Investigating the metabolic regulation of male fertility

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Abstract

Infertility is the inability to achieve pregnancy after 12 months of regular intercourse and is associated with male factors in 40% of couples. Currently, the diagnosis of male infertility is based on conventional semen analysis and management options involve assisted reproduction therapies for the female partner. The regulation of male fertility therefore remains under investigated.

Recurrent pregnancy loss is the loss of 3 or more pregnancies before the 24th week of gestation. As half of the female partners have normal investigations, their male partners may be contributing to a significant extent, but male partners are not commonly investigated. By using a direct chemiluminescence assay to measure semen oxidative stress, my data showed markedly elevated oxidative stress in the semen of male partners affected by recurrent pregnancy loss.

The main source of semen oxidative stress is leucocytes derived myeloperoxidase, a hyperactivated enzyme in chronic inflammatory states such as obesity. I have performed an in vivo study investigating the effect of a novel myeloperoxidase inhibitor on reproductive function in mice with diet-induced obesity.

Having established that obesity has detrimental effects on male fertility from previously published studies, I investigated the effects of weight loss on obese male. Caloric restriction for weight loss was given to obese men over a period of two months and their semen parameters were compared to a control group who received simple observations over the same time period.

In summary, I have identified a novel diagnostic marker for male partners of women with recurrent pregnancy loss. My weight loss study was the first randomised controlled study to identify the effects of caloric restriction in sperm function of obese men. These data have important implications for the diagnosis of male infertility and could provide an effective intervention for couples with male factor infertility associated with obesity.

Declaration of Contributors

Declaration of Originality: The work described in this thesis is my own. Any collaborations and assistance are detailed below. Contributors are within the Section of Investigative Medicine (Imperial College London) unless stated otherwise.

Chapter 2: The study was designed by Dr. Channa Jayasena. I performed this study with the assistance of Dr Utsav Radia, Mrs Monica Figueiredo, Mrs Larissa Franklin Revill, Mrs Maria Osagie, Mr Wayne Vessey, Professor Lesley Regan, Mr Rajendra Rai and Professor Waljit S Dhillo. Semen analysis and ROS measurement was performed by Mr Utsav Radia. All serum samples were analysed by the in the clinical biochemistry department of Charing Cross Hospital, Imperial College Healthcare NHS Trust.

Chapter 3: The study was designed by Dr. Channa Jayasena in collaboration by AstraZeneca. I performed this study with the assistance of Drs Channa Jayasena and Ebrahim Subbar. Dr. Bryn Owen sacrificed and dissected the mice. Semen analysis was performed by Ms Michelyn Duldulao. Mr Wayne Vessey performed semen ROS measurement.

Chapter 4: I designed the study with the assistance of Dr Channa Jayasena. Study visits were carried out by me with assistance from Drs. Adrian Brown, Aditi Sharma, Emad Sindi and Ebrahim Subbar. Cambridge weight plan products were supplied by the Cambridge weight plan Ltd in collaboration with Dr Anthony Leeds, Semen analysis was performed by the Andrology Department staff, Imperial College Healthcare NHS Trust. All serum samples were analysed by the in the clinical biochemistry department of Charing Cross Hospital, Imperial College Healthcare NHS Trust.

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This thesis is dedicated to Dimitris and my family for all their love and support.

Abbreviations

ACS	Average comet score
ART	Assisted reproductive technology
AUC	Area under the curve
BMI	Body mass index
CI	Confidence interval
CBS	Centre for Biomedical Services
CRN	Clinical research network
CWP	Cambridge weight plan
DNA	Deoxyribonucleic acid
FSH	Follicle-stimulating hormone
GTAC REC	Gene therapy advisory committee
HAS	Human albumin solution
HbA1c	Glycated haemoglobin
HDL	High-density lipoproteins
HFD	High fat diet
ICSI	Intracytoplasmic Sperm Injection
IVF	In vitro fertilisation
LDL	Low-density lipoproteins
LH	Luteinizing hormone
NCD	Normal chow control
OHSS	Ovarian hyperstimulation syndrome

RCT	Randomized controlled trial
ROC	Receiver operating characteristic
ROS	Reactive oxygen species
RYGB	Roux-en-Y Gastric Bypass
SCSA	Sperm Chromatin Structure Assay
SHBG	Sex hormone binding globulin
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labelling
UKNEQAS	United Kingdom National External Quality Assessment Service
WHO	World Health Organization

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Chapter 1

General Introduction

1.1 MALE FERTILITY: AN OVERVIEW

Infertility is the inability to achieve pregnancy after 12 months of regular unprotected intercourse ("Fertility Overview" 2016). During the last few decades, tremendous advances have been made in the treatment of women with reduced reproductive capacity for example in vitro fertilization therapy (IVF) and specialized clinics for recurrent miscarriage. However, poor sperm quality is the causative factor in 40% of infertile couples and is characterised by the term 'male factor infertility'. Therapies proven to increase sperm function in men with reduced reproductive capacity are limited. For example, gonadotropin therapy is recommended for organic hypogonadotropic hypogonadism (HH) and oestrogen-blockade for functional HH, including obesity or opiates. Consequently, the most commonly used therapeutic option for male factor infertility is assisted reproductive technologies (ART), such as IVF and intra-cytoplasmic sperm injection (ICSI) (Human Fertilisation and Embryology Authority 2013); these require daily hormonal injections to stimulate several egg follicles to grow in the female partner. The eggs are collected surgically from the ovaries before fertilized with sperm from the male partner, prior to incubation and re-implantation of embryos to the uterus. Although highly effective, IVF and ICSI are invasive and have uncommon but potentially life-threatening complications for the female partner such as ovarian hyper-stimulation syndrome (OHSS) ("The Management of Ovarian Hyperstimulation Syndrome" 2016). In 2013, over 20,000 ICSI cycles were performed in the UK for male factor infertility, costing a total of £120M (Human Fertilisation and Embryology Authority 2013). NHS funding for IVF and ICSI is restricted and diminishing. Therefore, there exists an important and unmet need to develop practical and cost-effective first-line therapies for male factor infertility.

