is considered to be a direct measure of the presence of endogenous nicks in both single and double DNA strands (Gorczyca, *et al* 1993). dUTP is incorporated at single-stranded and double-stranded DNA breaks in a reaction catalysed by the enzyme TdT. The DNA breaks with the incorporated dUTP are then labelled with a fluorescent tag and can be measured using bright field or fluorescent microscopy as well as flow cytometry (Gorczyca, *et al* 1993). Sperm are then classified as TUNEL positive or negative and expressed as a percentage of the total sperm in the population. In contrast to the SCSA test, TUNEL testing assesses the actual level of DNA damage in the sperm sample rather than susceptibility to damage. As a result, care must be taken in the interpretation of this test, as non-motile dead sperm undergoing apoptosis or necrosis would stain positive with this stain. Therefore, reference ranges may be significantly different dependent on whether the stain was performed on a semen sample or a washed preparation enriched for motile sperm.

1.5.3 Comet

Comet is a pH sensitive electrophoresis assay, which evaluates how well the DNA is packaged within the nucleus. Decondensed sperm are suspended in an agarose gel, subjected to an electrophoretic gradient, stained with fluorescent DNA-binding dye, and then imaged with imaging software. Low-molecular weight DNA, short fragments of both single-stranded and double-stranded DNA, will migrate during electrophoresis giving the characteristic comet tail (Klaude, *et al* 1996). High-molecular weight intact segments of DNA will not migrate and remain

in the head of the ‗comet'. Imaging software is then used to measure comet tail length and tail fluorescent intensity, which are increased in sperm with high levels of DNA strand breaks (Hughes, *et al* 1996, Schulte, *et al* 2009).

1.6   Relevance of sperm DNA damage to infertility and pregnancy

In the general population, elevated levels of sperm DNA damage have been associated with early pregnancy loss (Carrell, *et al* 2003). Furthermore, environmental factors such as cigarette smoke have been shown to induce DNA damage in the spermatozoa of smokers and thereby increasing the chances of childhood cancer in the offspring (Lewis and Aitken 2005). Therefore, the integrity of the paternal genome appears important for embryo and fetal development and also long-term health of the offspring. For ART, studies have also shown that increased sperm DNA damage is associated with decreased fertilisation rates (Lopes, *et al* 1998, Sun, *et al* 1997), embryo cleavage rates (Lopes, *et al* 1998, Saleh, *et al* 2003, Tomsu, *et al* 2002), impaired blastocyst development rates (Seli, *et al* 2004a), and decreased clinical pregnancy rates (Greco, *et al* 2005, Larson, *et al* 2000, Saleh, *et al* 2003, Tomsu, *et al* 2002, Virro, *et al* 2004). It has also been shown that elevated sperm DNA damage is associated with decreased clinical pregnancy rates following ART when ICSI was the insemination technique (Bakos, *et al* 2008). This same study showed that when routine IVF was the insemination technique, pregnancy rates were only marginally reduced when compared to ICSI. This highlights the potential that in ICSI, DNA-damaged sperm may be used to inject the oocytes.

1.7   Causes and mechanisms of sperm DNA damage

Several groups have reviewed this issue and put forward numerous theories. Many factors are associated with sperm DNA damage. These range from external environmental origins such as

cigarette smoking (Potts, *et al* 1999), irradiation (Arnon, *et al* 2001), chemotherapy (Chatterjee, *et al* 2000, Morris 2002), heat (Paul, *et al* 2008, Perez-Crespo, *et al* 2008a), prolonged abstinence periods prior to IVF (Bakos, *et al* 2008), mobile phones (Agarwal, *et al* 2009, De Iuliis, *et al* 2009, Fejes, *et al* 2005b), certain medical conditions such as leukocytospermia (Alvarez et al. 2002; Erenpreiss et al. 2002), varicocele (Saleh, *et al* 2003), and cancer (Kobayashi, *et al* 2001), as well as iatrogenic causes such as sperm cryopreservation (Donnelly *et al.* 2001; Zribi *et al.* 2010). Although the exact mechanism of sperm DNA damage is yet to be fully understood, there are currently three main non-mutually exclusive theories, which shall be reviewed: oxidative stress; chromatin packaging abnormalities; and apoptosis (Figure 1.4).

The most plausible theory in the literature is that oxidative stress causes sperm DNA damage. Spermatozoa are particularly susceptible to oxidative stress because their plasma membranes contain large quantities of polyunsaturated fatty acids (Alvarez and Storey 1995) and their cytoplasm contains low concentrations of scavenging enzymes (Aitken and Fisher 1994, de Lamirande and Gagnon 1995, Sharma and Agarwal 1996). Oxidative stress attacks not only the fluidity of the sperm plasma membrane, but also its DNA (Aitken 1999, Saleh, *et al* 2002).

In most mammals, the process of spermeogenesis renders the sperm relatively protected from external stress, as there is essentially complete replacement of the histones with protamines. However, human sperm chromatin is less compact than in other mammals, as approximately 15% of histones are retained in human sperm chromatin (Bench, *et al* 1996, Gatewood, *et al* 1987). Human sperm also contain two types of protamines: P1 and P2. P2 protamines contain fewer cysteine groups and thus contain less disulfide cross links (Corzett, *et al* 2002). Reportedly, infertile men have a higher histone to protamine ratio in their sperm chromatin (Steger, *et al* 2000), and DNA integrity has also been shown to be compromised in protamine deficient men (Aoki, *et al* 2005). Importantly, a feature that is unique to the sperm cell is that the shedding of

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the cytoplasm renders the sperm with little anti-oxidant mechanisms and therefore the sperm are almost entirely reliant on the protamination process to protect the sperm DNA from any damage.

It has been proposed that defective spermatogenesis is associated with errors in spermiogenesis leading to impaired chromatin remodelling. Consequently, the spermatozoa exhibit low levels of nuclear protamination. This condition secondarily creates a state of vulnerability in the chromatin such that the DNA becomes susceptible to various kinds of stress, particularly oxidative stress (Aitken and De Iuliis 2007a, Aitken and De Iuliis 2007c). The frequent observation that oxidative stress is associated with poor sperm motility (Aitken, *et al* 1989, Alvarez, *et al* 1987, Jones, *et al* 1979) might explain why motility and DNA damage are so often correlated in the patient population (Erenpreiss, *et al* 2008, Giwercman, *et al* 2003, Sills, *et al* 2004), and they are both dependent on stress induced by oxygen-free radical attack. It should also be mentioned that oxidative stress may lead to lipid peroxidation in spermatozoa, which may, in turn, lead to impaired motility.

The other theory as to the cause of DNA damage is referred to as the abortive apoptosis theory. Men with poor seminal parameters often display a large percentage of Fas-expressing sperm in the ejaculate (Sakkas, *et al* 1999). Fas is a protein expressed on the surface of the sperm that is thought to interact with the sertoli cell secreted Fas ligand (FasL). This has led to the theory which suggests that this interaction leads to apoptosis being initiated and then aborted. However, the full explanation of this theory remains a matter of debate in the literature.

Human spermatozoa are vulnerable to oxidative stress because:

• They contain high concentrations of unsaturated fatty acids

• Possess nuclear DNA that is incompletely protaminated and poorly compacted.

• Cannot effect DNA repair

• Cannot undergo apoptosis

• Possess low levels of cytosolic antioxidant enzymes

• Can generate reactive oxygen species via their mitochondria, specialized free radical generating systems (NOX5) and the redox cycling of xenobiotics

• Must spend several days as isolated cells in both the male and female reproductive tracts.

DNA damage

• Excessive ROS generation by virtue of:
　　Redox cycling xenobiotics
　　Aberrant mitochondrial function
　　Excess cytoplasmic retention
　　Inadequate control of ROS generation
　　Testicular heating
　　Cryopreservation
　　Centrifugation
　　Leukocyte contamination
• Inadequate antioxidant protection
• Impaired DNA repair

Lipid peroxidation and DNA damage

Poor fertilization

Aberrant DNA repair in the fertilized oocyte/early embryo

Impaired embryonic development　　Increased abortion　　Childhood disease

Figure 1.4: Oxidative stress as a major cause of DNA damage. Summary of the factors implicated in this theory. Taken from (Lewis and Aitken 2005).

1.8 Obesity and fertility

Obesity and its associated co-morbidities present significant health concerns throughout the world and, indeed, overweight and obesity are prevalent in the population of people trying to conceive. The increasing incidence of obesity in this population of people is cause for concern given the extensive literature highlighting the increased infertility with elevated BMI, although the focus has been mainly on women with less emphasis acknowledging the impact of male obesity on fertility.

Women who are overweight or obese (BMI≥25) have an increased time to pregnancy and a reduced probability of conceiving, relative to normal weight women (Hassan and Killick 2005, van der Steeg, *et al* 2008), as well as an increased risk of early and recurrent miscarriage (Lashen, *et al* 2004). For women undergoing ART, a progressive increased risk of spontaneous abortion and an impaired response to gonadotrophin treatment has been reported with increasing BMI (Fedorcsak, *et al* 2004, Wang, *et al* 2002), although this is not always the case (Dechaud, *et al* 2006). Even though women who are overweight or obese generally have more adverse outcomes when undergoing assisted reproduction (Ku, *et al* 2006, Lenoble, *et al* 2008, Loveland, *et al* 2001), they are less prevalent in younger women (Martinuzzi, *et al* 2008). Maternal obesity reduces the mean embryo grade and utilisation rate (Metwally, *et al* 2007). With evidence from oocyte donation studies suggesting that recipient obesity increases the risk of spontaneous abortion (implying a role for the endometrium) (Bellver, *et al* 2003), it appears that the combined effect of reduced oocyte quality and endometrial receptivity contribute to the infertility described for maternal obesity.

In comparison, factors affecting male fertility are relatively less well understood, with a small number of studies recently examining the effect of elevated BMI on sperm parameters and serum hormone levels. Couples with overweight and obese male partners had an increased odds ratio for increased time to conceive (Nguyen, *et al* 2007a, Ramlau-Hansen, *et*

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al 2007b). In normal men, increasing BMI is consistently associated with reduced serum sex hormone binding globulin (SHBG), testosterone and inhibin B levels (Aggerholm, *et al* 2008a, Winters, *et al* 2006a), and follicle stimulating hormone (FSH) (Pauli, *et al* 2008), an association also described for men seeking ART (Chavarro, *et al* 2010).

1.9 Male obesity and hormonal regulation

This area of research not only has received more attention but also reached more conclusive evidence in the literature compared to the effects on sperm parameters. The recent meta-analysis revealed convincing evidence that a negative relationship exists between male BMI and both serum testosterone and SHBG (MacDonald, *et al* 2010). It has been previously hypothesised that synthesis of excessive insulin may be involved in the inhibition of hepatic globulin in men with higher BMI (Pasquali, *et al* 2007).

1.10 Male obesity and basic semen analysis assessment

To date, 13 articles have investigated the effect of male obesity and basic sperm parameters, including sperm concentration, sperm motility and morphology, nine of which were published in the last three years. A recent meta-analysis concluded that there was no relationship between male obesity and these parameters (MacDonald, *et al* 2010). However, it was also highlighted that the relationship between obesity and basic semen analysis assessment has been a matter of contradiction in the literature. Furthermore, some studies that concluded differentially were not included in the meta-analysis findings. For a summary of the studies assessing the relationship between paternal BMI and sperm parameters, please refer to Table 1.2.

1.11 Male obesity and sperm concentration

The largest study examining the relationship between male obesity and sperm concentration, which included 1,989 European men from eight countries, concluded that there was no significant relationship between male obesity and sperm concentration (Aggerholm, *et al* 2008a). One limitation of this study was that BMI was self reported and not measured. Conversely, (Jensen, *et al* 2004) reported a significant negative relationship between BMI and sperm concentration. However, the study population of Jensen *et al.* was younger and therefore unlikely to be representative of a general population, plausibly explaining the differences between studies. (Qin, *et al* 2007) reported a positive relationship between BMI and sperm concentration. However, it should be mentioned that, again, in this study, obese men made up only a small proportion (1.7%) of the study population. A recent meta-analysis (of those studied eligible for inclusion) concluded that there is no significant relationship between male obesity and sperm concentration (MacDonald, *et al* 2010).

1.12 Male obesity and sperm motility

Some studies examining the effect of male obesity and sperm motility showed no significant relationship between the two measures (Chavarro, *et al* 2010, Fejes, *et al* 2005a, Jensen, *et al* 2004, Pauli, *et al* 2008, Qin, *et al* 2007).

Conversely, others have found a relationship between these outcomes (Hammoud, *et al* 2008b, Hofny, *et al* 2009, Kort, *et al* 2006, Magnusdottir, *et al* 2005). The reason for these discrepancies may be related to the methodology used to determine motility and also the populations selected.

1.13 Male obesity and sperm morphology

To date, nine studies have investigated whether there is any association between male obesity and sperm morphology. Three of these found an inverse relationship between increasing BMI and sperm morphology either directly or indirectly (Hammoud, *et al* 2008b, Hofny, *et al* 2009, Kort, *et al* 2006). Conversely, the majority of studies have reported no relationship between BMI and sperm morphology (Aggerholm, *et al* 2008a, Chavarro, *et al* 2010).

1.14 Male obesity and sperm DNA damage

Only two studies to date have assessed the effect of male obesity beyond the basic parameters of sperm motility, concentration and morphology. Both these studies have shown an association with male obesity and increased levels of DNA damage in sperm (Chavarro, *et al* 2010, Kort, *et al* 2006). However, a limitation of these human studies is that only correlative data have been established and there are often additional confounding lifestyle factors that make definitive conclusions difficult.

Table 1.2: Summary of the studies investigating paternal obesity and sperm parameters.

	Concentration	Motility	Morphology	DNA Damage
(Strain *et al.* 1982)	No	No	n/a	n/a
Ayers *et al.* 1985)	No	n/a	n/a	n/a
(Jensen, *et al* 2004)	Yes	No	No	n/a
(Magnusdottir, *et al* 2005)	Yes	Yes	n/a	n/a
(Fejes, *et al* 2005a)	Yes	No	No	n/a
(Koloszar, *et al* 2005)	Yes	n/a	n/a	n/a
(Kort, *et al* 2006)	Yes*	Yes*	Yes*	Yes
(Qin *et al.* 2007)	No	No	No	n/a
(Hammoud, *et al* 2008b)	Yes	Yes	Yes	n/a
(Pauli, *et al* 2008)	No	No	No	n/s
(Aggerholm *et al.* 2008)	No	No	No	n/a
(Nicopoulou, *et al* 2009)	Yes	n/a	n/a	n/a
(Hofny, *et al* 2009)	Yes	Yes	Yes	n/a
(Stewart, *et al* 2009)	Yes	n/a	n/a	n/a
(Chavarro, *et al* 2010)	No	No	No	Yes

*Significant for Normal Motile Sperm (NMS) = volume*concentration*%motility*%morphology.

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1.15 Reasons for discrepancy

The discrepancies seen in the literature may be due to several reasons. First, it is now known that numerous genetic causes of male infertility exist. Some obesity syndromes with known genetic defects are specifically associated with male infertility due to loss of function of a single gene responsible for both obesity and spermatogenesis, reviewed by (Mah and Wittert 2010)—for example, the Alms1 gene in Alström Syndrome (Arsov, *et al* 2006). Similarly, a number of genes in the chromosomal region (15q11-q13) lead to both obesity and defective spermatogenesis—for example, Prader-Willi Syndrome and Angelman Syndromes (Buiting, *et al* 2003). Second, it is well known that lifestyle factors such as cigarette and marijuana smoking impair sperm function and therefore complicate the analysis of a number of such studies. Third, the literature to date lacks controlled animal model studies, where the effect of obesity can be studied directly in relation to male fertility.

1.16 Animal models: Are there any assessing sperm parameters?

Only one study (published since the beginning of this thesis) in animals has examined the effects of male obesity and sperm parameters (Ghanayem, *et al* 2010). In this study, the investigators used genetically intact male C57Bl/6J mice, which were fed either a normal diet or a high fat diet for 30 weeks. Sperm from obese males exhibited decreased motility and reduced hyperactivated progression when compared with sperm from lean mice. However, this study is confounded by the fact that these males were not just obese but also relatively old and had higher serum glucose levels, hyperinsulinemia and were leptin resistant, which may be responsible for the changes seen rather than increased adiposity. The sperm molecular mechanisms involved in this impairment to sperm function were not investigated.

1.17 Paternal effects, embryo development and pregnancy outcomes

While the consequences of maternal obesity on conception and pregnancy have been examined extensively in animal models and in humans, the implications of paternal obesity for all aspects of embryo development and pregnancy are lacking. Understanding the repercussions of male obesity on fertility and pregnancy is pertinent given its increasing incidence, potentially influencing the medical advice for couples attempting to conceive and, of utmost concern, modulating the health and development of their offspring.

Perhaps the most well studied paternal effect on embryo development is the effect of heat. A delay in normal preimplantation embryo development has been reported for other paternal stresses in rodents, including diabetes (Kim and Moley 2008) and a mild scrotal heat stress (Paul*, et al* 2008, Zhu*, et al* 2004), paternal heat stress (Zhu*, et al* 2004) and advanced paternal age in humans (Luna*, et al* 2009).

Only one single study to date has assessed embryo health or quality in relationship to a paternal factor with paternal heat stress in mice shown to cause a reduction in blastocyst cell numbers (Zhu and Setchell 2004). So far no examination of male obesity on the development and quality of the preimplantation embryo exists. However, some assessment was conducted of embryo development following in-vitro fertilisation (IVF) or intra-cytoplasmic sperm injection (ICSI) with DNA-damaged sperm in animal models. For example, IVF using irradiated bovine sperm did not impair fertilisation but blocked development to the blastocyst stage by increasing apoptosis in embryos at the second or third cleavage division (Fatehi*, et al* 2006). Further, in the mouse, zygotes from irradiated mouse sperm (Shimura*, et al* 2002) or those generated from frozen-thawed (FT) sperm (Perez-Crespo*, et al* 2008a) cleaved and developed normally during early cleavage stages. However, the implantation rate was decreased with FT sperm (Perez-Crespo*, et al* 2008a) and a dose dependent decrease in fetal development was reported with increasing irradiation (Shimura*, et al* 2002). Further, in the mouse, the use of DNA damaged FT sperm followed ICSI, has been shown to be associated

with long term health and behavioural effects of adult offspring (Fernandez-Gonzalez, *et al* 2008). Finally, in humans there is convincing evidence that increased DNA fragmentation or damage is associated with poor development to blastocyst, reduced post-implantation development and decreased pregnancy rate (Bakos, *et al* 2008, Borini*, et al* 2006a, Seli, *et al* 2004a).

1.18 Seminal plasma composition: An effect on semen quality?

Human seminal plasma is composed of carbohydrates, including glucose and fructose, with the latter as the main carbohydrate present in semen, as well as ions such as calcium, magnesium, sodium, potassium and zinc. The focus of this review will be on the important metabolic carbohydrates (fructose and glucose) as well as insulin, because of their potential relevance in obesity and metabolic control of sperm function.

1.18.1   Glucose

Glucose is present in semen at a range of concentrations (0.22-16.37 mM); however, lower than fructose. It has previously been demonstrated that glucose transporter GLUT 3 is present in spermatozoa (Angulo, *et al* 1998, Burant and Davidson 1994, Haber*, et al* 1993). Glucose may be an important source of energy to spermatozoa (Martikainen*, et al* 1980, Peterson and Freund 1971). Its role in supporting motility and capacitation has been challenged, as other glycolysable sugars have been shown to be as effective in supporting these functions (Williams and Ford 2001). Its source of production is largely unknown. However, some may be present endogenously within the sperm as glycogen (Ballester*, et al* 2000), which is why it has been hypothesised previously that it may be important in maintaining motility and allow spermatozoa to capacitate in glucose-free media (Ford 2006). Glucose is supplied by the female reproductive tract (van der Linden*, et al* 1992) as well as culture media in human IVF.

1.18.2   Fructose

Fructose is secreted by the seminal vesicles and is present in semen at high concentrations (7.55-34.86 mM). It is important for semen coagulation (Montagnon, *et al* 1982). Fructose is also an important source of energy and the primary source of lactic acid. It is also thought to be important for sperm chromatin stability, reviewed by (Owen and Katz 2005). Low fructose in semen has been shown to be related to male infertility (Daudin, *et al* 2000, de la Taille, *et al* 1998, von Eckardstein, *et al* 2000). The fructose transporter in human spermatozoa is GLUT 5 (Burant, *et al* 1992). It is also supplied by the female reproductive tract (van der Linden, *et al* 1992); however, interestingly, fructose has not been used in human IVF culture media until recently (GIVF series 5). It remains unknown why fructose is present at much higher concentrations in the seminal plasma than glucose. In serum it has been well documented that glucose is the main carbohydrate. We hypothesise that fructose be the preferred carbohydrate when it comes to the source of sperm energy.

1.18.3   Insulin

Insulin in the seminal plasma from fasting non-diabetic donors has been shown to be significantly higher than in serum (19 ± 3 μU/mL vs. 7.5 μU/mL respectively) (Hicks, *et al* 1973). The function of insulin in the seminal plasma is largely unknown; however, it may be important in sperm acrosome reaction and motility parameters as evidenced by increased total and progressive motility as well as the sperm hyperactivation characteristics (Lampiao and du Plessis 2008).

An early study demonstrated quite convincingly that insulin concentration in the seminal plasma and serum insulin concentration were significantly correlated (Paz, *et al* 1977). The local origin of insulin is both prostatic and vesicular. However, it has also been shown to be secreted by sperm (Aquila, *et al* 2005). In split ejaculates, the concentration of insulin was

significantly higher in the second (seminal vesicles) fraction of the ejaculate (Paz, *et al* 1977). The same study concluded that an effect of exogenous insulin on glucose metabolism of washed spermatozoa was not substantiated. However, this finding contradicted a previous study in which the direct stimulatory effect of insulin on glucose metabolism by sperm was found (Hicks, *et al* 1973).