1.2 SPERMATOGENESIS, A KEY PROCESS TO MALE REPRODUCTION

Spermatogenesis occurs in the testes of men after puberty. It takes 64 ± 8 days (range 42-76) to produce mature sperm (Misell et al. 2006). The testicle has two main physiological compartments: the extratubular compartment (outside the seminiferous tubules) and the intratubular (inside the seminiferous tubules). Spermatozoa derive from pluripotent self-replenishing spermatogonial stem cells (SSCs) in the intratubular compartment. Spermatogenesis consists of three distinct stages (Johnson 2012):

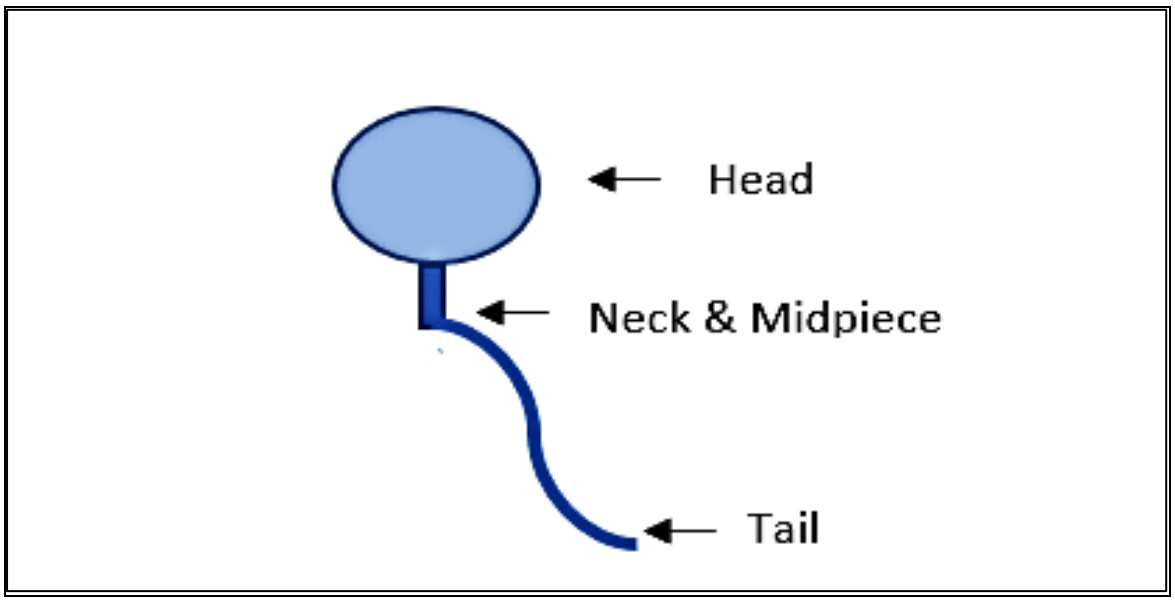
i. mitotic proliferation of SSCs to produce large numbers of spermatocytes,

ii. meiotic division of primary and secondary spermatocytes,

iii. cytodifferentiation of spermatocytes to round spermatids, elongated spermatids and mature spermatozoa.

Spermatozoa have three main structural regions (Figure 1.1).

Figure 1.1 Structure of human spermatozoon



The sperm head contains the nucleus packed with chromatin fibres and is surrounded anteriorly by the acrosome and its enzymes which are used to penetrate the oocyte. The midpiece has plenty of spiralled mitochondria for ATP production and the tail executes the lashing movements required for advancement. Spermatozoa undergo further maturation in the epididymis and express the capacity for motility when they are activated at ejaculation (Johnson 2012).