1.19 Research aims and hypothesis

As discussed the effect of paternal obesity on sperm function to date is limited and contradictory. There is also a distinct lack of animal studies assessing this question. Therefore the first aim of this study was to both establish an animal model of male obesity and determine the effect of paternal obesity on sperm function. For this aim it was hypothesised that sperm function would be impaired via mechanisms that are related to oxidative stress and sperm DNA damage.

The second aim of this study was to use this same animal model to determine the effect of paternal obesity on preimplantation embryo development. It was also hypothesised that embryo development would be impaired due to the hypothesised deleterious effects of sperm function.

In order to confirm or refute any findings from the animal model, a retrospective analysis was also conducted to determine the effect of paternal obesity on sperm, embryo and pregnancy outcomes as well as pregnancy loss and live birth rates following human assisted reproductive outcomes.

To further elucidate the causes for the observed effects on the sperm, seminal plasma composition in a cohort of obese patients and controls were analysed. The effect of glucose, insulin and other obesity-associated metabolites on sperm oxidative status as well as sperm DNA damage was also analysed.

Current IVF protocols involve the removal of the seminal plasma and the supplementation of fertilisation media instead. IVF media do not include many of the above-mentioned elements. Therefore, the final aim was to determine if the addition of one or more of these elements has any detrimental or beneficial effect on sperm function. The effect of different ratios of glucose and fructose was examined.

These studies should add substantially to the current knowledge of paternal obesity and fertility and specifically will address the knowledge gap as to how paternal obesity affects sperm function and the consequences of this to the establishment and health of the subsequent embryo.

Hassan W. Bakos

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2 The effect of paternal diet-induced obesity on sperm function and fertilisation in a mouse model

2.1 Introduction

The worldwide epidemic of obesity has numerous negative effects on health (Proietto and Baur 2004). Although paternal obesity has been associated with alterations in spermatogenesis, the relationship between obesity and male infertility has largely been ignored. This is in contrast to the widespread attention being devoted to the effect of female obesity and female fertility where studies have shown that overweight women are more likely to have reduced fertility (Bolumar, *et al* 2000, Gesink Law, *et al* 2007, Pasquali, *et al* 2006, Rich-Edwards, *et al* 2002).

The vast majority of studies conducted to date on male obesity and fertility have focused on hormonal effects of obesity such as the well-documented decrease in both free and total testosterone as well as sex hormone-binding globulin (reviewed, Mah and Wittert 2010). Some reports indicate that paternal obesity may negatively affect basic sperm parameters such as concentration and motility (Fejes, *et al* 2005a, Hammoud, *et al* 2008b, Magnusdottir, *et al* 2005). However, the literature shows considerable contradiction, with several other studies demonstrating that these same parameters are not affected by paternal obesity (Chavarro, *et al* 2010, Nicopoulou, *et al* 2009). Furthermore, a recently published meta-analysis concluded that paternal obesity was not associated with standard sperm function parameters (MacDonald, *et al* 2010).

The discrepancies seen in the literature may be due to several reasons:

- It is now known that there are a number of genetic causes of male fertility. Some obesity syndromes have known genetic defects which are specifically associated with abnormalities of spermatogenesis due to loss of function of a single gene responsible for both (reviewed, Mah and Wittert 2010).

- It is well known that lifestyle factors such as cigarette and marijuana smoking impair sperm function and therefore complicate the analysis of a number of studies.

- To date there are only two studies which have assessed the effect of obesity beyond the basic parameters of motility, concentration and morphology. Both these studies have shown an association with male obesity and increased levels of DNA damage in sperm (Chavarro, *et al* 2010, Kort, *et al* 2006). However, a limitation of the human studies is that only correlative data haves been established and there are often additional confounding lifestyle factors that make definitive conclusions difficult. To date the literature lacks controlled animal model studies, where the effect of diet induced obesity can directly be studied in relation to male fertility. A recent study has established that a mouse model of obesity in conjunction with hyperinsulinemia consistent with altered glucose metabolism in an aged male mouse is associated with reduced sperm motility (Ghanayem, *et al* 2010). The effect of a type II diabetic state on sperm is well established (Agbaje, *et al* 2007, Kim and Moley 2008, Shrilatha 2007).

However, to date there is no information on the effects of diet-induced obesity without the confounding effects of a diabetic state on sperm function and fertilisation capacity. Therefore, the aim of the current study was to determine the effect of diet-induced obesity on sperm function and the ability of the sperm to bind and fertilise the oocyte in the mouse. Furthermore, intracellular mechanisms that may be responsible for sperm function parameters were also elucidated.

2.2 Materials and methods

2.2.1 *Animals and diet*

All mice were obtained from the University of Adelaide Laboratory Animal Services, Adelaide, Australia. The animal ethics committee of The University of Adelaide approved all experiments, and the animals were handled in accordance with the Australian Code of Practices for the Care and Use of Animals for Scientific Purposes. All mice had free access to water and food, and were maintained at The University of Adelaide animal house at 24 ºC on a 14-h light, 10-h dark illumination cycle. Six-week old C57BL/6 male mice were allocated to two groups. Group 1 (n=18) received a control diet containing only 6% fat, 19% protein, and 64.7% carbohydrate (SF04–057 Specialty Feeds, Glen Forrest, Australia). Group 2 (n=18) received a high fat diet (HFD) (SF00-219, Specialty Feeds, Australia) providing 22% fat, 0.15% cholesterol, 19% protein, and 49.5% carbohydrate (SF00–219; Specialty Feeds), intended to resemble a Western style diet. These diets were matched for other nutritional content and were provided *ad libum*.

Males were housed in pairs and maintained on these diets for nine weeks. All assessments of sperm function were performed blinded to the diet group and were performed by the same individual throughout the study.

2.2.2 *Body measurements*

Body weight for individual males was measured weekly. At the completion of a nine-week period, mice were anesthetised and a blood sample was obtained by cardiac puncture. Body composition was assessed by measuring organ weights of testes, seminal vesicles, retroperitoneal and peritoneal fat, heart and liver.

2.2.3 Hormonal blood analysis

Testosterone was measured at the ANZAC Research Institute, Sydney, Australia, with a stable-isotope dilution LC-MS/MS method using an API 5000 instrument using atmospheric pressure photoionisation as previously described (Harwood and Handelsman 2009) to simultaneously quantify testosterone (T), dihydrotestosterone (DHT), 17-estradiol (E2) and estrone (E1) from serum 100 µL. The run was divided into two periods to allow estrogen detection in the negative ionisation mode and then switching to androgen detection in the positive mode. Sample preparation involved liquid-liquid extraction with 1 mL hexane:ethyl acetate 3:2 ratio containing deuterated internal standards.

Calibrants and quality control samples (3 levels) were prepared in 4% BSA. Accuracy was assessed by spiked recovery of serum pools, and imprecision was assessed using the quality control samples.

2.2.4 Metabolic profiling

The quantitative determination of plasma glucose was performed with a Hitachi 912 automated sample system using the Glucose HK assay kit, with the C.f.a.s. Calibrator, and quality controls: Precinorm U and Precipath U (Roche Diagnostics, NSW, Australia). The mean coefficient of variation was less than 3.3%.

The quantitative determination of plasma non-esterified free fatty acids was performed with a Hitachi 912 automated sample system using the NEFA-C Free Fatty Acid assay kit (Wako, Japan, through NovoChem, Australia) and quality controls: QCS 1 and 2 (Bio-Rad, Australia). The mean coefficient of variation was less than 4.6%.

The quantitative determination of plasma triglycerides was performed with a Hitachi 912 automated sample system using the TG assay kit, with the C.f.a.s. Calibrator, and quality controls: Precinorm U and Precipath U (Roche Diagnostics, NSW, Australia). The mean coefficient of variation was less than 3%.

The quantitative determination of plasma cholesterol was performed with a Hitachi 912 automated sample system using the CHOL assay kit, with the C.f.a.s. Calibrator, and quality controls: Precinorm U and Precipath U (Roche Diagnostics, NSW, Australia). The mean coefficient of variation was less than 4%.

2.2.5   Conventional semen analysis

The vas deferens and caudal epididymis were separated away and spermatozoa obtained by emptying the vas deferens and puncturing the caudal epididymis into a well containing 1 ml of GIVF medium (Vitrolife, AB, Gothenburg, Sweden). Sperm were incubated at 37°C for 5–10 min before motility assessment was performed.

Sperm motility was determined manually under 40 x magnification and expressed as a percentage of motile sperm in a given sample. Duplicate measures of two hundred sperm were counted for motility assessment. Sperm concentration was determined using an improved Neubauer haemocytometer and applying the appropriate dilution factor as recommended by the 1999 World Health Organisation laboratory manual for the examination of human semen and sperm-cervical mucus interaction (WHO 1999).

2.2.6   Sperm intracellular reactive oxygen species

Intracellular ROS levels were assessed in motile spermatozoa to ensure that analysis was conducted on live sperm, as this dye requires cleavage by intracellular esterases to detect ROS levels. The probe 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA), which fluoresces when it binds to intracellular ROS, was utilised as previously described (Lane, *et al* 2002, Nasr-Esfahani, *et al* 1990). The dye was added to GIVF medium at a final concentration of 5 µM. Motile sperm were incubated with the dye for 15 min at 37°C. Spermatozoa were then washed twice with PBS by centrifugation at 1000g for 1 min. Ten µl of spermatozoa were then smeared on polylysine-coated slides and examined using a fluorescent microscope with

photometer attachment. Each sperm was imaged individually and a fluorescence reading was obtained. The relative fluorescence for each spermatozoon was expressed as mean fluorescence units. A minimum of twenty motile sperm were measured per sample as this number of observations has previously been shown to have sufficient power to detect differences in ROS levels (Lane *et al.* 2002; Nasr-Esfahani *et al.* 1990). This assay was initially validated with hydrogen peroxide in a dose-dependent manner; and a positive control using UV light exposure was included in every replicate.

2.2.7 *Sperm mitochondrial reactive oxygen species*

Assessment of intracellular mitochondrial ROS production was performed on live motile sperm. This assay was performed as previously described (Koppers*, et al* 2008b) using the MitoSOX Red (MSR) assay (Molecular Probes). This dye rapidly and selectively targets the mitochondria. Once in the mitochondria, when MitoSOX™ Red reagent is oxidised by superoxide, it exhibits red fluorescence. Briefly, MSR was added to spermatozoa at a final concentration of 2 µM and allowed to incubate for 30 min at 37°C. The cells were washed twice in PBS at 1000g for 1 min. Ten µl of spermatozoa were smeared on polylysine-coated slides and covered with cover slips and immediately assessed using fluorescence microscopy (Menzel-Glaser, Braunschweig, Germany). For these assessments, excitation was achieved with an argon laser (488 nm). Red fluorescence was measured using a filter attached to the microscope (excitation 540–565 nm, emission 605–660 nm). Images were captured using IPLab software (version 3.61; Scanalytics, Inc., Rockville, MD, USA). Phase contrast images were also captured to determine the number of cells on the image. Results were expressed as the number of MSR-positive cells per total cells. A minimum of 50 motile sperm cells were counted for each sample.

2.2.8   Sperm DNA damage

Spermatozoa were smeared on polylysine-coated slides (Menzel-Glaser, Braunschweig, Germany), air-dried and fixed with methanol and acetic acid (ratio:1:3 respectively), for 1 h at room temperature. Smears were then permeabilised with 0.5% Triton X-100 in 0.5% sodium citrate for 1 h, washed twice with phosphate-buffered solution (PBS; JRH Biosciences, Lenexa, KS, USA) and incubated with the terminal deoxyribonucleotidyl transferase-mediated dUTP nick-end labelling (TUNEL). The assay was performed using a Cell Detection Kit (Roche, Mannhein, Germany) for 1 h at 37°C. Smears were then washed twice with PBS and stained with propidium iodide (PI, 1 mg/mL) to identify sperm nuclei. Smears were then washed with PBS twice and a drop of a pre-prepared mixture of gold antifade reagent (Molecular Probes, Eugene, OR, USA) and 99% Glycerol (Sigma Chemical Co., New South Wales, Australia) applied. Slides were examined using fluorescence microscopy. Two individual filters were utilised to capture the nuclear signal (PI; excitation 540–565 nm, emission 605–660 nm) and the TUNEL signal (FITC; excitation 465–495 nm, emission 515–555 nm). The two captured images were superimposed using IPLab software (version 3.61; Scanalytics, Inc., Rockville, MD, USA). The percentage of sperm DNA damage was calculated as the number of TUNEL-positive sperm from the total number of sperm nuclei. At least 200 sperm were counted for each sample as previously described by (Bakos, *et al* 2008).

2.2.9   Sperm binding and fertilisation rates

To assess sperm binding to the zona pellucida and fertilisation rates, mature cumulus-enclosed oocytes were collected from 4 week old F1 (C57BL/6XCBA) hybrid females 12 h following superovulation with an intraperitoneal injection of pregnant mares serum gonadotropin (PMSG) and human chorionic gonadotropin (hCG) administered 48 h apart as per (Lane and Gardner 2003a). Cumulus-enclosed oocytes (n≥45 oocytes per male) were placed in 80 µl drops of GIVF at 6%CO_2, 5%O_2 and 89%N_2 at 37°C. Sperm from males was

collected from the vas deferens and capacitated for 1 h in medium G-IVF (Vitrolife). After 1 h oocytes were inseminated with 1×10^6 sperm per ml and gametes co-incubated for 4 h. A sample of the sperm used for insemination was used for the assessment of capacitation and acrosome reaction.

A further sample of sperm was removed from the drops containing the cumulus and oocytes at 4 h post-insemination. At 4 h post-insemination, sperm binding to oocytes was assessed by incubation for 3 minutes in bisbenzamide ($25\mu g/ml$) followed by imaging under UV light. All analysis was performed blinded to the diet of the male. The number of sperm bound to the zona pellucida was assessed by counting sperm nuclei. At 6 h post-insemination, the number of fertilised embryos was determined by the presence of 2 pronuclei within the oocyte following staining with bisbenzamide. Fertilisation rates are expressed as the percentage of oocytes with 2 pronuclei from the total number inseminated.

2.2.10 *Sperm capacitation and acrosome reaction*

Capacitation and acrosome reaction were measured using Arachis hypogaea (peanut) agglutinin (PNA, Molecular Probes, Eugene, Oregon, USA) as previously described by (Baker, *et al* 2004). A sperm sample was used to determine the percentage of capacitating and acrosome sperm reacted at the time of insemination. Briefly, sperm were washed with PBS first at 1000g for 1 min twice. Sperm were then incubated with Lectin PNA Alexa antibody at a final concentration of $100\mu l/mL$ of the antibody for 30 min at room temperature. Samples were then washed with PBS at 1000g for 1 min twice and then incubated with PI (1 mg ⁄ mL) to identify sperm nuclei. Smears were then washed with PBS twice. Ten μl of spermatozoa were then smeared on polylysine-coated slides and examined using a fluorescent microscope Two individual filters were utilised to capture the nuclear signal (PI; excitation 540–565 nm, emission 605–660 nm) and the Lectin PNA signal (Conjugate Alexa Flour; excitation 465–495 nm, emission 515–555 nm). Sperm were categorised as either capacitated, non

capacitated or acrosome reacted as previously described using this method (Baker, *et al* 2004). A minimum of 200 sperm were counted per animal. At 4 h post insemination sperm were taken out of the insemination drops and the process repeated as above. Comparisons were then made between the two groups and the two time points.

2.2.11 *Statistical analysis*

All data presented are expressed as mean ± SEM. For all data collected, differences between males fed a control diet compared to a HFD were assessed using a generalised linear model where individual males were included in the model as a co-variate. Between treatment assessments were established using least significant difference. Proportional data were analysed using binary logistic regression analysis. A minimum power of 80% was used for assessment between treatment differences.

2.3 Results

2.3.1 Body weights and composition

Mice fed a HFD gained significantly more weight after the nine-week period compared with the mice fed the control diet (16.0g vs. 8.8g; $p<0.001$). The weight difference between the two cohorts became significantly different after three weeks on the diet and increased over the remaining six weeks, such that at the end of the nine week feeding period, males were significantly different in average body weight ($30.5g \pm 0.6$ vs. $26.8g \pm 0.5$, $p<0.001$; Figure 2.1).
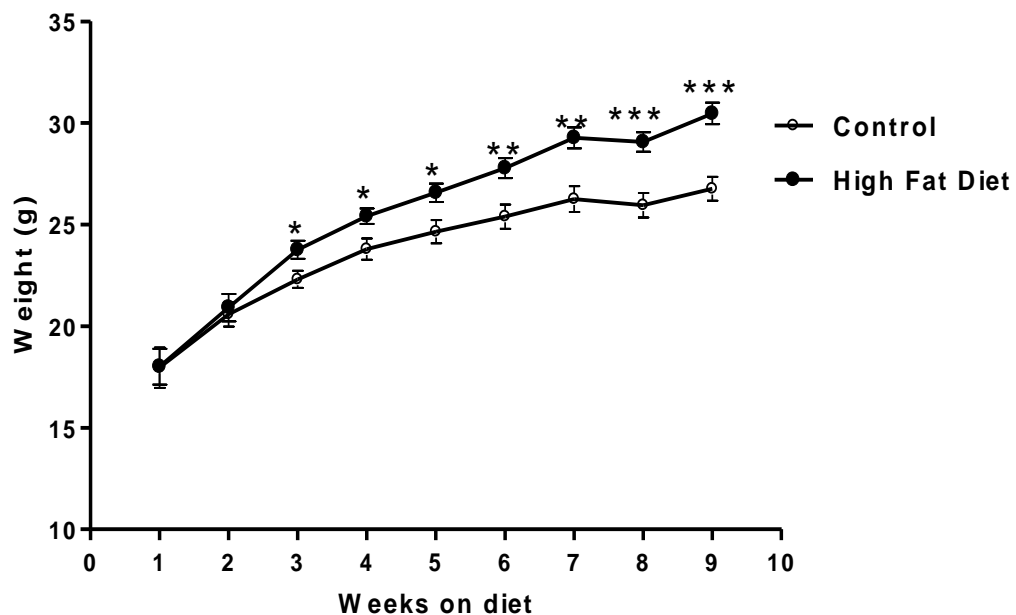
Figure 2.1: The effect of HFD on body weight gain.

Note: Data expressed as mean ± SEM.
N=12 males per treatment group.
*$p<0.05$, ** $p<0.01$, *** $p<0.001$.

Analysis of the body composition of the animals confirmed that there was increased adiposity in the males fed a HFD as demonstrated by significantly increased amounts of peritoneal and retroperitoneal fat (Table 2.1). This increase in adiposity did not normalise for

body weight indicating that there was a disproportionate increase in fat content in the males fed a HFD. The weights of the liver and pancreas were significantly increased in the mice fed a HFD compared with controls, although this was normalised when expressed as a percentage of total body weight (Table 2.1).

The weight of the testes and seminal vesicles were not significantly different between the two groups (Table 2.1).

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Table 2.1: The effect of HFD on body components.

	Right Testis (g)	Left Testis (g)	Seminal Vesicles (g)	Adipose Tissue (peritoneal) (g)	Adipose Tissue (retroperitoneal) (g)	Total Fat (g)	Liver (g)	Pancreas (g)
Control	0.09 ± 0.00	0.08 ± 0.01	0.27 ± 0.02	0.39 ± 0.03	0.15 ± 0.08	0.42 ± 0.07	1.49 ± 0.07	0.15 ± 0.01
HFD	0.09 ± 0.01	0.09 ± 0.01	0.30 ± 0.02	0.87 ± 0.13	0.29 ± 0.06	0.92 ± 0.16	1.81 ± 0.06	0.19 ± 0.01
Significance	NS	NS	NS	0.00	0.16	0.02	0.001	0.006

Note: Data expressed as mean ± SEM.
N=12 males per treatment group.

2.3.2   Hormonal blood analysis

Male mice fed the HFD had lower testosterone levels compared with the control group (1.66 ± 0.93ng/mL vs. 4.29 ±1.95ng/mL), although this difference did not reach statistical significance (Figure 2.2).
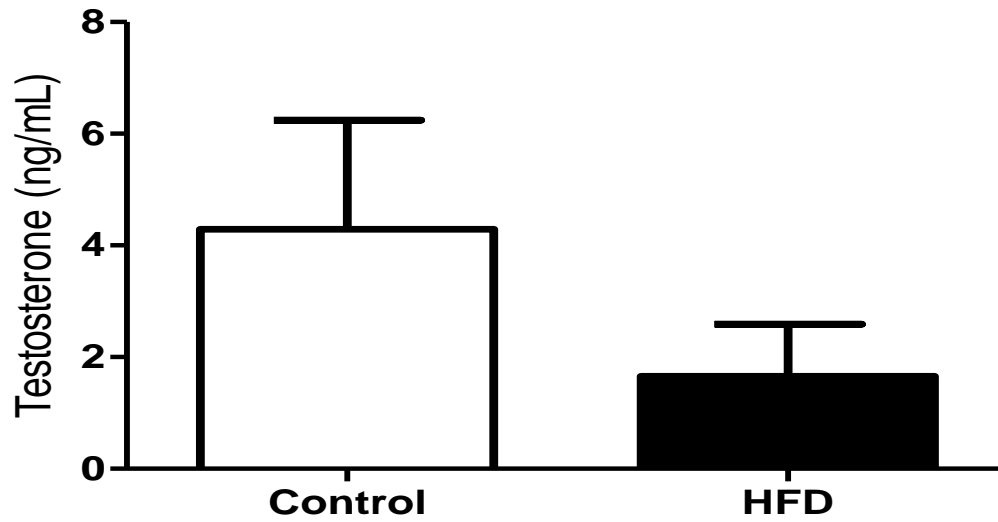
Figure 2.2: The effect of HFD on plasma testosterone levels.