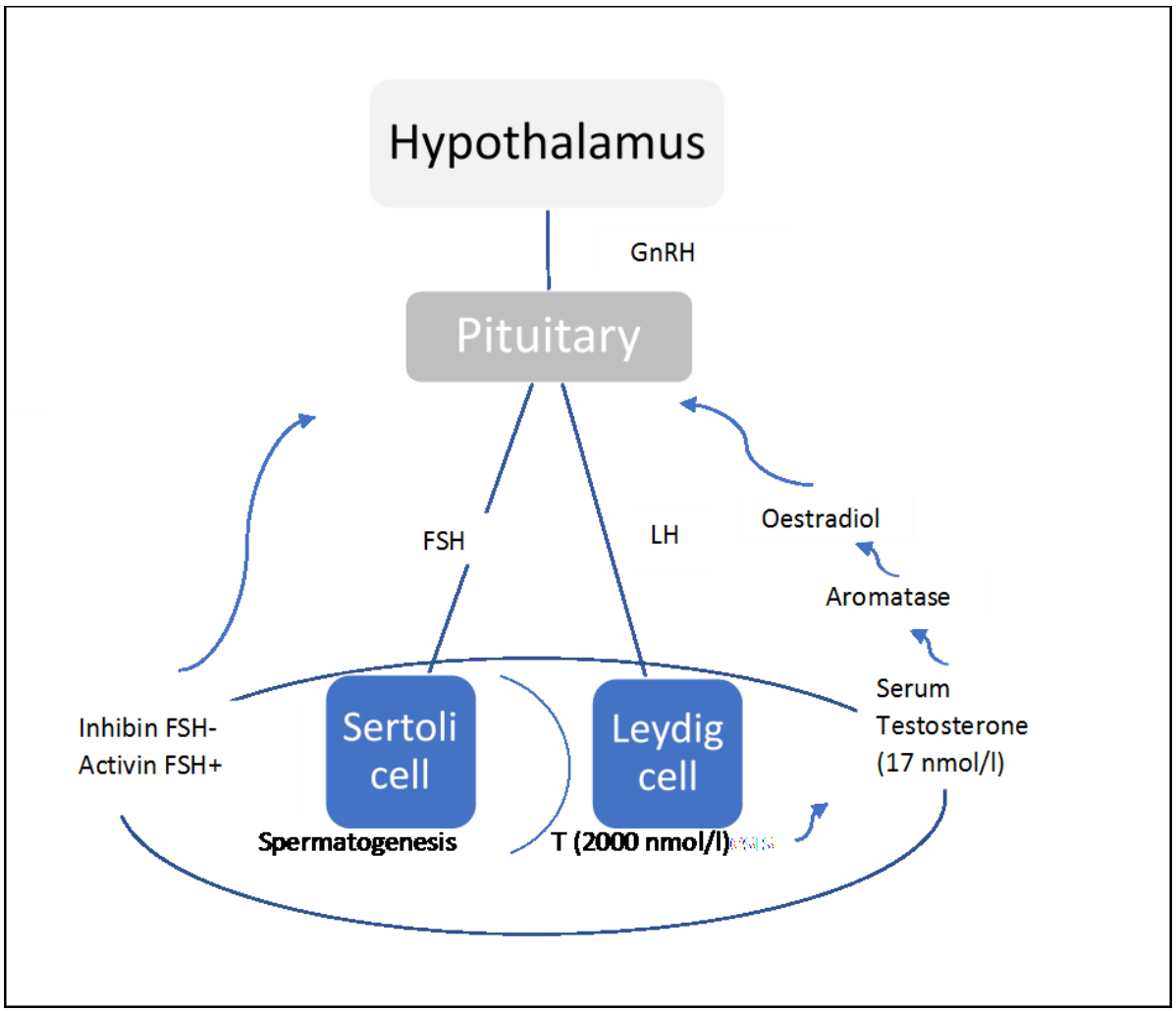
Cycles of spermatogenesis occur in adjacent regions along a seminiferous tubule. Cross-sections of the same tubule seem to 'cycle' together whilst nearby sections could be at a slightly advanced or delayed phase, giving the impression of a spermatogenic 'wave'. The rounds of spermatogenesis are organised by the Sertoli cells, which lie within the tubules. Each Sertoli cell supports a species-specific number of germ cells; in humans four germ cells are supported by one Sertoli cell. The result is production of large numbers of spermatozoa, approximately 300-600 per gram of testis per second. The number of spermatozoa in an ejaculate is a predictive component of pregnancy (Johnson 2012).

1.3 REGULATION OF SPERMATOGENESIS

1.3.1 Endocrine regulation of spermatogenesis

Spermatogenesis is controlled by the Hypothalamic-Pituitary-Gonadal (HPG) axis. This axis relies on the interaction between the hypothalamus that orchestrates reproduction, the pituitary gland and the testicles. Gonadotrophin-releasing hormone (GnRH) is synthesized and released via pulses into the portal circulation. GnRH reaches the anterior pituitary via the portal circulation, where it promotes luteinizing hormone (LH) and follicle-stimulating hormone (FSH) secretion. LH and FSH act synergistically in the testicles to achieve spermatogenesis (Figure 1.2).

Figure 1.2 The hypothalamic-pituitary-gonadal axis. Gonadotrophin-releasing hormone (GnRH) stimulates the secretion of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) from the anterior pituitary. FSH and testosterone (T) are both required for spermatogenesis [adapted from (Kathrins and Niederberger 2016)].



GnRH is a 10 amino acid peptide that is released into the hypophyseal-portal circulation in pulsatile manner (Comninos, Jayasena, and Dhillo 2014). The hypophyseal-portal circulation presents GnRH at the anterior pituitary gonadotroph cells, which are subsequently stimulated to release LH and FSH into the systemic circulation (Wildt 1981). FSH stimulates Sertoli cell function for sperm production and is additionally required for the synthesis of inhibin and activin. LH stimulates Leydig cells for testosterone production. This action results in 100-fold higher intratesticular testosterone concentration than

serum testosterone concentration, which is crucial to additionally stimulate Sertoli cells and initiate spermatogenesis.