Note: Data expressed as mean ± SEM.
N=12 males per treatment group.
No statistically significant difference was detected between groups.

2.3.3   Metabolic blood analysis

Males fed a HFD had significantly higher levels of cholesterol and triglycerides compared to the control fed males (Figure 2.3). No differences were detected for glucose or free fatty acids.
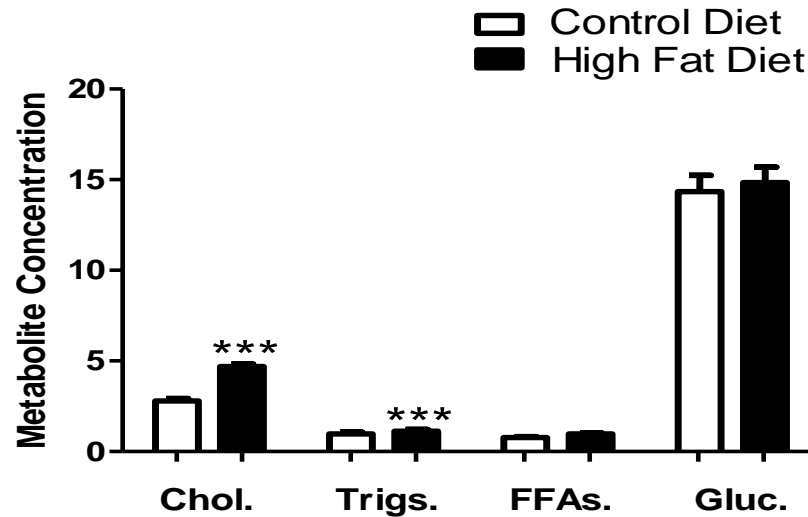
Figure 2.3: The effect of HFD on plasma metabolite levels.

Note: Cholesterol (Chol; mmol/L), Triglycerides(Trigs; mmol/L), Free Fatty Acids (FFAs; mEq/L) and Glucose (Gluc; (mol/L).
Data expressed as mean ± SEM.
N=9-10 males per treatment group.
*** p<0.001.

2.3.4   The effect of HFD on conventional semen analysis

No significant differences were found in relation to sperm concentration between the two diet groups (13.5 ± 1.7 x10^6 for HFD and 16.1 ± 3.4 x10^6 for control diet). In contrast, the percentage of motile spermatozoa was significantly decreased in the HFD group compared with controls ($36 \pm 2\%$ vs. $44 \pm 4\%$; $p < 0.05$).

2.3.5   The effect of HFD on sperm intracellular reactive oxygen species and mitochondrial reactive oxygen species

Intracellular ROS levels in the sperm were significantly elevated (692 ± 83 units vs. 409 ± 22 units, $p < 0.01$) from males fed a HFD compared to controls as measured by H_2DCFDA fluorescence (Figure 2.4). The percentage of MSR positive sperm was also increased ($33 \pm 3\%$ vs. $19 \pm 5\%$, $p < 0.05$) in the HFD group compared with controls (Figure 2.5).
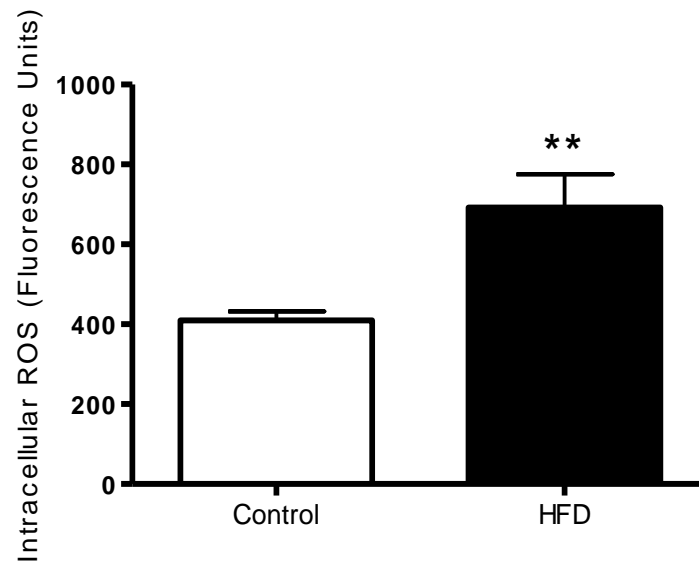
Figure 2.4: The effect of HFD on intracellular ROS.

Note: Data expressed as mean ± SEM.
N=12 per treatment group.
**p<0.01
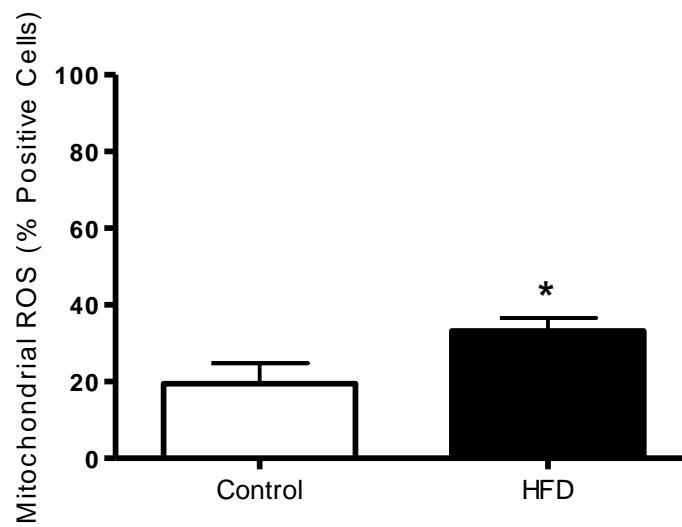
Figure 2.5: The effect of HFD on mitochondrial ROS.

Note: Data expressed as mean ± SEM.
N=10 per treatment group.
*p<0.05

2.3.6 The effect of HFD diet on sperm DNA damage

Only 5/12 (41.7%) mice fed a control diet exhibited sperm with any DNA damage compared with 10/12 (83.3%) in the HFD group (p=0.045). The percentage of sperm DNA damage (TUNEL positive) within the sperm samples of each animal was also increased (1.64±0.6% vs. 0.17±0.06%) in the HFD group compared with the control group (p<0.05; Figure 2.6).
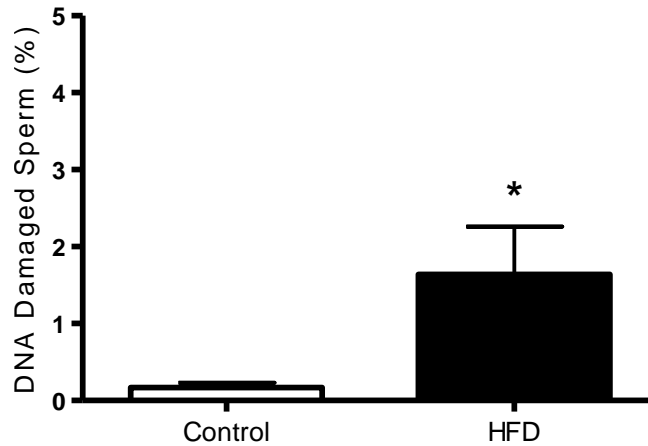
Figure 2.6: The effect of HFD on Sperm DNA damage.

Note: Data expressed as mean ± SEM.
N=12 per treatment group.
*p<0.05

2.3.7 The effect of HFD on sperm binding and fertilisation

Following 4 h of co-incubation with cumulus-enclosed oocytes the number of sperm bound to each oocyte was counted. Interestingly, the number of bound sperm was significantly lower (41.14±2.5 vs. 58.39±2.4; p<0.01) in the HFD group compared to controls (Table 2.2). Moreover, the percentage of fertilised oocytes was significantly lower (25.89% vs. 43.85; p<0.01) in the HFD group compared to controls (Table 2.2).

Table 2.2: The effect of HFD on sperm capacitation, acrosome reaction, sperm binding and fertilisation rates.

	Control	**HFD**	**Significance**
Capacitation at insemination (%)	21.1±5.1	12.3±2.5	<0.01
Capacitation 4 h post insemination (%)	36.8±3.3	34.6±2.1	ns
Acrosome reaction at insemination (%)	12.9±1.9	12.1±1.3	ns
Acrosome reaction 4 h post insemination (%)	45.1±2.8	49.6±4.1	ns
Sperm binding numbers 4 h post insemination	58.4±2.4	41.1±2.5	<0.01
Fertilisation rate (%)	43.9±4.2	25.9±4.7	<0.01

2.3.8 The effect of HFD on capacitation and acrosome reaction at insemination and following sperm binding

The percentage of non-capacitated sperm was significantly lower after 1 h of incubation in medium GIVF in males fed a HFD compared to controls (12.34% vs. 21.06%, p<0.01). However, the percentage of acrosome-reacted sperm did not differ between the groups (Table 2.2).

Following 4 h of co-incubation with cumulus-enclosed oocytes no difference was observed between the two groups in terms of capacitation or acrosome reaction.

2.4 Discussion

To establish the direct effects of diet-induced obesity on sperm function it is necessary to establish a model where the effects of diet-induced obesity can be determined in the absence of altered glucose homeostasis such as those changes associated with type-II diabetes. A diabetic state is known to cause significant effects on sperm. To date the current literature has only established the effects of obesity on sperm function in conjunction with significantly altered glucose homeostasis. In the current study C57/Bl6 mice were fed a HFD for nine weeks to facilitate a more direct assessment on both basic and molecular aspects of sperm function and capacity to fertilise an oocyte. Males fed this diet had significantly higher body weight; which was predominantly a result of an increase in adipose tissue. These males also had increases in levels of serum triglycerides and cholesterol; however, plasma glucose levels were not different.

The current study demonstrates that males fed a HFD for a period of nine weeks to induce obesity have impaired spermatozoa as evidenced by a reduction in motility, increased levels of intracellular and mitochondrial levels of ROS, increased sperm DNA damage, lower levels of capacitation and reduced ability to bind and fertilise an oocyte. These data therefore provide direct evidence that the metabolic health of the male can have a significant impact on sperm function parameters that are associated with infertility.

In humans, previous studies have focused on the effects of male BMI on standard semen analysis parameters. Limitation of previous studies in the human are confounding factors such as genetics, other lifestyle factors and the limitation to basic parameters of sperm health such as concentration and motility. While the majority of previous studies confirm a negative relationship between sperm concentration, motility and male obesity (Fejes, *et al* 2005a, Hammoud, *et al* 2008b, Hofny, *et al* 2009, Koloszar, *et al* 2005, Magnusdottir, *et al* 2005, Stewart, *et al* 2009), others reported different findings (Nicopoulou, *et al* 2009, Pauli, *et al* 2008).

Although the current data showed no significant reduction in sperm concentration, this may still be consistent with previous studies in the human (Nicopoulou, *et al* 2009, Pauli, *et al* 2008). Given that the mice in the current model were only on the diet for nine weeks, effects would not be expected to be seen that are associated with more extreme levels of obesity.

The reduction in motility observed in the current study is consistent with a recently published report using a mouse model of long-term HFD consumption in conjunction with hyperinsulinemia and elevated serum glucose (Ghanayem, *et al* 2010). This impairment to sperm motility may be due to sperm lipid peroxidation. The current data showed increased levels of intracellular ROS as well as increased production of mitochondrial ROS, which may suggest an increase in ROS leakage from complex III of the mitochondria (Chen, *et al* 2003). Therefore, it is hypothesised that this increase in ROS may have induced lipid peroxidation in spermatozoa, which may in turn impair sperm motility (Kadirvel, *et al* 2009).

A previous report has shown that overweight and obese humans demonstrate an increased level of endothelial oxidative stress (Silver, *et al* 2007). It is believed that this is the first report providing evidence that male obesity induces oxidative stress in spermatozoa. Spermatozoa are particularly susceptible to oxidative stress because their plasma membranes contain large quantities of polyunsaturated fatty acids (Alvarez and Storey 1995) and their cytoplasm contains low concentrations of scavenging enzymes (Aitken and Fisher 1994, de Lamirande and Gagnon 1995, Sharma and Agarwal 1996). Oxidative stress attacks not only the sperm's plasma membrane but also its DNA (Aitken 1999, Saleh, *et al* 2002). The current study hypothesises that the mice fed a HFD may also have an increase in DNA damage as a result of this increase in ROS levels.

The current results confirmed that mice fed a HFD did show an increase in the level of DNA damage in their sperm. Studies in humans have also reported that obese men are likely to have a higher level of DNA damage in the sperm (Chavarro, *et al* 2010, Kort, *et al* 2006). One clear difference in the results from the mouse and human is the levels of DNA damage in

the sperm. In the mouse, the percentage of DNA-damaged sperm are low, and mouse sperm are particularly resistant to DNA damage (Bennetts and Aitken 2005). As expected the majority of males did not have any DNA damage in the control group.

The results in relation to oxidative stress and DNA damage taken together are significant given that both of these parameters are closely related to fertilisation rate, embryo development, and pregnancy and miscarriage rates (reviewed, Aitken and De Iuliis 2007b). Furthermore, increased level of DNA damage in mouse spermatozoa has previously been shown to decrease the numbers of developing fetuses but also impair the long-term-behavioural outcomes of the offspring (Fernandez-Gonzalez, *et al.* 2008, Perez-Crespo, *et al* 2008a).

A possible mechanism for the effects of obesity seen in this study is heat. Obese mice may have increased adiposity around the testes, which may elevate scrotal temperature, and thus impair sperm function. Heat has previously been shown to impair sperm function, including elevated levels of sperm DNA damage in mice (Paul, *et al* 2008, Perez-Crespo, *et al* 2008a). Heat in itself has been proposed as a potential cause for increased levels of ROS in sperm. However, studies to confirm this suggestion are lacking (Setchell 1998a).

Another possible mechanism to explain male obesity and decreased fertility is the altered hormone levels whereby obesity may lead to a decrease in testosterone levels. Obese men consistently have been found to have lower serum testosterone and higher estrogen levels than their normal weight counterparts (Jensen, *et al* 2004, Pauli, *et al* 2008). However, while some studies have identified a relative hypogonadotrophic state in obese men (de Boer, *et al* 2005, Giagulli, *et al* 1994b, Pauli, *et al* 2008), others have reported serum LH and FSH levels compatible with normal weight controls (Aggerholm, *et al* 2008a, Jensen, *et al* 2004). Although in the current study testosterone levels were decreased in the HFD group, this did not reach statistical significance so that testosterone is an unlikely cause especially given that sperm concentration did not significantly decrease. A previous study similarly concluded that

associations between paternal BMI and semen quality were found to be statistically significant even after adjustment for reproductive hormones (Qin, *et al* 2007). This suggests that a hormonal explanation as the sole mechanism for the effects seen is unlikely. However, it should be emphasised that the current study only exposed the mice to the HFD for nine weeks. It is very likely that testosterone levels may significantly fall in the event of exposure to a longer period of time.

This study for the first time assessed the ability of sperm obtained from mice fed a HFD to undergo capacitation, the acrosome reaction and to bind and fertilise an oocyte. It was demonstrated that the percentage of capacitated sperm was lower compared to controls. This finding is consistent with the current finding of reduced motility and those of others where hyperactivity has also been shown to be reduced (Ghanayem, *et al* 2010). This clearly resulted in lower sperm binding to oocytes. Clinically, reduced sperm binding is an indicator of a sperm defect (WHO 1999). While a previous study (Ghanayem, *et al* 2010) has recently reported significant reduction in pregnancy rates, the current study for the first time reports a significant reduction in the ability of sperm from mice fed a HFD to fertilise an oocyte. This result formed the basis of Chapter 3.

In summary, this study has clearly demonstrated that paternal diet-induced obesity leads to significant impairment to many sperm function parameters, including decreased motility and capacitation, increased oxidative stress and DNA damage. Further, the ability of the sperm to bind and fertilise an oocyte was decreased as a result of diet-induced obesity. These results are an important addition to the emerging evidence of the relationship between obesity and male infertility.

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3 The effect of paternal diet-induced obesity on preimplantation embryo development in the mouse

3.1 Introduction

Obesity and its associated co-morbidities present significant health concerns throughout the world and, indeed, overweight and obesity are prevalent in the population of people trying to conceive. In Australia, 62% of the population aged 18 years and over are obese or overweight (National Health Survey 2007–2008), a figure reflective of many countries, including the United States (Health, United States 2008) and England (Health Survey for England 2007). More specifically, the prevalence of obesity in young, reproductive age men has tripled since the early 1970s (National Center for Health Statistics 2008). The increasing incidence of obesity in this population of people is cause for concern given the extensive literature highlighting the increased infertility with elevated body mass index (BMI), although the focus has been mainly on women with less emphasis acknowledging the impact of male obesity on fertility.

Women who are overweight or obese (BMI≥25) have an increased time to pregnancy and a reduced probability of conceiving, relative to normal weight women (Hassan and Killick 2005, van der Steeg, *et al* 2008), as well as an increased risk of early and recurrent miscarriage (Lashen, *et al* 2004). For women undergoing assisted reproduction (ART), a progressive increased risk of spontaneous abortion and an impaired response to gonadotrophin treatment has been reported with increasing BMI (Fedorcsak, *et al* 2004, Wang, *et al* 2002), although this is not always the case (Dechaud, *et al* 2006). Women who are overweight or obese generally have more adverse outcomes when undergoing ART (Ku, *et al* 2006, Lenoble, *et al* 2008, Loveland, *et al* 2001), although this is less prevalent in younger women (Martinuzzi, *et al* 2008). Maternal obesity reduced the mean embryo grade and utilisation rate (Metwally, *et al* 2007) and, with evidence from oocyte donation studies suggesting that recipient obesity increases the risk of spontaneous abortion (Bellver, *et al* 2003), it appears that the combined effect of reduced oocyte quality and endometrial receptivity contribute to the infertility described for maternal obesity.

In comparison, factors affecting male fertility are relatively less well understood, with a small number of studies recently examining the effect of elevated body weight on sperm parameters and serum hormone levels. Couples with overweight and obese male partners had an increased odds ratio in the time to conceive (Nguyen, *et al* 2007a, Ramlau-Hansen, *et al* 2007b). In normal men, increasing BMI is consistently associated with reduced serum sex hormone binding globulin (SHBG), testosterone and inhibin B levels (Aggerholm, *et al* 2008b, Winters, *et al* 2006b), and follicle stimulating hormone (FSH) (Pauli, *et al* 2008), an association also described for men seeking ART (Chavarro, *et al* 2010). A more pertinent model to the obesity prevalent in our population today is diet-induced obesity (DIO); however, studies of male fertility in such models are scarce. A reduction in the rate of plugging and the pregnancy rate has recently been described for DIO male mice (Ghanayem, *et al* 2010). Furthermore, there has been no examination of male obesity on the development and quality of preimplantation embryos. Therefore, the aim of this study was to establish and characterise the effect of paternal obesity on embryo development in a mouse model. In addition, the effect on cell lineage was also examined.

3.2   Materials and methods

3.2.1   Mice, dietary intervention and collection of reproductive material

All experimental procedures were conducted in accordance with the National Research Council's (NRC) publication *Guide for Care and Use of Laboratory Animals* and were approved by the University of Adelaide Animal Ethics Committee.

From 6 weeks of age, male C57Bl/6 mice (Laboratory Animal Services, Adelaide) were fed one of two diets for eight weeks duration. The high-fat diet (HFD, Obese group) provided 22% fat (0.15% cholesterol), 19% protein and 49.5% carbohydrate (SF00-219, Glen Forrest, Australia) and the matched control diet (CD, Control group) provided 6% fat, 19% protein and 64.7% carbohydrate (SF04-057, Specialty Feeds). The semi-pure diets were sterilised by gamma radiation. All males were housed in pairs and feed was available *ad libitum*.

Body weight was recorded for individual males at the commencement of the experiment, and weekly thereafter until the completion of the feeding period (12 males per group in total). Blood samples were taken from a subset of males (n=12–13 per diet group) via cardiac puncture under anaesthetic, and plasma metabolites were measured using enzymatic calorimetry and a COBAS Mira automated sample system. Plasma glucose, cholesterol, free fatty acids and triglycerides were measured using the assay kits, as described previously in Chapter 2. Following euthanasia by cervical dislocation, fat (retroperitoneal and peritoneal deposits) and testes were dissected, and weight was recorded as per Chapter 2.

3.2.2   Media composition

The media used for embryo culture were G1 and G2 sequential medium (Version 3, Vitrolife, Sweden, containing 10% human serum albumin), and G-MOPS was used as a collection and handling medium (Mitchell*, et al* 2009). All other chemicals were from Sigma (Seven Hills, Australia) unless specified otherwise.

3.2.3   *Embryo collection and culture*

From 14 weeks of age, control and obese males were paired with 21–26 day old, control-fed CBAxC57Bl/6 females over night. Females were treated with 5IU of eCG (Folligon; Intervet, Australia) via an intra-peritoneal injection, and 44 h later injected intraperitoneally with 5IU hCG (Pregnyl; Organon, The Netherlands) prior to housing with males. The presence of copulatory plugs was detected the following morning, and one-cell embryos were collected from the oviduct 22 h after hCG administration. One-cell embryos were cultured to the blastocyst stage in G1 and G2 sequential medium (Vitrolife, Sweden), for 48 h then 48 h respectively. All embryo culture media was equilibrated at $37^{o}C$ in 6% CO_2 5% O_2 89%N_2 at least 4 h before embryo culture. Embryo culture was conducted in 20µl drops under mineral oil with embryos grouped such that development could be determined for each male individually.