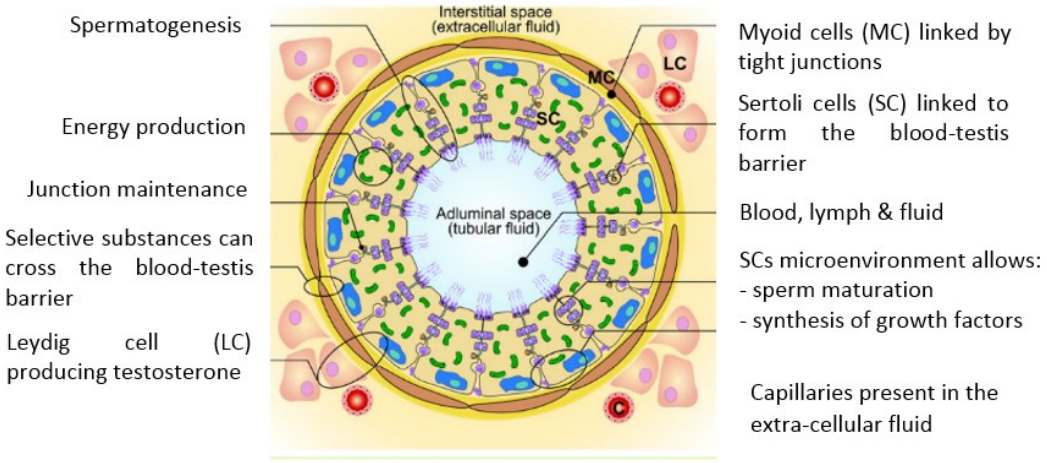
Inhibin B is a glycoprotein dimer, composed of α and β subunits. It is secreted by the testicular epithelium and reflects the proliferating activity of mature Sertoli cells. In contrast to inhibin, Anti-Müllerian hormone (AMH) is the dominant product of immature Sertoli cells and declines to low levels during puberty and adult male life (Kathrins and Niederberger 2016). Studies in the adult male rhesus monkey (macaca mulatta) demonstrate that in a physiological setting Sertoli cell number is the major determinant of circulating concentrations of inhibin B and is positively correlated with the levels of inhibin B (Ramaswamy et al. 1999). Inhibin B exerts negative feedback over FSH secretion by selective action on the anterior pituitary. Remarkably, inhibin B has no effect on LH secretion from gonadotrophs, and the mechanism by which it specifically inhibits FSH release is unknown. Contrary to inhibin, activin exerts positive feedback on FSH secretion for spermatogenesis and is antagonised by inhibin B. Activin is a dimer protein with two subunits also secreted by the testicular epithelium (Johnson 2012; Meachem, Nieschlag, and Simoni 2001).

LH binds to receptors on the Leydig cells located in the interstitial testicular fluid and stimulates testosterone production. Intratesticular testosterone levels are 100-fold higher than systemic testosterone levels and stimulate spermatogenesis by the adjacent Sertoli cells (Kathrins and Niederberger 2016). Testosterone also diffuses out of the Leydig cells into the systemic circulation bound by sex hormone binding globulin (SHBG). In an interventional study of five men with congenital HH, exogenous testosterone and FSH supplementation only led to a small increase in intratesticular testosterone. Nonetheless, systemic testosterone levels were found to be 2-fold higher than usual systemic testosterone levels leading to suppressed spermatogenesis (Schaison et al. 1993).

1.3.2 Metabolic regulation of testicular function

Sertoli cells play a pivotal role to spermatogenesis, offering physical and nutritional support to developing germ cells. They are responsible for the maintenance of the blood-testis barrier, which prevents toxins or autoimmune response triggers from reaching the germ cells (Rato et al. 2014). Sertoli cells create tight junctions to support the blood-testis barriers and form a microenvironment to promote maturation of the germ cells (Figure 1.3).

Figure 1.3 The role of Sertoli cell in spermatogenesis [adapted from (Crisóstomo et al. 2017) - license provided by Oxford University Press and Copyright Clearance Center. License Number: 4664330279375, License date Sep 08, 2019].



The conversion of nutrients to energy required for cellular processes as well as energy production within the testis share common pathways (Melendez-Hevia, Waddell, and Cascante 1996)(Figure 1.4). However, Sertoli cells rely on β-oxidation of fatty acids for their internal energy consumption (Rato et al. 2014). Conversely, germs cells rely on lactate generated from pyruvate to cover their energy requirements (Crisostomo et al. 2017). Germ cells mature to sperm cells, which rely on glucose for

energy production and partially on β-oxidation (Rato et al. 2014). Hence different metabolic reactions take place during distinct sperm cell developmental stages. It remains unknown how these reactions are regulated within various testicular compartments.