3.2.4   *Embryo morphological assessment*

Embryo morphology was assessed using a phase contrast microscope at the following time-points after hCG injection; 42 h (day 2), 66 h (day 3), 97 h (day 4) and 114 h (day 5). Embryos were classified using the following criteria; two-to-eight cells, morula (compact structure), early blastocyst (blastocoel cavity comprises less than one-third of the embryo), blastocyst (blastocoel cavity comprises greater than one-third, but less than two-thirds of the embryo) expanded blastocyst (blastocoel cavity comprises greater than two-thirds of the embryo), and hatching blastocyst (clear herniation of the zona pellucida).

3.2.5   *Blastocyst cell differential staining*

Blastocyst-stage embryos were examined using a differential staining protocol to determine the allocation of cells to the trophectoderm (TE) and inner cell mass (ICM), using the protocol of Gardner and colleagues (Gardner, *et al* 2000). Stained embryos were washed in 100%

ethanol then mounted in glycerol on a microscope slide and visualised using a fluorescent microscope under a UV filter. The TE (appearing pink) and ICM (appearing blue) cell nuclei were counted independently and reported as average cell number per blastocyst (blastocysts from 3–5 males per diet).

3.2.6 *Blastocyst DNA damage assessment*

A TUNEL (terminal deoxynucleotidyl transferase biotin-dUTP nick end labelling) technique was used to determine the number of DNA-damaged cells per blastocyst and expressed as a proportion of total cells (blastocysts from 3–5 males per diet) (Kamjoo, *et al* 2002).

3.2.7 *Statistical analysis*

Statistical analysis was undertaken using SPSS (Version 17, SPSS, Chicago, IL). Differences between paternal dietary treatments in body and organ weights, plasma metabolites, and blastocyst cell numbers were determined using a univariate general linear model and a least significant difference (LSD) test. Proportional embryo development on each day was determined using binary logistic regression analysis.

3.3   Results

3.3.1   *Physiology of control and HFD male mice*

The average body weight for male mice fed the control diet compared with those fed a high-fat diet (HFD) began diverging significantly after 3 weeks on the experimental diets (p<0.001). This difference persisted throughout the remaining feeding period (p<0.001).

3.3.2   *Paternal obesity and embryo development*

To establish the effect of male obesity on embryo development following in-vivo mating, 1-cell zygote stage embryos were recovered from female mice and cultured in vitro to the blastocyst stage. Embryo cleavage to the 2-cell stage was significantly reduced with paternal obesity (79.1% vs. 50.5% for control vs. HFD males respectively, p<0.001) (Table 3.1).

On day 3 of culture, the proportion of embryos that were on time (i.e. an 8-cell embryo with or without compaction) was significantly less when embryos were produced by HFD males (78.8% vs. 55.6% for control vs. HFD males, p<0.001) (Table 3.1).

This delay in development persisted when embryos were assessed on day 4, such that significantly more embryos from the HFD males were still at the morula stage (32.7% vs. 62.1% for control vs. HFD males respectively, p<0.001) (Table 3.1) and significantly less were at the early blastocyst, blastocyst, expanded or hatching blastocyst stage of development, compared with embryos from their control male counterparts (57.0% vs. 26.5% respectively, p<0.001) (Table 3.1).

Table 3.1: The effect of paternal HFD on embryo development on day 2, 3 and 4 of culture.

	Control	HFD	Significance
Day 2 cleavage (%)	79.1	50.5	***
Day 3 on-time (%)	78.8	55.6	***
Day 4 ≤ 8-cell (%)	10.3	11.3	NS
Day 4 morula (%)	32.7	62.1	***
Day 4 ≥ early blastocyst (%)	57.0	26.6	***

Note:
Day 2: Number of embryos cleaved on day 2 as a proportion of total one-cell embryos recovered.
Day 3: On-time embryos are those scored morphologically as 8-cells or greater on day 3, expressed as a mean proportion of those cleaved on day 2.
Day 4: Data expressed as mean proportion of embryos at each stage, as calculated from the number cleaved on day 2.
≤8-cells= 8-cells or less, morula stage, ≥ early blastocyst= includes early blastocyst, blastocyst, expanded or hatching blastocyst.
Data represents n=8 males per group (from 387 and 319 zygotes in total from control vs. obese males respectively).
Data expressed as mean. Superscript *** $p<0.001$ for significant differences between the control and obese groups.

On the final day of culture (day 5), 25.5% of embryos from HFD males were still at the morula stage or less developed, compared with 5.2% of embryos from control males ($p<0.001$, Table 3.2) Furthermore, only 25.5% of embryos from HFD males were at the hatching or hatched morphological stage compared with 44.1% of embryos from control males ($p<0.001$, Table 3.2).

Thus embryos from HFD males had delayed morphological development from the first cleavage stage throughout the preimplantation period compared with embryos from their control counterparts.

Table 3.2: The effect of paternal HFD on embryo development on day 5 of culture.

	Control	**HFD**	**Significance**
Day 5 ≤ morula (%)	5.6	25.5	***
Day 5 EB, B and ExpB (%)	45.2	49.1	NS
Day 5 ≥ HB (%)	46.0	25.5	***

Note:
Embryo development data expressed as mean proportion of embryos at each stage, as calculated from the number cleaved on day 2.
≤Morula =scored as morula stage or less, EB, B and ExpB= early blastocyst, blastocyst or expanded blastocyst, ≥HB= hatching or hatched blastocyst.

3.3.3   Paternal obesity and blastocyst cell numbers

For embryos that reached the blastocyst stage, the number of trophectoderm (TE), inner cell mass (ICM) and DNA damaged cells was determined as a measure of mitosis and differentiation. Blastocysts from HFD males had significantly less cells in total ($p<0.001$), primarily as a result of a decrease in cells forming the ICM cell lineage ($p<0.001$) but also due to a reduction in TE cells ($p<0.05$), although the proportion of these cells did not differ (Table 3.3).

3.3.4   Paternal obesity and blastocyst DNA damage

Further, blastocysts from HFD males had a greater apoptotic cell index (proportion of total cells that were apoptotic), such that 21.3% compared with 14.1% of cells were apoptotic for HFD and control males respectively ($p<0.05$, Table 3.3).

Table 3.3: The effect of paternal HFD on embryo development on day 5 of culture and average total, trophectoderm and inner cell mass cell number, and proportion of apoptotic cells in blastocyst stage embryos.

	Control	HFD	Significance
Total cell number	47.9 ± 2.0	34.7 ± 3.8	***
Trophectoderm (TE) cell number	32.1 ± 1.7	24.6 ± 3.1	**
Inner cell mass (ICM) cell number	15.8 ± 0.8	10.1 ± 1.0	***
Proportion ICM (%)	33.3	30.3	NS
Proportion apoptosis (%)	14.1	21.3	**

Note:

Cell number data represents embryos from n= 3-5 male mice per treatment (41 and 16 embryos from control and HFD males respectively).

Apoptosis data represents embryos from n= 3-5 male mice per treatment (34 and 12 embryos from control and HFD males respectively).

Proportion ICM= ICM number as a proportion of total cells, Proportion apoptosis= number of apoptotic cells as a proportion of total cells.

Data are mean ± SEM. Superscript *** $p<0.001$ and ** $p<0.05$ for significant differences between control and HFD groups.

3.4   Discussion

This was the first study to document preimplantation embryo development and quality in a mouse model of paternal DIO. The consequences of maternal obesity on conception and pregnancy have been examined extensively in animal models and in humans, yet the implications of paternal obesity for all aspects of embryo development and pregnancy are relatively under researched. Understanding the repercussions of male obesity on fertility and pregnancy is pertinent given its increasing incidence, potentially influencing the medical advice for couples attempting to conceive and, of utmost concern, modulating the health and development of their offspring.

In this study, it has been shown that paternal obesity reduced the cleavage rate of zygotes fertilised in vivo and development to the 8-cell stage on day 3 of in-vitro culture. Furthermore, this delayed development persisted throughout the culture period, such that on-time development on day 4 (ability to form an early blastocyst) was decreased and on day 5, a significant proportion of embryos remained at the morula stage, with significantly less embryos reaching the hatching blastocyst stage, compared with embryos from their control counterparts. This delay in normal preimplantation development has been reported for other paternal stresses in rodents, including diabetes (Kim and Moley 2008) and a mild scrotal heat stress (Paul*, et al* 2008, Zhu*, et al* 2004), paternal heat stress (Zhu*, et al* 2004) and advanced paternal age in humans (Luna*, et al* 2009).

Of those embryos that did reach the blastocyst stage, paternal obesity reduced the total number of cells, from both the inner cell mass and trophectoderm cell populations, with no significant change in the proportion of each cell lineage. Decreased blastocyst cell number is reflective of reduced viability (Kwong*, et al* 2000, Lane and Gardner 1997, Mitchell*, et al* 2009). To understand whether this decrease was related to decreased cell cycle progression or increased cell apoptosis, a TUNEL staining system was used to identify apoptotic cells. This showed that blastocysts from HFD fathers had a marked increase in the proportion of

apoptotic cells present. A similar increase has been reported for embryos exposed to various stresses, including diabetes and hyperglycaemic culture conditions (Pampfer, *et al* 1997), single embryo culture (Brison and Schultz 1997) and culture in media with elevated ammonium levels (Lane and Gardner 2003b). Although paternal heat stress in mice has been shown to cause a similar reduction in blastocyst cell numbers (Zhu and Setchell 2004), the majority of studies have examined blastocyst cell number and apoptosis when the embryo or oocyte has been exposed to a treatment, with few studies specifically examining the consequences of paternal-specific effects.

As discussed in Chapter 2, a potential mechanism responsible for the observed reduction in blastocyst development is increased DNA damage in sperm from HFD fathers. Increased DNA fragmentation in sperm was reported for overweight and obese men (Kort, *et al* 2006), and was recently confirmed by Chavarro and colleagues, who showed more sperm with DNA damage in obese men, using a different laboratory technique (Chavarro, *et al* 2010). There has been some assessment of embryo development following in-vitro fertilisation (IVF) or intra-cytoplasmic sperm injection (ICSI) with DNA-damaged sperm in animal models. For example, IVF using irradiated bovine sperm did not impair fertilisation but it blocked development to blastocyst by increasing apoptosis in embryos at the second or third cleavage stage (Fatehi, *et al* 2006). Further, in the mouse, zygotes from irradiated mouse sperm (Shimura, *et al* 2002) or those generated from frozen-thawed (FT) sperm (Perez-Crespo, *et al* 2008b) cleaved and developed normally during early cleavage stages. However, the implantation rate was decreased with FT sperm (Perez-Crespo, *et al* 2008b) and a dose-dependent decrease in fetal development was reported with increasing irradiation (Shimura, *et al* 2002). Finally, in humans there is convincing evidence that increased DNA fragmentation or damage is associated with poor development to blastocyst, reduced post-implantation development and decreased pregnancy rate (Bakos, *et al* 2008, Borini, *et al* 2006b, Seli, *et al* 2004b). As discussed in Chapter 2, it was determined that sperm from obese males used in

this study had elevated levels of DNA damage and reactive oxygen species (Bakos, *et al* In Press).

In summary, this study has shown that paternal obesity caused delayed embryo development from the first cleavage event throughout the preimplantation period and that blastocysts were of lower quality as assessed by reduced cell numbers and apoptosis. Both of these parameters are important indicators of implantation potential.

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4 The effect of paternal body mass index on embryo development and live birth rates following assisted reproductive technology

4.1 Introduction

Obesity and its health consequences are an increasing health burden in the developed world. In Australia, 62% of the population aged 18 years and over are obese or overweight (National Health Survey 2007–2008), with similar figures reported in other developed countries, including the United States (Health, United States 2008) and England (Health Survey for England 2007). It is now clear that maternal obesity reduces fertility, predominantly through actions on the oocyte that affect the health of the resultant pregnancy (Stothard, *et al* 2009). However, the potential role of male obesity in infertility has been essentially ignored until the last two to three years. This is surprising as 50% of reproductive age males are overweight (Begg, *et al* 2008). Several examples are provided of how paternal health around the time of conception affects the health of the offspring. One of the best characterised is the effects of paternal smoking, which has been associated with an increased prevalence of asthma, respiratory tract infection, diabetes and obesity in offspring (Chang 2009, Sorahan, *et al* 1997, Toschke, *et al* 2007). There is also a known causal association between paternal age and significant medical conditions in the offspring such as autism (Lazarou and Morgentaler 2005). Similarly, children born to fathers of specific occupations are shown to be at an increased risk of developing congenital abnormalities (Lin, *et al* 2008, Sung, *et al* 2009). Furthermore, there is also an association with increased paternal body mass index (BMI) around the time of conception with an increase in the BMI of the offspring (Danielzik, *et al* 2002, Li, *et al* 2009).

While, the effect of paternal obesity on sperm function has been studied (Jensen, *et al* 2004, Kort, *et al* 2006) and systematically reviewed (Macdonald, *et al* 2009), the effect of paternal obesity on embryo development and subsequent pregnancy and live birth outcomes has not been determined in the human. Therefore, the aim of the current study was to

determine whether or not there is an association between male BMI, fertilisation, embryo development, pregnancy and live birth rates following assisted reproductive technology (ART).

4.2 Materials and methods

Patients included in this study attended the infertility clinic, Repromed, Dulwich, South Australia, between January and May 2008. A total of 305 couples undergoing a fresh ART cycle were included in this study. Couples where the female partner was less than 38 years of age (as outlined by Reproductive Technology Accreditation Committee) at the time of oocyte collection were included to minimise the effect of maternal age as a confounding factor. Couples undergoing donor or frozen gamete treatment were excluded from the study. Frozen sperm cycles were excluded as cryopreservation has been shown to induce sperm oxidative stress and DNA damage (Thomson, *et al* 2009), which may result in poorer outcomes following ART. None of the men had any significant symptoms or signs of andrological dysfunction. A retrospective chart review was performed to obtain the patients' semen analysis, ART cycle outcomes, and paternal and maternal age. Approval was granted from Repromed's Institutional Review Board. No identifying information was used in this study.

4.2.1 *Paternal and maternal BMI*

Paternal and maternal height (meters) and weight (kilograms) were measured at the clinic prior to the treatment cycle and converted into a Body Mass Index (BMI = kg/ m^2). Couples were classified according to the following paternal BMI ranges: normal weight BMI 20–24.9 kg/m^2, overweight 25–29.9 kg/m^2, obese 30–34.9 kg/m^2 and morbidly obese $\geq$35 kg/m^2.

4.2.2 *Conventional semen analysis*

Evaluation of sperm samples for the initial diagnosis prior to the treatment was performed and reported according to the World Health Organisation methods and criteria (WHO 1999). After abstinence of 1–6 days, semen samples were collected by masturbation in sterile containers. When necessary, samples were transported to the laboratory maintained at room temperature.

Samples were analysed within 1 h after collection in all cases and as recommended by the 1999 World Health Organisation Laboratory Manual for the Examination of Human Semen and Sperm-Cervical Mucus Interaction. Sperm motility was determined manually under 40 x magnifications and expressed as a percentage of motile sperm in a given sample. Duplicate measures of two hundred sperm were counted for motility assessment. Sperm concentration was determined using an improved Neubauer haemocytometer and applying the appropriate dilution factor. The percentage of morphologically normal sperm was also calculated using the Quick Diff staining method as described by the 1999 WHO manual. Two hundred sperm were counted for each sample (WHO 1999).

4.2.3   Sperm isolation using density gradient separation

Motile sperm were separated from semen samples using 40% and 80% Sperm Grad (Series III, Vitrolife AB, Gothenburg, Sweden). Semen was layered onto the gradient and centrifuged at 100g for 20 min. The pellet was removed and washed once in medium G-Sperm (Series III, Vitrolife AB, Gothenburg, Sweden) at 300g for 10 min and resuspended in medium G-sperm (Series III, Vitrolife AB). All sperm was maintained for a minimum of 3 h at 37°C in 6% CO_2 in air before use.

4.2.4   Ovarian stimulation

All female partners in this study underwent controlled ovarian stimulation using recombinant FSH (Puregon, Schering-Plough, Sydney, Australia; Gonal F, Serono, Sydney, Australia) and a GNRH antagonist on day 2 of the cycle. rFSH (125-300IU daily) was administered based on the women's age prior IVF response, and ovarian reserve status assessed by antral follicle count and serum Anti-Mullerian Hormone measurement. The GnRH antagonist (Orgalutran, Schering-Plough, Sydney, Australia) was given from day 7 of the cycle and continued until the day of hCG administration. Follicle development was monitored using both ultrasound

and blood levels of estrogen and progesterone. Ovulation was induced by hCG administration (5000 IU Pregnyl, Schering-Plough, Sydney, Australia; 250 gm Ovidrel, Serono, Sydney, Australia) when 2 or more follicles $\geq$ 17 mm in diameter were present (Tremellen and Lane 2010). Cumulus oocyte complexes were aspirated from the ovarian follicles under trans-vaginal ultrasound at 36 h following the administration of the hCG

Luteal support was provided by a combination of daily vaginal progesterone (Crinone 8%, Serono, Sydney, Australia) and a single injection of 500 IU hCG (Pregnyl, Schering-Plough, Sydney, Australia).

4.2.5 Fertilisation assessment, embryo culture, cleavage and grading

Oocytes were collected and either inseminated using conventional IVF or ICSI as per Schoolcraft *et al.* (1999). Insemination occurred between 4 and 6 h after oocyte collection. For conventional IVF, oocytes were placed in 50µl drops of fertilisation medium with 30,000 spermatozoa. For ICSI, a single motile spermatozoon was selected and injected into the oocyte. Fertilisation was assessed the following morning (16–18 h post-insemination) by the presence of 2 pronuclei and 2 polar bodies (2PN). All 2PNs were cultured in groups of 2 to 4 in G1 medium (Series III, Vitrolife AB). Cleavage-stage embryo morphology assessment was based on embryo cell number and the degree of fragmentation. For day 3 embryos assessed at 66 h post-insemination, a grade 1 embryo was considered to have 8 cells and no fragmentation. Grade 1 and 2 embryos had a minimum of 7 cells with less than 10% fragmentation and lacked multi-nucleated cells. Blastocyst quality was determined by the degree of blastocyst expansion and by assessment of the inner cell mass and trophectoderm as previously described (Schoolcraft, *et al* 1999). All decisions about which embryos to transfer were based on morphology. Good quality embryos were defined as those receiving scores of either grade 1 or 2, as these were considered suitable for freezing.

4.2.6 Embryo transfer and pregnancy outcomes

Embryos were transferred in 10 µL of EmbryoGlue medium (Vitrolife) using a Sydney IVF Transfer Catheter (Cook Australia, Queensland, Australia) under ultrasound guidance. Biochemical pregnancy was determined when the serum ßhCG of 2 consecutive blood tests was >20 IU from day 14 following embryo transfer. Clinical pregnancy was determined by presence of a viable fetal heartbeat on ultrasound 4–6 weeks after embryo transfer. Implantation rates were measured by the presence of fetal sac. Live birth outcomes were received from the external obstetrician as required for the Australian and New Zealand Assisted Reproduction Database (ANZARD) regulatory reporting, including date of delivery, number of babies, weight, gender and complications. Pregnancy loss was defined as any loss occurring between a positive ßhCG test and live birth.

4.2.7 Statistical analysis

Retrospective analysis was carried out using SPSS. Generalised linear modelling was utilised using either univariate analysis for male only measures, or multivariate analysis for cycle outcomes including both male and female BMI. Pearson's coefficient was used to assess linear relationships between embryo development and pregnancy rates with paternal BMI. A P value of 0.05 was considered significant.

4.3 Results

4.3.1 *Cohort characteristics*

For this analysis patients were characterised into four groups based on paternal BMI (Table 4.1). There was no difference in paternal age across the groups. Similarly, there was no difference in maternal age. However, while there was no difference in maternal BMI for the male groupings of normal, overweight or obese, there was a significantly higher maternal BMI in the morbidly obese male BMI group compared to the normal weight group (p<0.01). For all four groups, the percentage of patients seeking treatment for male infertility was similar (Table 4.2). The percentage of patients within the respective BMI categories was 20.7%, 48.5%, 20.3% and 10.5% respectively.

Table 4.1: Characteristics of the study population according to paternal body mass index (N =305).

	Normal N=63	Overweight N=148	Obese N=62	Morbidly Obese N=32	Significance
Paternal age (yrs)	35.5±0.7	36.3±0.4	35.5±0.7	34.7±0.7	NS
Maternal age (yrs)	32.9±0.5	33.1±0.3	32.0±0.4	31.9±0.8	NS
Maternal BMI (kg/m^2)	23.9±0.7[a]	24.6±0.4[a]	26.8±0.7[a]	33.1±1.6[b]	p<0.05
Number of previous IVF cycles	0.86±0.2[a]	1.22±0.2[a]	1.08±0.2[a]	2.0±0.6[b]	p<0.05
Average number of embryos transferred	1.1±0.05	1.1±0.04	1.1±0.05	1.1±0.06	NS

Note: a-b different superscripts are significantly different p<0.05. Results expressed ± SEM.

Table 4.2: The diagnosis of infertility according to the categories of paternal body mass index.

	Normal N=63	Overweight N=148	Obese N=62	Morbidly Obese N=32	Significance
Male Factor (%)	43.4	49.2	46.6	52.3	NS
Endometriosis (%)	7.9	9.3	4.1	9.1	NS
Tubal/Pelvic Disease (%)	9.2	3.3	12.3	9.1	NS
Ovulation/ovarian defect (%)	17.1	6.0	8.2	6.8	NS
Unexplained (%)	0.0	0.5	1.4	0.0	NS
Other (%)	22.4	31.7	27.4	22.7	NS

Note: a-b different superscripts are significantly different $p<0.05$. Results expressed $\pm$ SEM.

4.3.2 Paternal BMI and conventional semen analysis

For all males, a semen analysis was performed prior to the ART cycle as part of a standard investigation process. Sperm concentration for the normal weight group was significantly higher than the three other groups ($p<0.05$). In contrast, motility was only reduced in males that were morbidly obese ($p<0.05$). There was no effect of male BMI on sperm morphology (Table 4.3).

Table 4.3: Semen quality parameters according to paternal body mass index levels.

	Normal N=63	Overweight N=148	Obese N=62	Morbidly Obese N=32	Significance
Concentration (million/ml)	78.8±12.1[a]	57.8±4.3[b]	50.4±6.4[b]	58.2±13.1[b]	p<0.05
Motility (% progressive motile)	47.7±2.6[a]	46.0±1.5[a]	46.9±2.2[a]	32.6±3.9[b]	p<0.05
Morphology (% normal)	15.1±1.8	13.4±1.1	14.0±1.9	13.9±3.6	NS

Note: a-b different superscripts are significantly different p<0.05. Results expressed ± SEM.

4.3.3 Paternal BMI and fertilisation rates and embryo development in vitro

Fertilisation rates, either overall or after conventional IVF or ICSI separately, were not altered by paternal BMI. In this study, it was not possible to establish the effects of morbid obese state on conventional IVF fertilisation as only three patients had this insemination method in the morbidly obese group (Table 4.4).

To establish whether paternal BMI influenced the quality of the embryos, the grade of embryos was assessed at both the cleavage stage and the blastocyst stage. There was no effect of paternal BMI on day 3 cleavage rates or the morphological grade of embryos. In contrast, there was a significantly linear reduction in expanded blastocyst development rates per 2PN with increasing paternal BMI (p<0.05; Table 4.4).

4.3.4 Paternal BMI and pregnancy and live birth outcomes

Initial pregnancy rates were assessed by the presence of a positive ßhCG from day 14 post embryo transfer. There was a highly significant linear reduction in pregnancy rate with

increasing paternal BMI from normal to obese males (p<0.01). This was further decreased for males who were morbidly obese; however, this latter reduction in pregnancy rate was a combined effect of both high paternal and maternal BMI (p<0.01). Implantation rate (as measured by the presence of fetal sac) decreased as paternal BMI increased (Table 4.5). Conversely, there was a significant overall increase in pregnancy loss with increasing paternal BMI (p<0.05; Table 4.5).

Similarly, viable on-going pregnancy rates (as measured by the presence of fetal heart at the 4–6 week scan) decreased as paternal BMI increased (Table 4.5).

Live birth rates were significantly reduced by increasing paternal BMI (p<0.05; Table 4.5). There was no difference in gestation length (range from 36.2±0.6 to 36.4±0.4) or baby weight (range 3054g to 3212g) across the four groups nor any effect on sex ratios.

Table 4.4: Embryo development outcomes according to paternal body mass index.

	Normal N=63	**Overweight N=148**	**Obese N=62**	**Morbidly Obese N=32**	**Significance**
IVF Fertilisation Rate (%)	67.2	58.5	60.2	ND	NS
ICSI Fertilisation Rate (%)	75.0	72.5	78.7	65.4	NS
Day 3 Grade 1 and Grade 2 embryos /2PN (%)	55.1±4.6	61.5±2.8	61.3±4.6	42.1±7.4	NS
Day 5 On-time blastocyst development /2PN (%)	29.3±4.3	27.8±3.1	20.3±3.9	18.7±5.7	$p<0.05^{+}$
Day 5 expanded blastocyst / 2PN (%)	17.9±3.3	15.2±2.2	10.7±2.9	8.5±4.2	$p<0.05^{+}$

Note: [+]Linear decrease in blastocyst development with increasing BMI $p<0.05$. Results expressed ± SEM.

Table 4.5: Pregnancy and live birth outcomes according to paternal body mass index.

	Normal N=63	Overweight N=148	Obese N=62	Morbidly Obese N=32	Significance
ßhCG/OPU (%)	46.03^a	36.49^b	35.48^b	15.15^c	$p<0.05+$
ßhCG/ET (%)	50.88^a	41.54^{ab}	38.60^b	20.83^c	$p<0.05+$
SAC/OPU(%)	44.44^a	31.76^b	32.26^b	12.12^c	$p<0.05+$
SAC/ET (%)	49.12^a	36.15^b	35.09^b	16.67^c	$p<0.05^+$
HEART/OPU (%)	42.86^a	29.73^b	25.81^b	12.12^c	$p<0.05^+$
HEART/ET (%)	47.37^a	33.85^b	28.07^b	16.67^c	$p<0.05^+$
Pregnancy Loss (%)	10.3^a	38.5^b	36.4^b	20.0^{ab}	$p<0.05$
Live Birth/OPU (%)	41.3^a	26.4^b	22.6^b	12.12^c	$p<0.05^+$

Note:
Data is expressed both per oocyte pick up (OPU) and also per embryo transfer (ET).
a-b different superscripts are significantly different $p<0.05$. $^+$Linear decrease with increasing BMI $p<0.05$.

4.4 Discussion

This study represents the first attempt in the literature to determine the effect of paternal obesity on embryo development, pregnancy and live birth outcomes following assisted reproductive technology. This study demonstrates that as paternal BMI increases, several outcomes are impaired, including reduced blastocyst development, reduced clinical pregnancy rates and decreased live birth outcomes, as well as increased risk of miscarriage rates.

The first surprising result was that 79.4% of male patients in this study were either overweight, obese or morbidly obese compared to only 20.5% of those who were in the normal weight category. This was highly surprising especially given that the selection criteria for this study was primarily based around the female age (<38 yrs). This figure is also higher than the general population estimates where, in Australia, 62% of the population aged 18 years and over are either obese or overweight (National Health Survey 2007–2008). This provides evidence that overweight and obese males are perhaps more common in a subfertile population. Interestingly morbidly obese patients tended to have more ICSI treatment compared to the other three groups. This may be due to the decreased sperm motility in this group as shown in Table 4.3.

It has now been demonstrated that the incidence of oligozoospermia triples from 5.32% in men of a normal BMI to 15.62% in obese men (Hammoud, *et al* 2008b). Similarly, the percentage of males with low numbers of progressively motile sperm increased from 4.52% to 13.28% in normal BMI compared to obese men (Hammoud, *et al* 2008b). In a study investigating factors associated with semen quality among couples who visited an assisted reproduction clinic, the prevalence of obesity among men with male factor infertility (defined as at least two findings of density less than 10 million, total count less than 20 million, motility less than 30%) was three times greater than among male partners of couples with idiopathic or female factor infertility (Magnusdottir, *et al* 2005). Similar to other studies, this

study also showed that sperm count declined in males that were overweight, obese or morbidly obese.

It is generally accepted that obesity can be associated with reduced sperm concentrations; however, this relationship has been challenged as obese men may still have normal sperm concentrations and there is a lack of a linear relationship (Hammoud, *et al* 2008a). There is some evidence to suggest that the accumulation of adipose tissue in men is associated with decreased serum levels of total and free testosterone (Giagulli, *et al* 1994a) and an increase in serum levels of estrogen (Schneider, *et al* 1979). The conversion of androgens to estrogen by aromatase in adipose tissue is hypothesised to be responsible for this altered hormone profile. This imbalance is thought to result in alterations in spermatogenesis such as reduced sperm concentration. The reduction in sperm concentration and total sperm count may be related to both the decrease in serum testosterone, sex hormone binding globulin (SHBG) and inhibin B and increased estradiol levels with increasing BMI (Jensen, *et al* 2004).

Studies of the relationship between male obesity and sperm motility have been less certain and show conflicting results. While some studies have shown no association between paternal BMI and the percentage of motile sperm (Fejes, *et al* 2005a, Jensen, *et al* 2004), others have shown paternal BMI to be negatively correlated with motile sperm count (Kort, *et al* 2006). This may be due to the different classifications used to determine BMI groupings between studies. In this current study an effect on sperm motility was found only in males that were classified as morbidly obese.

The majority of studies investigating the relationship between male obesity and sperm morphology have reported no relationship (Aggerholm, *et al* 2008a, Chavarro, *et al* 2010, Fejes, *et al* 2005b, Jensen, *et al* 2004, Pauli, *et al* 2008, Qin, *et al* 2007). Similarly, the current study also found no relationship between sperm morphology and BMI. Therefore, it appears there are no increases in major structural abnormalities with increasing BMI. Rather, it is apparent that molecular changes such as DNA damage are more prevalent (Chavarro, *et al*

2010, Kort, *et al* 2006). Until recently, no studies have examined accessory gland function or composition regarding paternal obesity. However, it has recently been reported a negative correlation exists between paternal BMI and seminal plasma neutral α-glucosidase levels (Martini, *et al* 2010). Therefore, these data collectively suggest that obesity-induced manifestations affect testicular and/or epididymal milieu to induce damage to the sperm post spermiogenesis events.

Moreover, increased testicular temperature may also play a role in obesity related impaired spermatogenesis, as obese men tend to be more sedentary. The sitting position has been shown to produce up to a 2 degree Celsius increase in scrotal temperature compared to a standing position (Jung, *et al* 2005). It is therefore plausible that the effects seen on sperm function may be due to heating of the testicles as spermatogenesis is very sensitive to increases in temperature (Ivell 2007). Animal models would certainly support this theory (Setchell 1998b).

Our data in relation to embryo quality suggests no correlation between paternal BMI and cleavage-stage embryo development up until day 3. This is perhaps not surprising, as it is generally accepted that the paternal genome is not activated until the 4–8 cell stage in humans and therefore the influence of paternal BMI on sperm health and function would be limited prior to this stage (Artley, *et al* 1992). In contrast, at the later stages of embryo development after activation of the embryonic genome, the data in the current study showed a significant decrease in the proportion of expanded blastocysts (per 2PN) as paternal BMI increased. This is consistent with other studies, where other paternal factors such as sperm DNA damage have similarly shown no association with early embryo development (Bakos, *et al* 2008, Benchaib, *et al* 2003, Greco, *et al* 2005, Sun, *et al* 1997), but negatively associated with blastocyst development (Seli, *et al* 2004a). Interestingly, there have been two recent studies demonstrating that increased paternal BMI is associated with increased sperm DNA damage

(Chavarro, *et al* 2010, Kort, *et al* 2006). Therefore, it is hypothesised that the decrease in blastocyst development observed in the current study may be due to increased DNA damage.

This study demonstrated that paternal obesity at the time of conception is associated with reduced pregnancy rates and live birth rates following ART treatment. This observation is consistent with a recent study on IVF outcomes and epidemiological studies where obese fathers have been shown to have a decreased chance of fathering a pregnancy (Jokela, *et al* 2008, Jokela, *et al* 2007, Keltz, *et al* In Press, Nguyen, *et al* 2007b, Ohwaki, *et al* 2009, Ramlau-Hansen, *et al* 2007a, Sallmen, *et al* 2006). For example, Nguyen *et al*. showed that the odds ratio for infertility was 1.20 for overweight men (BMI 25–29.9) and 1.36 for obese men (BMI 30–34.9) relative to men with normal BMI (20.0–22.4) (Nguyen, *et al* 2007b). These epidemiological studies show that this decrease is not mediated by sexual dysfunction in heavier men (Nguyen, *et al* 2007b), perhaps suggesting that sperm molecular mechanisms are more likely to be the cause.

The effect of maternal obesity on fertility has been described in several studies in recent years (Robker 2008). The results of the current study coupled with results in animal models (Bakos, *et al* In Press, Chavarro, *et al* 2010) suggest that equally paternal obesity affects fertility and pregnancy outcomes. Also, in the current study it is clear that a male partner with a very high BMI was significantly more likely to seek ART treatment with an obese female partner. However, none of the studies examining the effects of maternal BMI on pregnancy outcome and IVF have accounted for paternal BMI. This study therefore highlights a likely confounding factor and may possibly explain, at least in part, some of the discrepancy reported in recent reviews in relation to maternal BMI effects (Robker 2008).

Weight loss in women is associated with the return of spontaneous ovulation and a reduced likelihood of requiring induction of ovulation (Clark, *et al* 1995). In fact, a recent meta-analysis in relation to maternal obesity and assisted conception stated that ―weight loss should be considered in overweight women (i.e. BMI ≥25) before initiating assisted

reproduction" (Maheshwari*, et al* 2007). Clearly, this highlights the importance in determining the effect of weight loss programs on paternal reproductive health and fecundity.

One limitation of the current study is that it does not take into consideration any genetic confounding factors. Some obesity syndromes with known genetic defects are specifically associated with male infertility due to loss of function of a single gene responsible for both obesity and spermatogenesis, (Mah and Wittert 2009); for example the Alms1 gene in Alström Syndrome (Arsov*, et al* 2006). Similarly, a number of genes in chromosomal region (15q11–q13) lead to both obesity and defective spermatogenesis; for example Prader-Willi Syndrome and Angelman Syndromes (Buiting*, et al* 2003). Therefore, future studies would benefit from some genetic testing to determine if there are any genetic factors associated with the increased prevalence seen in an obese and infertile population.

Finally, this study represents the first steps in determining the effects of paternal obesity on subsequent reproductive health beyond sperm parameters, including embryo, pregnancy and live birth outcomes. Larger multi-centre studies are required to confirm the results observed in this study. However, enquiries made during this study have revealed that the majority of units do not routinely capture this information. ART units are therefore encouraged to consider adding male weight and height to their standard assessment parameters. Clearly this study highlights the importance in determining the effect of weight loss on paternal reproductive health and fecundity.

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5 Seminal plasma composition: Obesity metabolic markers and human sperm function

5.1 Introduction

Constituents of seminal plasma have a vital role in the promotion of sperm survival, conditioning of the female tract to tolerate the conceptus and the molecular and cellular changes in the endometrium required to facilitate pregnancy (Robertson 2005). However, the area of seminal metabolites in relation to sperm function has received little attention in the literature. This is especially surprising given that many metabolic markers have been shown to be present in seminal plasma at the same concentration or higher than serum. Until recently, no studies have examined seminal plasma composition with regard to paternal obesity. Recently, however, it has been shown that male BMI correlates with seminal plasma neutral α-glucosidase levels (Martini, *et al* 2010) suggestive of a relationship between seminal plasma composition and male obesity.

A typical example of a seminal plasma metabolite is insulin, which has been shown to exert a stimulatory effect on sperm glucose metabolism (Hicks, *et al* 1973). Insulin in the seminal plasma from fasting non-diabetic donors has been shown to be significantly higher than that of serum (19 ± 3 µU/mL vs. 7.5 µU/mL respectively) (Hicks, *et al* 1973).

Glucose, another seminal plasma metabolite, is present in semen at a range of concentrations (0.22–16.37 mM), reviewed by Owen and Katz (2005). Glucose is suggested to be an important source of energy for spermatozoa (Martikainen, *et al* 1980, Peterson and Freund 1971). Its role in supporting motility and capacitation has been challenged, however, as other glycolysable sugars have been shown to be as effective in supporting these functions (Williams and Ford 2001). Its source of production is largely unknown. However, some may be present endogenously within the sperm as glycogen (Ballester, *et al* 2000), which is why it has been hypothesised previously that it may be important in maintaining motility and allow spermatozoa to capacitate in glucose-free media (Ford 2006). Glucose is supplied by the female reproductive tract (van der Linden, *et al* 1992) as well as culture media in human IVF.

Clearly, levels of cholesterol, free fatty acids (FFAs) and triglycerides have been implicated in obesity-induced pathologies as shown in Chapter 1; where males fed a high fat diet had significantly higher levels of plasma cholesterol and triglycerides compared with the control-fed males.

In order to further elucidate the causes for the observed perturbations of sperm outlined in Chapter 4, seminal plasma composition in a cohort of patients was studied in relation to male BMI. The objectives of the current study were therefore to evaluate semen from men grouped by their BMI in relation to: (1) sperm quality parameters such as volume, concentration, motility, morphology and DNA damage as well as (2) the levels of obesity metabolic markers in the seminal plasma such as insulin, glucose, FFAs, cholesterol and triglycerides.

5.2   Materials and methods

5.2.1   Cohort characteristics

Semen samples were obtained from fasted male members of couples being studied for infertility who attended the Andrology laboratory at Repromed, Adelaide, South Australia (n=69). This prospective study was performed during 2007–2008, and written informed consent was obtained from all participants. Ethical approval was obtained from the University of Adelaide and Women's and Children's Hospital in Adelaide, South Australia. Approval was also obtained from Repromed's Scientific Advisory committee. A chart review was performed to obtain the paternal age and length of abstinence. All assessments of sperm function and seminal metabolites were performed blinded to the BMI group.

5.2.2   Body mass index

Paternal height (meters) and weight (kilograms) were measured on the day of producing the sample (BMI = kg/ m^2). Waist to hip ratio was also obtained. Men enrolled in this study were classified into four groups by BMI: normal (18.5–24.9), overweight (25–29.9), and obese (30–34.9) and morbidly obese (=>35).

5.2.3   Semen analysis

Evaluation of sperm samples on the day of study was performed according to the World Health Organisation criteria. After abstinence of 1–6 days, semen samples were collected by masturbation in sterile containers. When necessary, samples were transported to the laboratory while maintained at room temperature. Samples were analysed within 1 h after collection in all cases as recommended by the 1999 World Health Organisation Laboratory Manual for the Examination of Human Semen and Sperm-Cervical Mucus Interaction. Sperm motility was determined manually under 40 x magnifications and expressed as a percentage of

motile sperm in a given sample. Duplicate samples of two hundred sperm were counted for motility assessment. Sperm concentration was determined using an improved Neubauer haemocytometer and applying the appropriate dilution factor. The percentage of morphologically normal sperm was also calculated using the Quick Diff staining method as described by the 1999 WHO manual. Two hundred sperm were counted for each sample (WHO 1999).

5.2.4   Sperm DNA damage

Spermatozoa were smeared on polylysine-coated slides (Menzel-Glaser, Braunschweig, Germany), air-dried and fixed with 4% paraformaldehyde in phosphate-buffered solution (PBS; JRH Biosciences, Lenexa, KS, USA), for 1 h at room temperature. Smears were then permeabilised with 0.5% Triton X-100 in 0.1% sodium citrate for 1 h, washed twice with phosphate-buffered solution (PBS; JRH Biosciences, Lenexa, KS, USA) and incubated with the terminal deoxyribonucleotidyl transferase-mediated dUTP nick-end labelling (TUNEL). The assay was performed using a Cell Detection Kit (Roche, Mannhein, Germany) for 1 h at 37°C. Smears were then washed twice with PBS and stained with propidium iodide (PI, 1 mg ∕ mL) to identify sperm nuclei. Smears were then washed with PBS twice and a drop of a pre-prepared mixture of gold antifade reagent (Molecular Probes, Eugene, OR, USA) and 99% Glycerol (Sigma Chemical Co., New South Wales, Australia) applied. Slides were examined using fluorescence microscopy. Two individual filters were utilised to capture the nuclear signal (PI; excitation 540–565 nm, emission 605–660 nm) and the TUNEL signal (FITC; excitation 465–495 nm, emission 515–555 nm). The two captured images were superimposed using IPLab software (version 3.61; Scanalytics, Inc., Rockville, MD, USA). The percentage of sperm DNA damage was calculated as the number of TUNEL positive sperm from the total number of sperm nuclei. At least two hundred sperm were counted for each sample as previously described by Bakos *et al.* (2008).

5.2.5   Metabolic profiling

For the measurement of seminal plasma metabolites, semen was centrifuged at 1000g for 5 min. Seminal plasma was then removed and frozen at –20ºC until the samples were thawed and analysis conducted for each metabolite as described below.

Seminal plasma was measured for insulin using a commercially available radioimmunoassay kit specific for porcine insulin (Linco, Abacus ALS). Standard (100 µl) and unknown (100 µl) plasma samples were pipetted into tubes, ^{125}I-insulin (100 µ) and then 100 µl of antibody was added and allowed to incubate on a shaking rack for 2 h at room temperature. Double antibody suspension (2 ml) was then added and allowed to incubate for a further ½ h at room temperature. Samples were centrifuged for 10 min at 1500g. Tubes were then decanted and left upside down on absorbent paper for approximately 10 min, being careful not to dislodge the pellet at the bottom of the tube. The radioactivity of the pellet was determined using a gamma counter. The mean coefficient of variation was less than 5%.

The quantitative determination of seminal plasma glucose was performed with a Hitachi 912 automated sample system using the Glucose HK assay kit, with the C.f.a.s. Calibrator, and quality controls: Precinorm U and Precipath U (Roche Diagnostics, NSW, Australia). The mean coefficient of variation was less than 3.3%.

The quantitative determination of seminal plasma non-esterified FFAs was performed with a Hitachi 912 automated sample system using the NEFA-C FFA assay kit (Wako, Japan, through NovoChem, Australia) and quality controls: QCS 1 and 2 (Bio-Rad, NSW, Australia). The mean coefficient of variation was less than 4.6%.

The quantitative determination of seminal plasma triglycerides was performed with a Hitachi 912 automated sample system using the TG assay kit, with the C.f.a.s. Calibrator, and quality controls: Precinorm U and Precipath U (Roche Diagnostics, NSW, Australia). The mean coefficient of variation was less than 3%.

The quantitative determination of seminal plasma cholesterol was performed with a Hitachi 912 automated sample system using the CHOL assay kit, with the C.f.a.s. Calibrator, and quality controls: Precinorm U and Precipath U (Roche Diagnostics, NSW, Australia). The mean coefficient of variation was less than 4%.