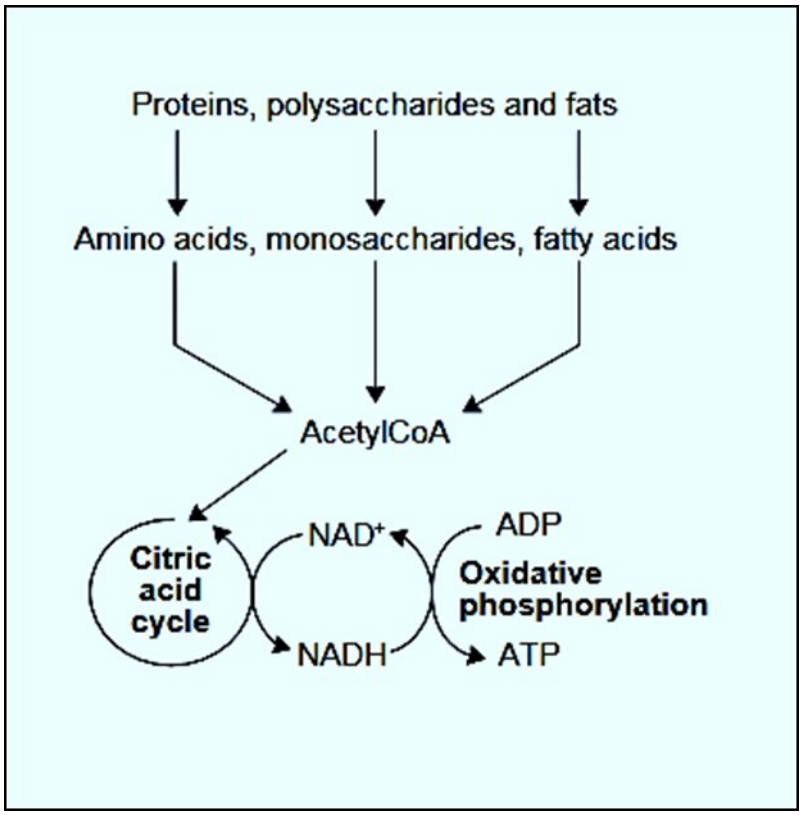


Figure 1.4 Energy metabolism. Proteins, carbohydrates and fats are catabolised according to the following simplified outline. Firstly, proteins are catabolised to amino acids, which are oxidised with the removal of the amino group. The amino group is left deaminated to form a keto acid. Several keto acids are intermediates to the citric acid cycle, whilst the glucogenic amino acids are converted into glucose by gluconeogenesis. Secondly, carbohydrates are broken down into glucose to undergo glycolysis, where glucose is converted to pyruvate. Pyruvate is then converted to acetyl-coenzyme A (acetyl-CoA) by aerobic glycolysis and fed into the citric acid cycle. The acetyl group on the CoA is oxidised to water and carbon dioxide into the citric acid cycle, releasing energy that is stored by reducing the coenzyme nicotinamide adenine dinucleotide (NAD+) into NADH. In anaerobic conditions glycolysis produces lactate which is also able to produce NADH. Thirdly, fats are catabolised to free fatty acids by hydrolysis. Fatty acids undergo beta oxidation to release acetyl-CoA, which then is fed into the citric acid cycle [adapted from (Melendez-Hevia, Waddell, and Cascante 1996)].

It is probable that peptide hormones such as leptin and ghrelin regulate testicular as well as sperm function. Leptin is mainly produced by adipocytes and plays a permissive role in GnRH secretion by transmitting energy status information to the hypothalamus (Livadas and Chrousos 2019). Neuromodulators such as Kisspeptin are thought to mediate the subsequent effects on hypothalamic function, including modulating GnRH pulse generation to enable optimal LH and FSH activity (Comninos, Jayasena, and Dhillo 2014). In Sertoli cells, leptin decreases acetate, which is a combined acetic acid salt, usually bound to coenzyme A (CoA) to form acetyl-CoA. Acetyl-CoA is a central coenzyme to the metabolism of carbohydrates, fats and proteins (Figure 1.4). Leptin also binds to receptors located at the tail of human spermatozoa to facilitate sperm motility (Elfassy et al. 2018).

Ghrelin is a peptide hormone mainly released by the stomach during hunger. Ghrelin acts alongside leptin at the testicle to regulate testicular metabolism (G. Alves et al. 2016). It is demonstrated that ghrelin decreases alanine and acetate production, however it remains unclear how decreased acetate production regulates testicular metabolism (Crisostomo et al. 2017).

In addition to leptin and ghrelin, insulin secreted by pancreatic β-cells stimulates hypothalamo-pituitary function. Several studies demonstrate that insulin replacement increases pulsatile LH secretion in rodent models with diabetes (Dong et al. 1991). Insulin is also expressed in the testes and regulates Leydig cell function by promoting DNA synthesis and steroidogenesis during puberty. Insulin is crucial to Sertoli cell function, as it mediates lactate synthesis an important substrate for germ cells (Mita et al. 1985). According to a previous *in vitro* study, washed human spermatozoa treated with insulin and leptin have significantly increased motility and acrosome reaction compared to non-treated spermatozoa (Lampiao and Du Plessis 2008). In conclusion, insulin increases LH secretion from the pituitary and is important for the development of germinal epithelium as well as the movement of mature sperm.

1.4 EXPERIMENTAL METHODS FOR ASSESSING SPERM FUNCTION IN MEN

1.4.1 Conventional semen analysis

Concentration of spermatozoa in the human semen, sperm motility and sperm morphology are standards parameters of conventional semen analysis. Sperm concentration, motility and morphology to a lesser extent are associated with a couple's ability to achieve pregnancy within 12 months of unprotected sexual intercourse. That is unsurprising, as ejaculated spermatozoa during intercourse are carried to the female genital tract via the seminal fluid produced by the male accessory glands. WHO has set up reference values for each one of these parameters in order to provide a standardised guide for male fertility status (Edition 2010). The reference values originated after assessment of 4500 men in 14 different countries on four continents with proven fertility, as their partners were able to conceive within 12 months. Consequently, an individual's semen analysis report can be assessed in comparison to a reference population (Table 1.1).

Table 1.1 Lower Reference limit and their 95% CI for semen parameters from fertile men whose partners had a time-to-pregnancy of 12 months or less (Cooper et al. 2010).

Parameter	Normal range & Lower reference limit (95% CI)
Semen volume (ml)	>1.4 1.5 (1.4–1.7) ml
Sperm concentration (10^6 per ml or M/ml)	>15 15 (12–16) M/ml
Total motility (PR* + NP**, %)	>39 40 (38–42) %
Progressive motility (PR, %)	> 31 32 (31–34) %
Sperm morphology (normal forms %)	>3.9 4 (3.0–4.0) %
PR* progressively motile, NP** non-progressively motile	

There is specific nomenclature in relation to semen quality, which is summarised in the table below (Table 1.2).