5.2.6   Statistical analysis

Retrospective analysis was carried out using SPSS. Generalised linear modelling was used to determine differences between across the four BMI groups with least significant difference comparison procedure used to assess for differences between individual treatments. A P value of 0.05 or less was considered significant. Values are expressed as mean ± SEM.

5.3 Results

5.3.1 Cohort demographics

For this analysis patients were characterised into the four groups based on paternal BMI. There was no difference in paternal age or abstinence across the groups (Table 5.1).

5.3.2 BMI and conventional sperm parameters

Overall, sperm concentration decreased significantly as BMI increased (117.1 ± 20.5, 21.1 ± 11.4, 62.8 ± 23.4, 32.8 ± 36.9, $p<0.01$) in the normal, overweight, obese and morbidly obese groups respectively (Table 5.1). Sperm motility, on the other hand, did not differ across the groups in the normal, overweight, obese and morbidly obese groups respectively (Table 5.1). Similarly, seminal plasma volume did not differ across the four groups (Table 5.1). However, there was a strong trend towards a decreased total motile progressive sperm numbers (140.7 ± 22.7, 48.7 ± 12.9, 67.9 ± 25.9, 41.8 ± 40.9, $p=0.08$). Further, the normal BMI group had significantly higher total progressive motile sperm numbers when compared to the other three groups (Table 5.1). The percentage of normal morphology sperm did differ between some groups; however, there was no trend associated with BMI.

5.3.3 BMI and sperm DNA damage

Sperm DNA damage did not differ across the four BMI groups in the normal, overweight, obese and morbidly obese groups respectively (Table 5.1).

5.3.4 BMI and seminal plasma insulin

Overall, fasted insulin seminal plasma concentrations increased as BMI increased (17.5 ± 4.5, 28.9 ± 4.5, 41.5 ± 2.5 and 36.1 ± 8.1, $p<0.05$) in the normal, overweight, obese and morbidly obese groups respectively (Table 5.2).

5.3.5   *BMI and seminal plasma glucose*

A trend was observed such that fasted seminal plasma glucose concentrations increased as BMI increased (2.9±0.5mM, 3.5±0.3mM, 3.5±0.5mM and 5.2±0.8mM, p=0.1). Further, there was a statistically significant difference between the normal weight group compared with the morbidly obese group (p<0.05, Table 5.2)

5.3.6   *BMI and seminal plasma FFAs, triglycerides and cholesterol*

No significant differences were observed across the four BMI categories in relation to seminal plasma levels of FFAs, triglycerides or cholesterol (Table 5.2).

Table 5.1: Characteristics and semen quality parameters of the study population according to male body mass index.

	Normal BMIN=13	Overweight N=42	Obese N=10	Morbidly Obese N=4	Significance
Age (yrs)	36.6.5±2.5	39.3±39.4	36.0±2.9	39.2±4.6	NS
Abstinence (days)	3.9±0.4	3.6±0.2	3.3±0.4	4.0±0.7	NS
Volume (mL)	3.6±0.5	3.4±0.3	3.1±0.6	3.7±0.9	NS
Concentration $(x10^6)$	117.1±20.5	21.1±11.4	62.8±23.4	32.8±36.9	p<0.01
Motility (% progressive motile)	44.2±3.1	39.8±1.8	47±3.5	41.5±5.6	NS
Total progressive motile sperm $(x10^6)$	140.7±22.7[a]	48.7±12.9[b]	67.9±25.9[b]	41.8±40.9[b]	p=0.08
Morphology (% normal)	11.5±2.0a	6.61.1b	13.9±2.2a	9.0±3.5b	NS
DNA damaged sperm (%)	40.9±7.3	46.7±4.1	36.6±8.4	44.5±13.2	NS

Note: (N =69).
a-b different superscripts are significantly different p<0.05.
Results expressed as mean ± SEM.

Table 5.2: Metabolic seminal plasma components of the study population according to male body mass index.

	Normal BMI N=13	Overweight N=42	Obese N=10	Morbidly Obese N=4	Significance
Glucose (mM)	2.9±0.5[a]	3.5±0.3	3.5±0.5	5.2±0.8[b]	p=0.1
Insulin (µU/mL)	17.5±4.5[a]	28.9±4.5 [b]	41.5±2.5 [b]	36.1±8.1[b]	p<0.05
Cholesterol (mmol/L)	0.66±0.74	1.1±0.4	0.7±0.8	0.8±1.3	NS
Triglycerides (mmol/L)	1.0±0.1	0.9±0.1	0.9±0.1	0.8±0.2	NS
Free Fatty Acids (mEq/L)	0.1±0.01	0.1±0.01	0.1±0.01	0.1±0.02	NS

Note: (N =69).
a-b different superscripts are significantly different p<0.05.
Results expressed as mean ± SEM.

5.4 Discussion

This study demonstrated that some seminal plasma components, including glucose and insulin, are increased as male BMI rises. Interestingly, however, other metabolites such as FAAs, triglycerides and cholesterol did not differ across the four BMI groups. Sperm concentration significantly decreased with increased BMI and a trend towards decreased total progressive motile sperm as BMI increased was also observed. Further, sperm DNA damage did not differ across the groups.

An early study demonstrated quite convincingly that insulin concentration in the seminal plasma and serum insulin concentration were significantly correlated (Paz, *et al* 1977). However, insulin is present at higher concentrations in semen than in serum (Povoa, *et al* 1972). On the other hand, it has been suggested previously that the concentrations of insulin per unit of total protein in rete testis fluid (RTF) and blood plasma to be similar, but because the concentration of total protein is only about 1% of that in blood plasma, the actual concentration of insulin in RTF is much lower. In any case, much of the RTF is reabsorbed in the epididymis and therefore insulin from the testes is not likely to contribute significantly to the amount in semen (B.P. Setchell and D.M. Anderson, unpublished observations in Setchell [1974]). The local secretion of insulin is predominantly both prostatic and vesicular in origin (Paz, *et al* 1977). In split ejaculates, where the first portion is of prostate origin and the second is seminal vesicles origin, the concentration of insulin was significantly higher in the second fraction of the ejaculate (Paz, *et al* 1977). Furthermore, it has also been shown recently that insulin is released by sperm (Aquila, *et al* 2005).

The function of insulin in the seminal plasma is largely unknown; however, it may be important in sperm acrosome reaction and motility parameters as evidenced by increases in total and progressive motility as well as sperm hyperactivation when insulin is added (Lampiao and du Plessis 2008). However, Paz *et al.* (Paz, *et al* 1977) concluded that an effect of exogenous insulin on glucose metabolism of washed spermatozoa was not substantiated.

Yet, this finding contradicted a previous study in which a direct stimulatory effect of insulin on glucose metabolism by sperm was found (Hicks, *et al* 1973).

The data in the current study in relation to insulin concentrations in the seminal plasma are similar to those reported by others (19 ± 3 μU/mL) (Hicks, *et al* 1973); however, this was only true for this study's normal weight group. Here it is reported, for the first time, that there is a significant increase in seminal plasma insulin levels as BMI increases. This increase also occurred despite decreasing total progressive motile sperm, and one would hypothesise an opposite trend if the source of insulin was the sperm as previously suggested (Aquila, *et al* 2005). Blood plasma insulin has been shown to be elevated in obese individuals (Pasquali, *et al* 1991, Seidell, *et al* 1990).

The current study also demonstrated that seminal plasma glucose levels increased as BMI increased. The results in relation to glucose levels are also interesting and pose the question as to whether this elevated glucose has the potential to influence sperm metabolism and function. The effects of elevated glucose levels on sperm are, to date, unclear.

Interestingly, sperm DNA damage did not differ across the four BMI groups. This is somewhat in contradiction to previous reports where sperm DNA damage has been shown to increase as BMI increases (Chavarro, *et al* 2010, Kort, *et al* 2006). Data in a similar cohort of patients confirmed this study's results where it was shown that DNA damage did not increase as a result of increased BMI (Tunc, *et al* In Press). However, seminal oxidative stress was increased (Tunc, *et al* In Press), potentially suggesting that reactive oxygen species (ROS) levels are more appropriate and sensitive markers than DNA damage. It should also be noted that Kort *et al* (2006) measured DNA damage using the SCSA assay, which only measures the susceptibility of sperm to DNA damage (reviewed in Chapter 1), whereas Chavarro *et al* (2010) measured it using the comet assay, which measures how well the chromatin is compacted within the nucleus. Conversely, in the present study and that of Tunc *et al.* (In Press), the TUNEL assay was employed, which is a direct measure of DNA integrity. Further,

it should also be mentioned that, in the current, study sperm was prepared for assessment by gradient washing prior to DNA damage assessment. As this process selects for motile sperm, this washing step may have resulted in a more even distribution across the groups. Overall, the current literature in relation to obesity and sperm DNA damage, including the present study, should be interpreted with caution. Further studies are required to assess the different DNA damage methods as well as the differences between raw semen and washed sperm preparations.

This study's data in relation to cholesterol, FFAs and triglycerides are surprising given their elevation in blood plasma in an obese state (see Chapter 1). However, this may suggest a local secretion or at least in part local regulation.

Although the composition of seminal plasma may be largely made up of secretions from the seminal vesicles and the prostate, it is unknown if constitutes of the seminal plasma represent the testicular and epididymal milieu, which would clearly have an impact on the sperm as they undergo spermatogenesis and sperm maturation respectively. This may explain the observed decrease in total progressive motile sperm associated with increased BMI in this study. Studies in relation to epididymal and testicular composition (Tuck, *et al* 1970) are currently lacking (Wales, *et al* 1966) and provide an interesting question as to what the environment is that the developing sperm encounters and if this impacts on sperm development and function.

In summary, this study demonstrated, for the first time, that seminal plasma insulin and glucose levels are elevated as male BMI increases. Other seminal plasma metabolites appear to be unaffected as BMI increases. Interestingly, sperm DNA damage did not increase as male BMI increased. Further evidence is required to confirm or refute this study's data in relation to DNA damage, and more detailed investigation into seminal plasma composition and male obesity is clearly warranted.

5.5   References

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6 Differential effects of hexoses on human sperm metabolism and function in vitro

6.1 Introduction

Currently there is a lack of information regarding how human spermatozoa regulate their energy metabolism. This is surprising considering that carbohydrate metabolism is a vital point for the understanding of sperm function and that aberrant metabolism is likely a source of reactive oxygen species (ROS) (Armstrong, *et al* 1999, Silver, *et al* 2007).

Sperm DNA damage caused by oxidative stress (reviewed in Chapter 1) is an emerging factor in the aetiology of male infertility. Sperm DNA damage has been shown to affect pregnancy outcome in the general population (Carrell, *et al* 2003) as well as fertilisation rates and embryo development following assisted reproductive technology (Bakos, *et al* 2008). There are three proposed main causes of sperm DNA damage, namely abortive apoptosis, poor protamination and increased levels of reactive oxygen species. The most plausible theory in the literature is that oxidative stress causes sperm DNA damage. Spermatozoa are particularly susceptible to oxidative stress because their plasma membranes contain large quantities of polyunsaturated fatty acids (Alvarez and Storey 1995) and their cytoplasm contains low concentrations of scavenging enzymes (Aitken and Fisher 1994, de Lamirande and Gagnon 1995, Sharma and Agarwal 1996). Oxidative stress attacks not only the fluidity of the sperm plasma membrane but also its DNA (Aitken 1999, Saleh, *et al* 2002).

A free radical attack on these polyunsaturated fatty acids generates peroxyl and aloxyl radicals that, in order to stabilise, abstract a hydrogen from an adjacent carbon generating a corresponding acid or alcohol. This attempt to stabilise results in a carbon-centred radical that combines with dioxygen, which, in turn, creates another lipid peroxide, resulting in a propagative process of lipid peroxidation throughout the cell membrane (Figure 6.1).
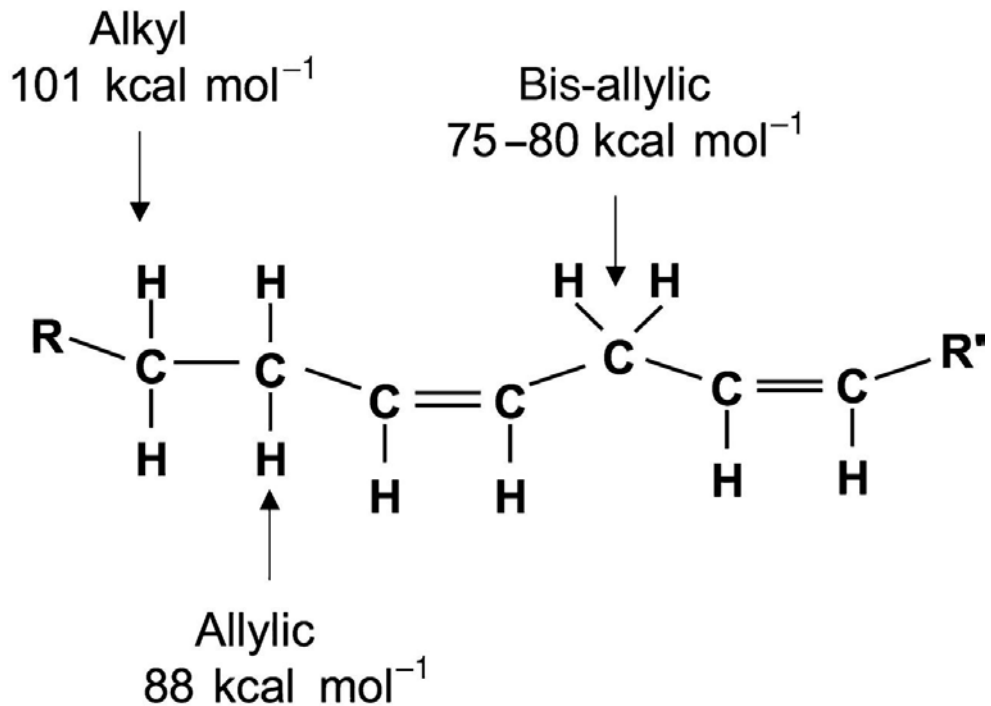
Figure 6.1: The vulnerability of polyunsaturated fatty acids to the hydrogen abstraction step in the lipid peroxidation cascade. The lowest dissociation energy is associated with the carbon in the bis-allylic position, which is particularly vulnerable to free radical attack. Figure adopted from (Aitken*, et al* 2007); Figure originally proposed by (Wagner*, et al* 1994).

It has been suggested earlier in this thesis and also by others (Aitken*, et al* 2007, Koppers*, et al* 2008a, Koppers*, et al* 2010) that mitochondria may be the source of a ROS in pathological conditions such as obesity and chemical treatment. In sperm cells, the mitochondria are localised to the midpiece, and the production of ROS in this section of the sperm is likely to result in an increase in lipid peroxidation, loss in membrane fluidity and a reduction in motility. However, currently there is little understanding as to how metabolism is controlled in sperm and whether altered provision of carbohydrates can cause this loss in metabolic control and oxidative stress.

There are two main carbohydrates within the seminal plasma: fructose and glucose. What is unique about these two sugars is that, unlike serum where glucose is present at higher levels; levels of fructose in the seminal plasma are much higher than glucose (7.55-34.86mM

vs. 0.22-16.37mM respectively) (Montagnon, *et al* 1982, Owen and Katz 2005). The reason for this difference is largely unknown. Both carbohydrates have been shown to have transporters within the sperm (Purcell and Moley 2009). Therefore, the aim of this study was to determine if altered carbohydrate metabolism affects the oxidative stress status of the sperm. In order to determine the level of oxidative stress three markers were used: (1) Sperm DNA damage; (2) Reactive oxygen species; and (3) Lipid peroxidation.

6.2 Materials and methods

All assessments of sperm were performed blinded to the concentration of metabolites in the media.

6.2.1 Sperm origin and isolation

Sperm samples were obtained from healthy fertile donors (age = 29 ±1.8 yrs), with normal semen parameters as assessed by the WHO; n=6. Samples were used either fresh or frozen using standard protocols for later analysis. Motile spermatozoa were isolated using 40 and 80% Sperm Grad gradients (Nicadon laboratories AB, Gothenburg, Sweden). Semen was layered onto the gradient and centrifuged at 100 g for 20 min. The pellet was removed and washed once in a carbohydrate-free medium at 300 g for 10 min (base medium as per Appendix 1) and re-suspended in the same medium. Each sample was then divided into nine aliquots and made to a final concentration as per Table 6.1; using the media outlined in Appendix I. Samples from all nine treatments were stored for 18 h at 37ºC in 6% CO_2 in air before function assessments of sperm motility, DNA damage, ROS and lipid peroxidation were conducted. Glucose and fructose uptakes as well as motility were measured at 3 and 6 h post incubation.

6.2.2 Sperm motility

Sperm motility was determined under x40 magnification and expressed as a percentage of motile sperm in a given sample. Duplicate measures of 200 sperm were counted for motility assessment, as recommended by the 1999 World Health Organisation laboratory manual for the examination of human semen and sperm-cervical mucus interaction (WHO 1999). Motility was assessed at 3, 6 and 18 h post incubation.

6.2.3 Media composition and treatment groups

Washed spermatozoa samples were allocated to nine media treatments with varying fructose:glucose ratios (Table 6.1 and Appendix I). Sperm were incubated for 18 h at 37ºC in 6%CO_2 in air before function assessments were conducted. Samples for glucose and fructose uptakes were taken at 3 and 6 h incubation. The rationale for using these concentrations is to reflect concentrations present in culture media (historical or current) and/or seminal plasma composition and/or pre-diabetic/diabetic status.

Table 6.1: The nine treatment groups used for these experiments.

	Fructose (mM)		
Glucose(mM)	0.0F:0.0G (1)	2.5F:0.0G (2)	24.0F:0.0G (3)
	0.0F:2.5G (4)	2.5F:2.5G (5)	24.0F:2.5G (6)
	0.0F:24.0G (7)	2.5F:24.0G (8)	24.0F:24.0G (9)

Note: Spermatozoa were incubated for either 18 h to determine the effect of varying fructose (F: mM) to glucose (G: mM) ratio concentrations on sperm DNA damage, ROS, lipid peroxidation and motility; or additionally for 3 and 6 h to determine uptake and motility.

6.2.4 Sperm DNA damage

This protocol was performed as previously described by (Bakos, *et al* 2008). Spermatozoa were smeared on polylysine-coated slides at 18 h post incubation. Slides (Menzel-Glaser, Braunschweig, Germany) were air-dried and fixed with 4% paraformaldehyde in phosphate-buffered solution (PBS; JRH Biosciences, Lenexa, KS, USA), for 1 h at room temperature. Smears were then maintained in PBS overnight at 4ºC and then permeabilised with 0.5% Triton X-100 in 0.1% sodium citrate for 1 h. Following washing twice with PBS, the smears were incubated for terminal deoxyribonucleotidyl transferase-mediated dUTP nick-end labelling (TUNEL). The assay was performed using a Cell Detection Kit (Roche, Mannhein, Germany) for 1 h at 37ºC. Smears were then washed twice with PBS and stained with propidium iodide (PI, 1 mg∕mL) to identify sperm nuclei, washed with PBS twice and a drop of a pre-prepared mixture of gold antifade reagent (Molecular Probes, Eugene, OR, USA) and 99% Glycerol (Sigma Chemical Co., New South Wales, Australia) was applied. Slides were

examined using fluorescence microscopy. Two individual filters were utilised to capture the nuclear signal (PI; excitation 540–565 nm, emission 605–660 nm) and the TUNEL signal (FITC; excitation 465–495 nm, emission 515–555 nm). The two captured images were superimposed using IPLab software (version 3.61; Scanalytics, Inc., Rockville, MD, USA). The percentage of sperm DNA damage was calculated as the number of TUNEL positive sperm from the total number of sperm nuclei. At least 200 sperm were counted for each sample.

6.2.5 Intracellular reactive oxygen species

Intracellular ROS levels were assessed in motile spermatozoa. A previously described protocol was used with the probe 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA), which fluoresces when it binds to intracellular ROS (Lane, *et al* 2002, Nasr-Esfahani, *et al* 1990). The dye was added to GIVF medium at a final concentration of 5 μM. Motile sperm were incubated with the dye for 15 min at 37°C. Spermatozoa were then washed twice with PBS by centrifugation at 1000g for 1 min. Ten μl of spermatozoa were then smeared on polylysine-coated slides and examined using a fluorescent microscope with photometer attachment. Each sperm was imaged individually, and a fluorescence reading was obtained. The relative fluorescence for each spermatozoon was expressed as mean fluorescence units. A minimum of twenty motile sperm were measured per sample. This assay was initially validated with hydrogen peroxide in a dose-dependent manner; and a positive control using UV light exposure was included in every replicate.

6.2.6 Lipid peroxidation

Lipid peroxidation is known to be a major factor in the aetiology of defective sperm function. Although techniques for measuring lipid peroxidation are available, they are relatively insensitive (Aitken, *et al* 2007). Recently, a novel fluorescence assay has been developed for

detecting lipid peroxidation. This technique has been utilised to detect lipid peroxidation (Naguib 1998) in equine (Ball and Vo 2002), bovine (Brouwers and Gadella 2003), ovine (Brouwers, *et al* 2005, Christova, *et al* 2004) and more recently human spermatozoa (Aitken, *et al* 2007). This assay is dependent upon the sensitivity of the fluorophore BODIPY® 581/591 C_{11} to oxidation by radicals (peroxyl and alkoxyl) formed from lipid hydroperoxides (Aitken et al., 2007; Drummen et al., 2002). This probe readily incorporates into biological membranes and responds to free radical attack with a spectral emission shift from red to green. This technique was performed according to (Aitken, *et al* 2007) with some minor modifications. BODIPY® 581/591 C_{11} (D3861, Molecular Probes) was added to 2 x 10^6 spermatozoa at a final concentration of 5 µM and incubated for 30 min at 37°C. The cells were washed twice at 1000 g for 10 min. 10µl of spermatozoa were smeared on polylysine-coated slides, covered with cover slips and immediately assessed using fluorescence microscopy (Menzel-Glaser, Braunschweig, Germany). Red fluorescence was measured using a filter attached to the microscope (excitation 540–565 nm, emission 605–660 nm). Images were captured using IPLab software (version 3.61; Scanalytics, Inc., Rockville, MD, USA) and the intensity of the signal in individual sperm was assessed using the same software.

6.2.7 *Carbohydrate uptake*

Carbohydrate uptake from the media was measured using fluorometric analysis where the levels of fluorescence are linearly related to the concentration of the substrate.

6.2.8 *Glucose*

The levels of glucose uptake was assessed using a two-step equation utilising the conversion of the non-fluorescent NADP+ to the fluorescent NADPH (Passonneau and Lowry 1993) (Figure 6.2).

Glucose

$$\text{Glucose + ATP} \xrightarrow{\text{hexokinase}} \text{glucose-6-P + ADP}$$

$$\text{Glucose-6-P + NADP}^+ \xrightarrow[\text{dehydrogenase}]{\text{glucose-6-P}} \text{6-P-gluconolactone + NADPH + H}^+$$
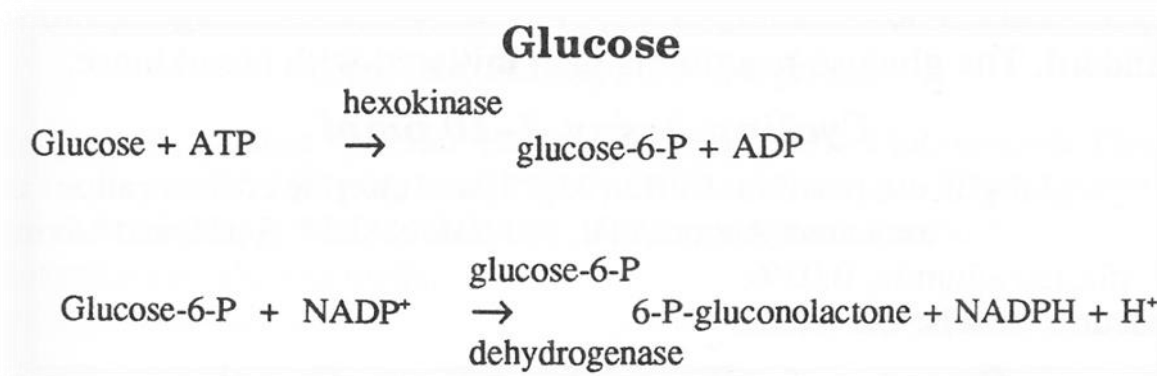
Figure 6.2: The two-step equation illustrating the conversion of the non-fluorescent NADP+ to the fluorescent NADPH. Figure adopted from (Passonneau and Lowry 1993).

A 20µl drop of substrate buffer containing all co-factors and enzymes (lacking substrate, as per Appendix I) was placed onto a siliconised slide under mineral oil. The fluorescence of this drop was recorded under UV light using a fluorescent microscope with a photometer attachment. A sample of the medium surrounding the sperm was added to the drop and the reaction was allowed to reach equilibrium by incubating at room temperature for 5 min. The fluorescence of the drop was again recorded and the difference in fluorescence compared to a standard curve performed with each analysis. The regression co-efficient (r^2) for all standard curves was >0.98. For each experimental run, a no sperm control was used to establish the starting levels of glucose. Glucose uptake was expressed as the amount of glucose taken from the media for each time point. A regression analysis was performed for each sample to establish a linear rate of glucose depletion from the media by the sperm and was expressed as nmol glucose uptake/10^7 sperm/h.

6.2.9 Fructose

The levels of fructose uptake were assessed using a three-step equation utilising the conversion of fructose-6-P to glucose-6-P, and then of the non-fluorescent NADP+ to the fluorescent NADPH (Passonneau and Lowry 1993) (Figure 6.3).

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Fructose

$$\text{Fructose} + \text{ATP} \xrightarrow{\text{hexokinase}} \text{fructose-6-P} + \text{ADP}$$

$$\text{Fructose-6-P} \xrightarrow{\text{P-gluco-isomerase}} \text{glucose-6-P}$$

$$\text{Glucose-6-P} + \text{NADP}^+ \xrightarrow{\text{glucose-6-P dehydrogenase}} \text{6-P-gluconolactone} + \text{NADPH} + \text{H}^+$$
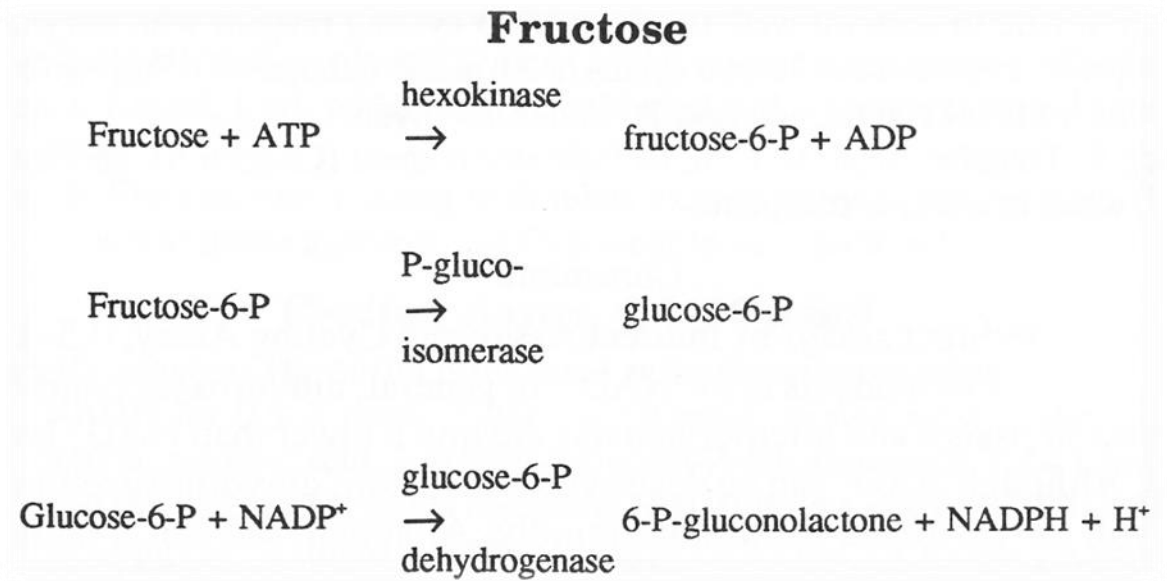
Figure 6.3: The three-step equation illustrating the conversion of fructose-6-P to glucose-6-P, and then of the non-fluorescent NADP+ to the fluorescent NADPH. Figure adopted from (Passonneau and Lowry 1993).

Validation steps were performed to establish the kinetics of the different steps of the assy. Initial analysis of Michaelis-Menton kinetics established that the assay reached completion after 5 min and was stable for up to 15 min. Saturation curves of both steps of the assay were also determined to optimise the dilutions for buffers and samples. A 20μl drop of substrate buffer containing all co-factors and enzymes (lacking substrate, as per Appendix I) was placed onto a siliconised slide under mineral oil. The fluorescence of this drop was recorded under UV light using a fluorescent microscope with a photometer attachment. A sample of the medium surrounding the sperm was added to the drop, and the reaction was allowed to reach equilibrium by incubating at room temperature for 5 min. The fluorescence of the drop was again recorded when glucoisomerase was added. This was to ensure that any glucose remaining in the media had reacted and the difference in fluorescence compared to a standard curve performed with each analysis. The regression co-efficient (r^2) for all standard curves was >0.98. For each experimental run, a no sperm control was used to establish the starting levels of glucose. Fructose uptake was expressed as the amount of fructose taken from the media for each time point. A regression analysis was performed for each sample to establish a

linear rate of fructose depletion from the media by the sperm and was expressed as nmol fructose uptake/10^7 sperm/h.

6.2.10 Statistical analysis

Analysis of all parameters was assessed using GraphPad Prism. For motility, differences between treatments at discrete time points were assessed by ANOVA. Assessment of changes in motility within treatments over time was assessed by a repeated measure two way ANOVA followed by Tukey-Kramer's test for multiple comparisons.

Differences and interactions in ROS and DNA damage as a result of glucose and/or fructose were assessed by two-way ANOVA. Between treatment, differences were assessed by Tukey-Kramer's test.

Differences in lipid peroxidation were assessed by ANOVA. Between treatment, differences were assessed by Tukey-Kramer's tests.

6.3 Results

6.3.1 Sperm motility

Motility was no different between any of the treatment groups at 3, 6 or 24 h of incubation (Figure 6.4), although for all treatment groups motility was substantially reduced after 24 h ($p < 0.01$).
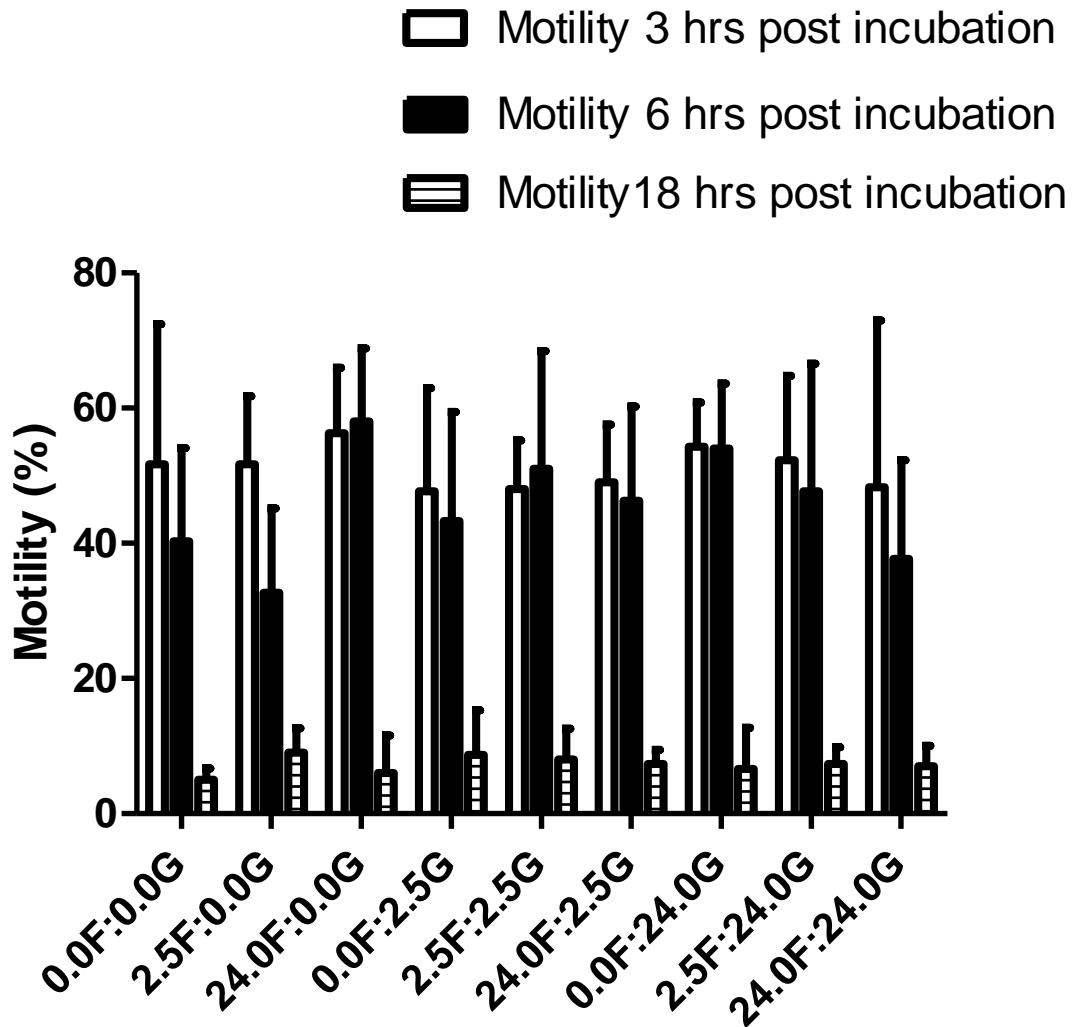

Figure 6.4: Sperm motility as assessed at 3, 6 and 24 h post incubation of the nine different media conditions.

Note: No significant differences were found across the nine treatment groups at the same time point. However, motility decreased significantly following 18 h within each treatment ($p < 0.01$).

6.3.2 Sperm DNA damage

Assessment of the number of TUNEL positive cells at 18 h of incubation increased with glucose concentration when glucose was the sole substrate in the medium, although this did not reach statistical significance. In contrast, increases in fructose concentrations did not alter the number of TUNEL positive cells (Figure 6.4). There appeared to be no interaction between the presence of glucose and fructose on sperm DNA damage.

6.3.3 Intracellular reactive oxygen species

At 18 h of incubation there were increased levels of ROS production as glucose concentration increased from 2.5mM to 25mM in the medium ($p<0.05$). In contrast, increases in fructose concentrations in the medium did not alter intracellular ROS levels in the sperm (Figure 6.5). There appeared to be no clear interaction between the presence of glucose and fructose on ROS levels. Interestingly, ROS levels were at their highest when no carbohydrate was present in the media (0.0F:0.0G). This was significantly higher than all other groups.
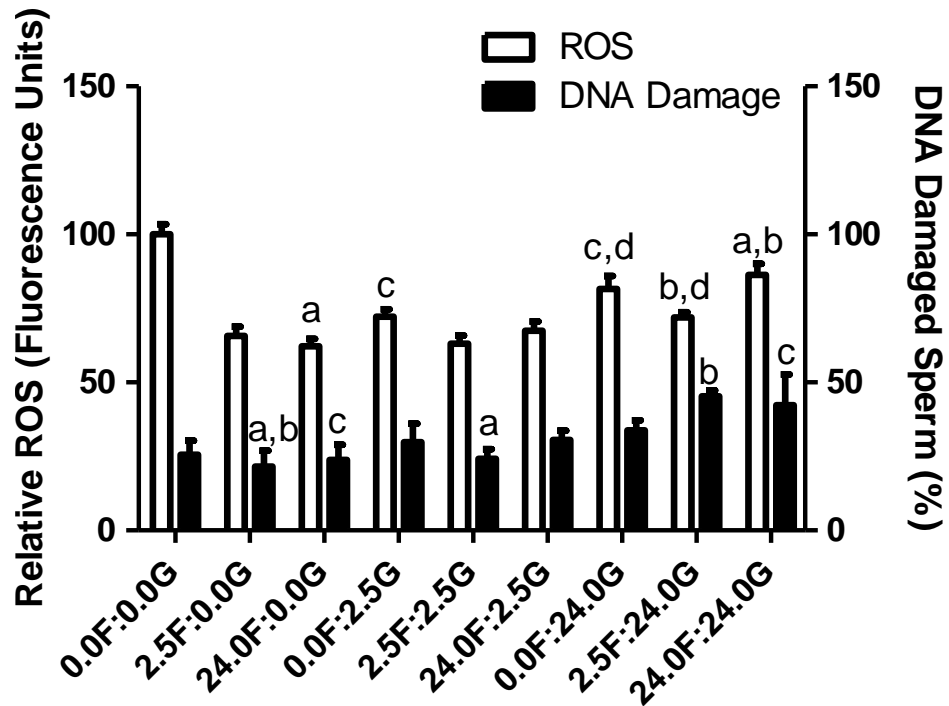
Figure 6.5: The effect of varying glucose and/or fructose concentrations on sperm DNA damage and intracellular ROS following 18 h of incubation.

Note; ROS: All treatments were different to treatment 1 (0.0F:0.0G).
[a,b] like pairs are significantly different (p<0.05).
[c,d] like pairs are significantly different (p<0.01).
DNA damage:
[a,b] like pairs are significantly different (p<0.05).
[c] like pairs are different (p=0.06).

6.3.4 Lipid peroxidation

At 18 h of incubation there were increased levels of lipid peroxidation production as glucose concentration increased from 2.5mM to 25mM in the medium (p<0.05). In contrast, there appeared to be no effect of increasing the fructose in the medium of levels of lipid peroxidation (Figure 6.6).
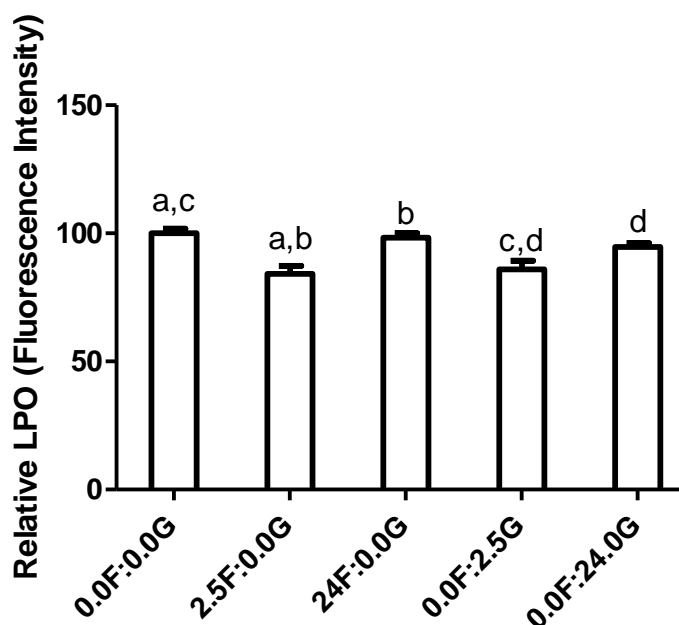
Figure 6.6: The effect of increasing glucose or fructose concentrations on sperm lipid peroxidation (LPO) following 18 h of incubation (p<0.01).

Note: [a,b] like pairs are significantly different ($p<0.01$).
[c] like pairs are significantly different ($p<0.05$).

6.3.5 Glucose and fructose uptake

6.3.5.1 Glucose uptake in the absence or presence of fructose

Glucose uptake by sperm was increased when the concentration of glucose in the medium increased from 2.5mM to 25mM ($p<0.01$). The addition of 2.5mM fructose to the medium at either glucose concentration did not alter glucose uptake by sperm (treatments 7 and 8). In contrast, by increasing the fructose concentration to 25mM there was a significant reduction in glucose uptake when glucose was present at either 2.5mM (14.4%, Table 6.2, treatment 6) or 25mM (30.3%, Table 6.2, treatment 9).

6.3.5.2 Fructose uptake in the absence or presence of glucose

Fructose uptake by sperm was similarly increased when the concentration of fructose in the medium was increased from 2.5mM to 25mM (p <0.01). The addition of glucose to the

medium at either 2.5mM or 25mM resulted in a slight increase in fructose uptakes (Table 6.3).

Table 6.2: Glucose uptake by sperm in the presence or absence of fructose in the media.

Glucose Uptake (nmol/h/10^7 sperm)						
Treatment	4	5	6	7	8	9
Glucose concentration (mM)	2.5	2.5	2.5	24.0	24.0	24.0
Fructose concentration (mM)	0.0	2.5	24.0	0.0	2.5	24.0
Mean glucose uptake	49.6 ±2.9	48.5 ±6.4	38.5 ±9.9	298.8 ±36.9	292.3 ±13.5	184.0 ±35.1
% of respective control group by fructose	Control	96.9 ±7.5*	76.6± 17.9*	Control	100.6 ±12.5**	69.7 ±11.7**

Note: * % of group (4); ** % of group (7). N=3. Data expressed as mean ± SEM.

Table 6.3: Fructose uptake by sperm in the presence or absence of glucose in the media.

Fructose Uptakes (nmol/h/10^7 sperm)						
Treatment	2	5	8	3	6	9
Glucose concentration (mM)	0	2.5	24.0	0	2.5	24.0
Fructose concentration (mM)	2.5	2.5	2.5	24.0	24.0	24.0
Mean fructose uptake	16.2 ±0.7	17.2 ±5.9	19.5 ±3.0	195.3 ±95.0	272.0 ±78.4	286.5 ±148.3
% of respective control group by glucose	Control	108.2 ±38.3*	119.7 ±16.3*	Control	121.3 ±20.8**	150.0 ±34.2**

Note: * % of group (2); ** % of group (3). N=3. Data expressed as mean ± SEM.

6.3.5.3   Total carbohydrate uptake

When either fructose or glucose were present in the medium at 2.5mM, carbohydrate uptake (glucose and fructose uptakes) was highest for glucose. When both glucose and fructose were present at 2.5mM, the glucose:fructose ratio was increased significantly, indicating a preference for glucose. In contrast, when fructose was present at 25mM this trend was reversed (Table 6.4).

Table 6.4: Total carbohydrate uptake nmol/h/10^7 sperm and the ratio of glucose to fructose uptake.

Treatment	Glucose concentration (mM)	Fructose concentration (mM)	Total carbohydrate uptake(nM)	Ratio of glucose to fructose
2	0	24.0	16.2	-
3	0	24.0	195.3	-
4	2.5	0	49.6	-
5	2.5	2.5	65.7	2.8
6	2.5	24.0	310.5	0.1
7	24.0	0	298.8	-
8	24.0	2.4	311.8	15.0
9	24.0	24.0	470.5	0.6

Note: N=3

6.4 Discussion

The results of this study have demonstrated that increased glucose concentration in the medium surrounding the sperm leads to an increased level of ROS production, which, in turn, results in elevated lipid peroxidation. Conversely, increased levels of fructose did not cause a similar increase in ROS levels and also did not perturb DNA damage. This result, therefore, indicates that this effect may be specific to the uptake of glucose and not simply an impact of an increased carbohydrate uptake. It also appears that glucose was preferentially taken up by spermatozoa, unless fructose was present at high concentrations.