Table 1.2 Nomenclature related to semen quality (Adopted from WHO manual for the examination of human semen)

Term	**Definition**
Asthenozoospermia	percentage of progressively motile (PR) spermatozoa below the lower reference limit
Teratozoospermia	percentage of morphologically normal spermatozoa below the lower reference limit
Cryptozoospermia	spermatozoa absent from fresh preparations but observed in a centrifuged pellet
Leukocytospermia	sperm disorder defined as >1 million myeloperoxidase-positive leukocytes per ml of semen

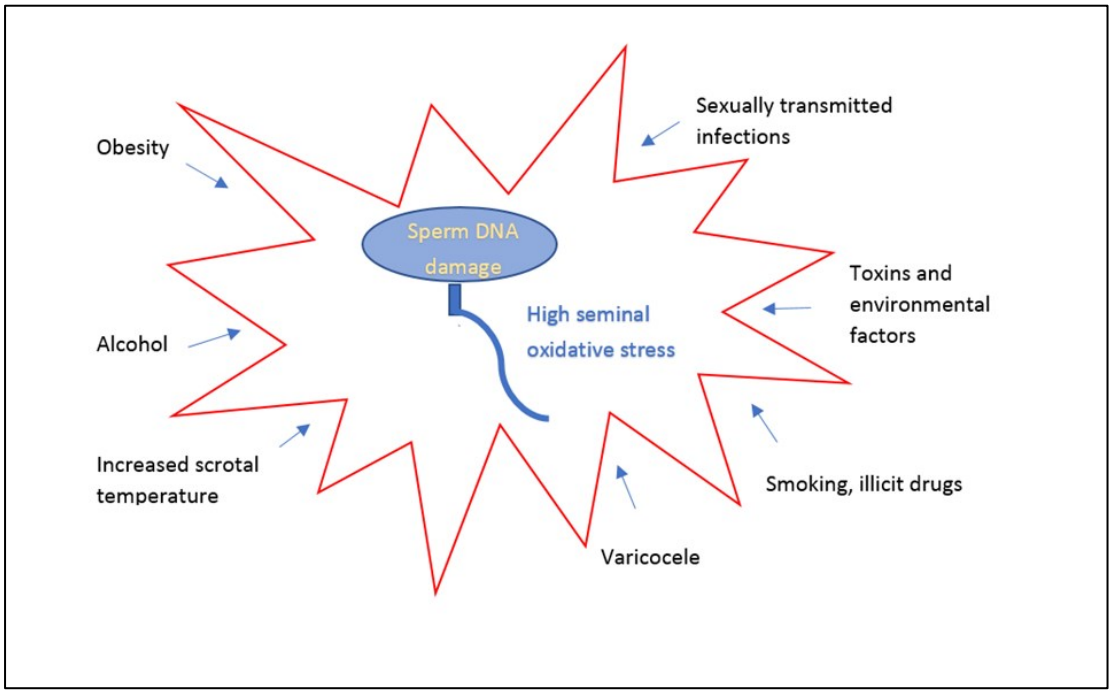
The degree of male infertility can be assessed by subdividing sperm count present in the ejaculate into four groups: i) aspermia which is the failure to produce semen, ii) azoospermia which refers to the absence of sperm in the ejaculate, iii) cryptospermia when sperm count is <1 million/ejaculate and iv) severe oligospermia which is the term used for sperm counts between 1-10 million/ejaculate (Punab et al. 2017). These four groups can assist diagnosis and contribute towards the management of infertility. For example, aspermia points towards erectile dysfunction or ejaculatory disorders. However, the number of sperms in the ejaculate, in combination with motility and morphology represent an indication of a man's fertility status and values above these limits do not guarantee fertility. It is notable that semen analysis results below the reference range do not imply infertility (WHO Edition 2010).

Conventional semen analysis fails to detect fertility status changes in 15% of men, who have semen parameters within range but remain unable to conceive after a year of regular unprotected intercourse (Sandro C. Esteves 2011). It is therefore clinically important to develop novel diagnostic methods for men with difficulty in conception. Over the last few years, the role of seminal reactive oxygen species (ROS) has gained considerable interest, as early diagnosis of high oxidative stress could potentially guide couples to effective therapeutic approaches. The quality of spermatozoa required for conception can be influenced by one additional factor, the sperm DNA fragmentation index that has recently attracted increasing interest. Detailed semen analysis is essential to monitor spermatogenesis and assess male fertility status.

1.4.2 Reactive Oxygen Species

Reactive Oxygen Species (ROS) are unstable molecules found in seminal plasma. ROS originate from endogenous sources, such as mitochondria of immature spermatozoa or leukocytes, which are physiologically present in the human ejaculate (Kessopoulou et al., 1992). ROS are physiologically required for sperm maturation, movement and fertilisation of the oocyte. The ultimate process to make sperm competent for fertilisation is capacitation. Capacitation is facilitated by ROS which trigger signalling cascades and result in membrane fluidity and sperm-oocyte fusion (Ashok Agarwal et al. 2018). However, in some men, disproportionate levels of oxidative stress ROS originate from exogenous sources, mainly associated with lifestyle factors such as excessive smoking, alcohol consumption, radiation and toxins (Ashok Agarwal et al. 2014) (Figure 1.5).

Figure 1.5 Lifestyle and other factors associated with sperm DNA damage by high seminal reactive oxygen species (ROS) [adapted from (Agarwal et al. 2014)].



The plasma membrane of spermatozoa contains extraordinarily high amounts of polyunsaturated fatty acids (PUFAs), which contribute to membrane fluidity. Sperm membrane fluidity is essential to acrosome reaction, capacitation, sperm count and motility. Questionnaire-based studies demonstrate a dose-dependent relationship between high saturated fat intake and decreasing sperm count, motility as well as morphology (Hayden, Flannigan, and Schlegel 2018). Spermatozoa with imbalanced PUFAs content are highly susceptible to oxidative damage (Gharagozloo et al. 2016). For example, BMI has been negatively associated with levels of sperm docosahexaenoic acid that has additionally been found to have a negative association with sperm DNA fragmentation index (Andersen et al. 2016). Deficiency in free radical antioxidant scavengers or amplified ROS production due to toxins, stress and inflammation can disrupt the balance between ROS and antioxidants. As a result, ROS attack PUFAs in the sperm membrane and a cascade of lipid peroxidation is triggered, which leads to impaired sperm function. Sperm DNA becomes susceptible to damage via fragmentation, both in the nucleus and the mitochondria (Makker, Agarwal, and Sharma 2009).

Seminal oxidative stress measurement represents a critical tool for the evaluation of the infertile male. At present, ROS measurement is considered a costly diagnostic test in the absence of a widely accepted and reliable method. Routine semen analysis could suggest the presence of oxidative stress and assist clinicians to make an initial diagnosis (Dutta, Majzoub, and Agarwal 2019). For instance, asthenozoospermia, abnormal sperm morphology and detection of leukoyctes are well known to be associated with oxidative stress. However, direct and indirect assays are available for ROS measurement in addition to routine semen analysis, to allow quantitative and efficient assessment of seminal oxidative stress.

ROS generated by sperm can be measured by direct assays, such as chemiluminescence. Chemiluminescence assays measure the imbalance between ROS production and the antioxidant concentration in semen. Other direct ROS measurements can be performed with quantification of nitroblue tetrazolium (NBT) activity, measurement of cytochrome C reduction, flow cytometry with the use of fluorescent probes or electron paramagnetic resonance spectroscopy (Vessey et al. 2014). In general, direct ROS assays can be problematical and their use is currently limited. For example, the half-life of free radicals in electron paramagnetic resonance spectroscopy is very short. Therefore, experiments must be performed at low temperatures which makes electron paramagnetic resonance spectroscopy impractical.

ROS is also measured by indirect assays, which assess normality of sperm chromatin or evaluate DNA fragmentation instead of actual ROS levels (Ko, Sabanegh, and Agarwal 2014). Other indirect methods measure seminal chemokines, levels of lipid peroxidation or seminal antioxidant capacity. Antioxidant capacity in the semen is measuring the effect of an oxidative reagent such as hydrogen peroxide, on a specific substrate. Measurement of a specific antioxidant does not provide the total antioxidant capacity. Measurement of the total antioxidant capacity could be determined by individual testing for

catalase, glutathione peroxidase, reductase and superoxide dismutase activity and the cost-efficiency of such testing is not proven yet (Ko, Sabanegh, and Agarwal 2014). Recently a novel technology based on galvanostatic measure of electron movement was developed to assess seminal ROS. It is known as Male Infertility Oxidative System (MiOXSYS) and measures transfer of electrons from a reductant (antioxidant) to an oxidant and thereby assesses seminal oxidation–reduction (redox) potential (Dutta, Majzoub, and Agarwal 2019).

Although seminal ROS is difficult to measure, it represents overall sperm function (Ashok Agarwal, Saleh, and Bedaiwy 2003). Chemiluminescence assays based on luminol are overall easier to use and were used for my research the Andrology Department, Hammersmith Hospital (Vessey et al. 2014).

1.4.3 DNA fragmentation Index

Damaged sperm DNA could be indicative of male subfertility regardless of routine semen parameter values. Sperm with fragmented DNA may lead to fertilisation however, it could cause defects in embryo development, childhood disease or pregnancy loss (Ribas-Maynou et al. 2012). It appears that the probability of fertilization via assisted reproduction is close to zero if the proportion of sperm cells with DNA damage exceeds 30% (Mona Bungum et al. 2004). Hence, tests evaluating DNA damage are clinically relevant when evaluating male fertility (Aly and Polotsky 2017).

In clinical practice there are three main techniques groups to assess sperm DNA fragmentation (Sandro C. Esteves 2011):

a) sperm chromatin structural probes using nuclear dyes. The most commonly referred method from this group is the sperm chromatin structure assay. This assay can be performed within

minutes and involves spermatozoal staining with acridine orange. Acridine orange is a dye that stains broken DNA as red fluorescence and intact DNA as green fluorescence (Lewis et al. 2013).

b) direct assessment of DNA fragmentation. The most commonly referred methods from this group are the TUNEL and COMET assays. TUNEL assay uses an enzyme to catalyse the attachment of fluorescent deoxynucleotides to 3'- hydroxyl-termini of DNA double strand breaks (García-Peiró et al. 2013). COMET assay relies on microscope slides where spermatozoa are lysed within agarose gel. DNA from lysed spermatozoa is decondensed in high salt to form supercoiled loops of intact DNA or less coiled strands of broken DNA. The slides are placed in an electrophoretic field, where electrophoresis results in movement of supercoiled DNA loops towards one pole of the electric field and less coiled strands of broken DNA towards the other pole of the electric field. The observed structures resemble comets on fluorescent microscopy. The intensity of the comet tail relatively to the head reflects the number of DNA breaks (Lewis et al. 2013).

c) sperm nuclear matrix assays which include sperm chromatin dispersion tests such as Halo testing. The Halo test is simple and inexpensive to perform. The spermatozoa are stained with propidium iodine and spermatozoa with intact DNA appear to have a 'halo' under bright-field microscopy. Consequently, the Halo test measures the absence of damage in sperm DNA rather than the actual damaged sperm DNA that only appears visually smaller (Lewis et al. 2013).