Much of the ATP required by sperm is used for motility (Storey 2008). In sperm cells, the mitochondria are localised to the midpiece – the site of oxidative metabolism – whereas the head and tail are sites of cytoplasmic glycolytic activity (Ford 2006, Storey 2008). Sperm metabolism can proceed through either glycolysis, mitochondrial oxidative phosphorylation or the pentose phosphate pathway (PPP); the predominant pathway used depends, at least in part, on hexose availability (Storey 2008). Interestingly, it has been suggested that there may be some built-in back-up mechanisms in the absence of one or more of these pathways providing some reserve mechanism for metabolism (Purcell and Moley 2009). Additionally, sperm are able to utilise intracellular glycogen metabolism to support their energy requirements (Ballester, *et al* 2000). It is likely that this adaptive behaviour is the reason why sperm were still able to maintain motility even with no hexose available (treatment 1).

When glucose was the sole energy source, it was observed that increasing the levels of glucose in the environment surrounding the sperm lead to an increase in ROS levels. It was hypothesised that this increase in glucose levels may drive glycolysis and therefore oxidative metabolism leading to an increase in ROS levels (Urner and Sakkas 2005). The excessive intracellular ROS seen in the high media glucose concentrations is also likely to have caused the increased lipid peroxidation levels observed. Oxidative stress has been shown previously to increase lipid peroxidation (Aitken 1995, Aitken, *et al* 1989).

Interestingly, the presence of increasing fructose concentrations did not induce perturbation in ROS levels, as observed with glucose. This may shed some light on the long-standing question as to why fructose is present in the seminal plasma at higher concentrations than glucose. The current study suggests that fructose may play a protective role by being present at high concentrations. However, the mechanism by which fructose achieves this is currently unclear. Nevertheless, a protective role for fructose has previously been suggested, as the utilisation of fructose prevents the acrosomal breakdown of the plasma membrane while in the seminal fluid, preventing premature activation of the spermatozoa (Rogers and Perreault 1990). Furthermore, the addition of fructose to a synthetic saline medium has been shown to better mimic the rete testes fluid in relation to oxygen uptake by ram spermatozoa. Interestingly, oxygen uptake increased more significantly when fructose was added compared with glucose (Evans and Setchell 1978). This further highlights the importance of fructose in sperm metabolism.

The data in the current study adds to the current knowledge, as fructose appears to produce less oxidative stress compared to glucose; however, it is required to be present at higher concentrations than glucose to be able to compete with glucose for uptake into the sperm. It has previously been demonstrated that both GLUT 3 (Angulo, *et al* 1998, Burant and Davidson 1994, Haber, *et al* 1993) and GLUT 5 (Angulo, *et al* 1998, Burant, *et al* 1992) are present in spermatozoa. These are responsible for glucose and fructose uptake respectively. However, it is known that the V_{max} for fructose (220 µmol/mg/min) is almost twice as great as for glucose, and the K_m for fructose is four to six times greater (700 µM) (Passonneau and Lowry 1993). Therefore, it would seem likely that the ratio of these two transporters is responsible for the increased preference for glucose when the carbohydrates are present at lower concentrations.

The absence of glucose from the incubation medium has been shown previously to impair sperm function by decreasing the number of human sperm that bind to the oocyte and impair

in-vitro fertilisation rates (Hoshi, *et al* 1991, Mahadevan, *et al* 1997). However, this role has been challenged in a more recent study where other sugars such as fructose, mannose and galactose have been shown to exert similar effects to glucose on sperm function by increasing the sperm penetration rates of zona-free hamster oocytes (Williams and Ford 2001). The data in the current study indicate that the absence of any carbohydrate impairs sperm function, as evidenced by the significant increase in ROS levels compared to all other groups. However, the addition of glucose is more detrimental to sperm than fructose, at least in relation to ROS levels, when glucose is present at high concentration.

The current study, in relation to decreased sperm function in the presence of high glucose concentrations, supports the results from Chapter 5, where obese patients had elevated levels of glucose in the seminal plasma as well as impaired sperm parameters. This suggests that spermatozoa in the testicular and epididymal milieu may be exposed to an altered carbohydrate environment.

From a practical point of view, current sperm-washing media for sperm preparation for ART do not contain fructose (HTF, Cook, Global, Medicult), with the exception of its recent addition to G-IVF™ and G-IVF™ PLUS (www.vitrolife.com). Furthermore, fertilisation culture media rarely contain fructose. More experiments are required to validate the role of fructose in the protection of the sperm against cellular damage and whether the addition of fructose could lead to improvements in sperm function in vitro and therefore fertilisation and embryo development.

As far as is known, this is the first report providing evidence that altered carbohydrate metabolism may induce ROS production, lipid peroxidation and increase the number of sperm exhibiting DNA damage. This concept is novel and requires further investigation due to both its in-vivo and in-vitro implications.

6.5   References

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7 Final discussion

Sperm function constitutes the single most common cause of infertility. Therefore, factors influencing the health of the sperm and the underlying mechanisms behind any pathology are paramount.

There are many lifestyle factors affecting male health that ultimately have an impact on the sperm and therefore male infertility. Well-described examples of this include smoking (Chang 2009, Sorahan, *et al* 1997, Toschke, *et al* 2007) and advanced age (Sartorius and Nieschlag 2010). For years it has been demonstrated that these lifestyle factors could have a detrimental effect on the health of the sperm, and more recently there is the growing understanding that the legacy of these factors may have implications for the health of the offspring.

Obesity, on the other hand, has received only limited attention until very recently in relation to its impact on male fertility. While there is a consensus view regarding obese males having reduced sex hormones such as testosterone and SHBG (MacDonald, *et al* 2010, Mah and Wittert 2010), the outcomes for sperm health could only be described as correlative. Furthermore, these outcomes are confounded by other lifestyle factors such as smoking and other effects of obesity such as hyperglycemia and insulin resistance as well as potential genetic factors. This has led to this study developing a mouse model to reduce the confounding factors present in the previous human studies.

In each set of experiments two groups of mice were fed either a control diet or a high fat diet intended to resemble a Western fast food diet. This model was able to mimic some of the aspects of male obesity such as increased adiposity and altered serum metabolites (Chapter 2). Surprisingly, significant effects were seen on almost every sperm and embryo outcome measured.

At the sperm level, function was negatively affected by diet-induced obesity of the male (Chapter 2). Regarding standard sperm characteristics it was evident that sperm motility was reduced by obesity. At a molecular level, sperm from the obese males had increased levels of

intracellular reactive oxygen species. Interestingly, the sperm from these males also had increases in the numbers of sperm positive for mitochondrial-produced ROS implicating poor mitochondrial function as a source of the elevated ROS. These males also had an increase in sperm DNA damage, which is likely a consequence of the increase in oxidative stress in the sperm. It is now well established that increases in oxidative stress cause increases in DNA damage in sperm (Aitken and Fisher 1994, Aitken 1999, de Lamirande and Gagnon 1995, Saleh, *et al* 2002, Sharma and Agarwal 1996). Significantly, one of the additional consequences of increased ROS is an increase in lipid peroxidation, which would result in a decrease in membrane fluidity thereby affecting sperm motility. These findings, outlined in Chapter 2, therefore implicate oxidative stress as a major factor in the causation of the effects of male obesity on sperm function.

In addition to the effects on the molecular integrity of sperm, the functional studies also demonstrated that sperm function from obese males was significantly compromised (Chapter 2). The number of sperm bound to an oocyte was significantly reduced, which resulted in considerably reduced fertilisation rates (number of 2 PN) in the high fat diet group compared with control males. It is likely that these reduced functional measures are an effect of the reduced motility of the sperm. However, there was also evidence that the complex changes that occur within the sperm during capacitation were also altered in the sperm from the high fat males. Therefore, there may be separate mechanisms involved, which together resulted in reduced fertilisation of oocytes by these sperm. These sets of experiments conducted in Chapter 2 demonstrate both novel and significant findings in relation to paternal obesity and sperm function (Figure 7.1).
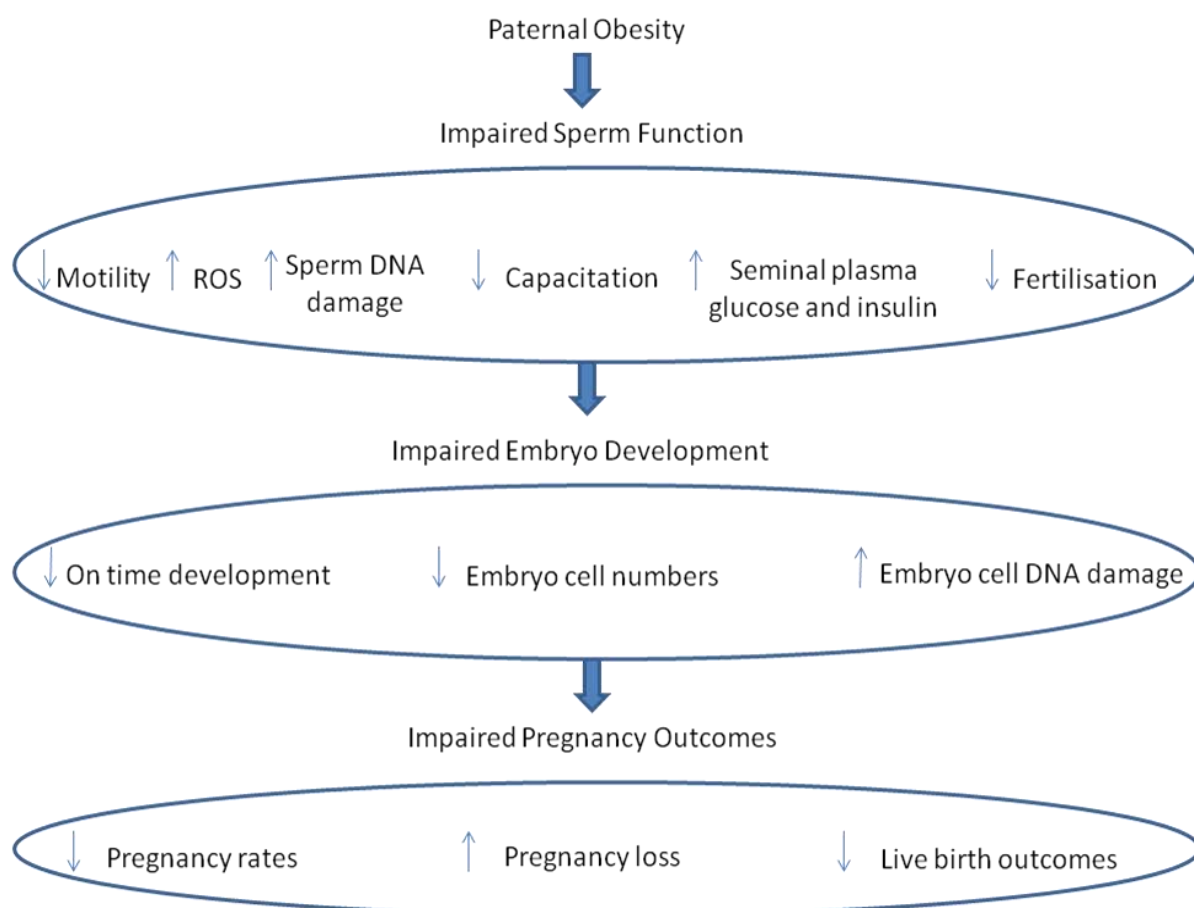
Figure 7.1: Summary of the effects of paternal obesity on sperm function, embryo development and pregnancy outcomes.

To establish further the impact that male obesity may have on natural mating and subsequent embryo development, further experiments were undertaken (Chapter 3). When the males were naturally mated to control females there was a tendency for fewer retrievable fertilised oocytes, confirming the effects on fertilisation that were observed in Chapter 2. However, a highly significant finding was that embryo development was considerably reduced in the resultant fertilised embryos. Embryo development was assessed at time-points corresponding to embryonic genome activation, and therefore activation of the paternal genome. Large reductions were found both on day 3 at the cleavage stage as well as subsequent blastocyst development. Quality assessments of the embryos by determining both cell numbers and differentiation into the inner cell mass and the trophectoderm further

showed that the embryos that did develop were significantly compromised showing fewer cells and substantial higher levels of DNA damage within the embryos (Figure 7.1). All of these features have been associated with reduced capacity to result in a viable fetus (Kwong, *et al* 2000, Lane and Gardner 1997). Indeed, recent laboratory experiments in our laboratories have shown that when these embryos are transferred to pseudo-pregnant recipient mothers, embryos generated from male mice fed a HFD showed substantial reductions in their ability to implant and develop into a viable fetus (publication 2 arising from this thesis).

Therefore, by developing a model system for the study of male obesity, fundamental changes were established to the molecular structure of the sperm from an obese male. Most significantly it was demonstrated that these changes have an impact on the ability of the male to father a normal healthy pregnancy.

However, what is ultimately of interest is how this relates to the human. Although human studies have proven to be controversial, it must be noted that almost all studies conducted to date (MacDonald, *et al* 2010) have assessed the relationship between male obesity and sperm function. In this study, on the other hand, the findings from the mouse model led to further studies assessing the relationship between paternal obesity and not only sperm function, but also human embryo development in vitro as well as pregnancy and live birth outcomes. To do this a population of couples attending infertility treatment at a private IVF unit in South Australia were studied (Chapter 4). In essence, this study confirmed the findings of the mouse model. In agreement with the previous studies, the effects on the standard parameters of a semen analysis of count, motility and morphology were minor. However, motility was reduced somewhat in morbidly obese patients. More significantly was the confirmation that male obesity affected the quality of the embryos that the couples produced with significant reductions in blastocyst development. In addition, for the first time it was demonstrated that both clinical pregnancy outcomes as well as live birth outcomes were significantly reduced as paternal BMI increased (Figure 7.1). The importance of this study is that these changes were

irrespective of maternal BMI and in actual fact, appeared to be additive to the effects of maternal BMI. Interestingly, in all the studies in the literature where the effects of maternal obesity were analysed in relation to embryo development there has been no consideration as to the effects of the paternal BMI on the outcomes (Robker 2008). Findings from this thesis will no doubt lead to a significant amount of debate as to the relative contribution of maternal and paternal BMI to fetal health. However, at a minimum these data will promote a new awareness of the impact of male health at the time of conception on the health of the pregnancy. This is an area that will need to receive more attention and more public awareness in the future.

Future studies should be conducted in conjunction with some analysis of genetic background to further dissect the relationship between obesity and male fertility. This coupled with some dietary information may determine if indeed the effects seen are additive (i.e. genetic and lifestyle). Some obesity syndromes with known genetic defects are specifically associated with abnormalities of spermatogenesis due to loss of function of a single gene; or a number of genes in a chromosomal region responsible for both obesity and male infertility (Mah and Wittert 2010).

The work presented in this thesis highlights another highly neglected area, namely the effects of weight loss on sperm function and beyond. While the effects of weight loss on other aspects of men's health have received some attention, studies examining the effect of dietary interventions or weight loss are virtually non-existent for sperm function and male fertility. This is in contrast to the widespread recommendations that weight loss is now advocated for women before seeking assisted conception.

The work conducted in chapters 5 and 6 is also important in beginning to unravel some of the mechanisms behind how and when sperm may be affected by paternal obesity. The first approach to this question was to examine the seminal plasma of the males to begin to understand if there may be some changes as a result of paternal obesity to the environment to

which the sperm may be exposed. Indeed, it was confirmed that metabolic factors such as glucose and insulin in semen tended to increase as paternal BMI increased (Figure 7.1). This observation provides some evidence that sperm are not necessarily exempt to the negative effects of the metabolic markers associated with obesity.

Chapter 6 showed that elevated glucose levels in culture can induce oxidative stress. This is consistent with the work conducted in the human (Chapter 5) where obese men were shown to have elevated fasted glucose levels. Obese men tend to be more hyperglycemic and hyperinsulinemic. It is therefore hypothesised that obese men and mice may be in a pre-diabetic state with some alterations in their metabolic state, which may be inducing oxidative stress in spermatozoa. The effect of diabetes has been examined more thoroughly in relation to male fertility in both animal models and the human (Agbaje, *et al* 2007, Agbaje, *et al* 2008, Kim and Moley 2008, Mallidis, *et al* 2007, Shrilatha 2007), and it may well be that the effects of pre-diabetes could impair sperm function before the clinical onset of diabetes.

The study in relation to fructose is also interesting, as fructose at elevated levels was shown to be not detrimental to sperm function, which may, at least in part, account for a long-standing question as to why fructose is secreted and present at much higher levels than glucose in semen. The in vitro work conducted in Chapter 6 demonstrates that the only way to increase fructose uptake by sperm over that of glucose is by having fructose present at much higher concentrations than glucose.

Future studies should also consider the concept of transgenerational effects of paternal obesity. There are now several examples of how paternal health around the time of conception affects the health of the offspring. The best characterised is the effect of paternal smoking, which has been associated with an increased prevalence of asthma, respiratory tract infection, diabetes and obesity in the offspring (Chang 2009, Sorahan, *et al* 1997, Toschke, *et al* 2007). There is also a known causal association between paternal age and significant medical conditions in the offspring such as autism (Sartorius and Nieschlag 2010). It is currently not

known whether paternal obesity could exert similar effects on the health and fertility of the offspring.

One of the questions still to be answered is where these molecular changes to the sperm from obese males may take place and how are they manifest. While these experiments would be difficult to conduct in the human, there is some indirect evidence to suggest that a prolonged period of sexual abstinence is associated with increased levels of sperm DNA damage implicating spermiogenesis and the epididymis as the site of damage (Bakos, *et al* 2008, Levitas*, et al* 2005, Richthoff*, et al* 2002). However, the only way to determine these effects is to conduct a series of animal-based experiments, whereby sperm can be obtained and compared from the various stages of spermatogenesis.

It should also be noted that work on epigenetic regulation such as histone modifications, chromatin remodelling and DNA methylation suggests that the correct setting of the epigenome is required for successful male reproductive function, which may also be important for embryonic development and the prevention of disease (Carrell and Hammoud 2010, Godmann*, et al* 2009, Miller*, et al* 2010). However, evidence in relation to the epigenetic status during spermatogenesis is extremely limited in relation to paternal obesity (Okada*, et al* 2010) and should receive more attention in future studies.

This thesis attempts to balance the knowledge concerning the effects of paternal obesity on fertility with the volume of studies in relation to female fertility. In summary, the current studies have shed some light on some long-standing questions regarding the effects of paternal obesity and fertility. Much work is required in order to elucidate the mechanisms of these effects (and their potential reversibility) at least to the same level of knowledge to that of the effect of maternal obesity on fertility. This study has shed some much needed light on a major knowledge gap and will undoubtedly stimulate further interest in this very important area of applied science and medicine.

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8 Appendix I

Unless otherwise indicated, all reagents were purchased from Sigma Chemical Co. (St. Louis, MO USA).

Nine media solutions were prepared according to the following:

Base medium:

1. Dissolve in 500 ml of MilliQ chemicals according to the following table:

Chemical	Weight (g)
NaCl	4.332
KCl	0.420
$NaHPO_4$	0.070
$MgSO_4$	0.444
L-Lactate	1.17
$NaHCO_3$	2.102
Pyruvate	0.035
Taurine	0.0125

2. Add:

10mL of non-essential amino acids and 5mL of glutaMAX (Gibco)

3. Weigh 0.147 g of $CaCl_2$ into 10 mL of Milli Q and dissolve completely.

4. Add the $CaCl_2$ into into medium prepared in (1).

5. Add milli Q to make up 1 mL in total.

6. To make up the nine different treatment groups take 100 mL of the base medium prepared above and add:

Chemical	Chemical	Weight (g)
Treatment 1	NaCl	0.175
Treatment 2	NaCl Fructose	0.175 0.0568
Treatment 3	NaCl Fructose	0.0875 0.54
Treatment 4	NaCl Glucose	0.175 0.0568
Treatment 5	NaCl Glucose Fructose	0.175 0.0568 0.0568
Treatment 6	NaCl Fructose Glucose	0.0875 0.54 0.568
Treatment 7	NaCl Glucose	0.0875 0.54
Treatment 8	NaCl Glucose Fructose	0.0875 0.54 0.0568
Treatment 9	Glucose Fructose	0.54 0.54

Composition (mM) of Culture Medium used in Chapter 6

Component	1	2	3	4	5	6	7	8	9
NaCl	96.6	95.1	80.1	95.1	93.6	80.1	80.1	80.1	65.1
KCl	4.1	4.1	4.1	4.1	4.1	4.1	4.1	4.1	4.1
NaPO4	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
MgSO4	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0
CaCl2	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
NaHCO3	25.0	25.0	25.0	25.0	25.0	25.0	25.0	25.0	25.0
Lactate	5.87	5.87	5.87	5.87	5.87	5.87	5.87	5.87	5.87
Glucose	0	0	0	3.0	3.0	3.0	30.0	30.0	30.0
Fructose	0	3.0	30.0	0	3.0	30.0	0	3.0	30.0
Pyruvate	0.32	0.32	0.32	0.32	0.32	0.32	0.32	0.32	0.32
Glycl-glutamine	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Alanine	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Asparagine	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Aspartate	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Glutamate	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Glycine	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Proline	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Serine	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
HSA*	5	5	5	5	5	5	5	5	5

*Mg/ml

9 Published version of chapter 2

10 Published version of chapter 3

11 Published version of chapter 4