The Comet assay is in receiver operating characteristic (ROC) curve analysis, followed by the TUNEL assay. The next higher area below the curve was shown by the Halosperm assay and followed by the sperm chromatin structural assay (García-Peiró et al. 2013).

1.5 AETIOLOGY AND TREATMENT OF MALE INFERTILITY

1.5.1 Causes of male infertility

Infertility is becoming increasingly common (Ashok Agarwal et al. 2015). It has been estimated that in 2010, 48.5 million couples across 190 countries were unable to have a child after a five-year period of being in an intimate relationship, not using contraceptives and having regular intercourse (Mascarenhas et al. 2012). Male infertility may be caused by multiple factors such as age, coexistence of chronic disease, exposure to toxins, environmental factors and some specific but relatively uncommon disorders which are broadly divided into three categories (Table 1.3).

Firstly, there are endocrine disorders associated with male infertility and these are encompassed by the term hypogonadotrophic hypogonadism (HH). Endocrine disorders are related to hypothalamic or pituitary disease resulting in low testosterone with low LH and FSH secretion. Pituitary disease accounts for most cases of HH but endocrine disorders may also arise from hypothalamic failure due to congenital or acquired GnRH deficiency (Boehm et al. 2015). Functional hypothalamic GnRH deficiency may also be associated with obesity, chronic disease, opioid or androgen misuse and severe weight loss.

Genetic abnormalities account for 10–15% of male factor infertility and can be broadly classified into chromosomal abnormalities, Y-chromosome microdeletions, X-linked gene mutations, autosomal gene mutations, polymorphisms and epigenetic errors. The most common chromosome abnormality associated with male infertility is Klinefelter's syndrome caused by the 47,XXY karyotype or mosaics that result in testicular failure during early adulthood. The Y-chromosome also contains genes which regulate spermatogenesis and microdeletions in the Yq11 region are prevalent in severe oligospermia.

Mutations to the androgen receptor gene can additionally lead to androgen insensitivity syndrome, which leads to difficulty in sperm production. Oligospermic men due to cryptorchidism have mutations in the 'insulin-like 3' gene on chromosome 19 and its receptor 'relaxin/insulin-like family peptide receptor 2' gene (Jayasenna, McCredie, and Brenton 2016).

Finally, less than 2% of male infertility cases are related to the absence of sperm caused by obstruction arising anywhere between the testis and the ejaculatory ducts (National Collaborating Centre for Women's and Children's Health 2013). The absence of sperm due to obstruction is termed obstructive azoospermia and can be caused by absence of the vas deferens, trauma, mumps, prostatitis, previous radiotherapy and surgery. These patients may have intact spermatogenesis and sperm can be retrieved surgically from the testes or epididymis, so that later used for treatment with intracytoplasmic sperm injection (Jayasenna, McCredie, and Brenton 2016).

The primary cause of male infertility is identified in 40% of men attending fertility clinics, whereas no aetiological factor is identified in the remaining 60% of infertile men (Punab et al. 2017).

Table 1.3 Causes of male infertility [adapted from (Jayasenna, McCredie, and Brenton 2016)].

I. Endocrine and systemic disorders (GnRH, LH and FSH deficiency)

- Hypothalamic or pituitary disease (secondary hypogadism) which could be congenital or acquired
- Chronic illness, nutritional deficiencies or obesity

II. Genetic disorders of testicular failure

- Chromosomal abnormalities associated with Klinefelter's syndrome
- Y chromosome microdeletions or gene mutations
- Polymorphisms and epigenetic errors

III. Obstructive infertility

- Congenital absence of the vas deferens (Cystic fibrosis transmembrane conductance regulator gene mutations)
- Infections such as prostatitis, mumps or sexually transmitted diseases, drugs, hyperthermia, trauma or torsion.

IV. Idiopathic or non-classifiable

1.5.2 Treatments for male infertility

Treatment strategies for male factor infertility are guided by the underlying cause, but this can be challenging as most causes of male infertility are difficult to identify. Men with hypogonadotrophic hypogonadism are offered gonadotrophin therapy, and obstructive azoospermia is treated with correction of epididymal blockage or surgical sperm retrieval. Urogenital infections are treated with antibiotics. Lastly, non-obstructive azoospermia is managed with donor insemination (National Collaborating Centre for Women's and Children's Health 2013).

Despite improvements in the diagnostic evaluation of infertile couples the cause remains unexplained in 40% of cases. Unexplained male factor is therefore common, and most couples are advised to enhance sperm function with healthy lifestyle changes. Several lifestyle factors may impair male fertility such as cigarette smoking, illicit drug use, alcohol, high fat diet or increased scrotal temperature due to intense cycling for instance (Agarwal et al.2018). Couples are advised to eliminate suboptimal lifestyle choices and continue to try to conceive for at least two years before assisted reproduction is considered (National Collaborating Centre for Women's and Children's Health 2013). Other therapies for unexplained male infertility include vitamins or antioxidants. However, these are most frequently ineffective and couples fail to conceive naturally (Tournaye, Krausz, and Oates 2016).

Subfertile couples who fail to conceive naturally require assisted reproductive technologies (ART) such as, in vitro fertilisation (IVF) or intracytoplasmic sperm injection (ICSI) to conceive (Inhorn and Patrizio 2014). IVF involves the administration of reproductive hormones to mature ova, which are collected and fertilized with the male partner's sperm in vitro. High quality embryos may then be transferred back to the uterus for implantation (National Collaborating Centre for Women's and Children's Health 2013). ICSI is treatment which can be reserved for cases in which IVF fails and involves extracting

sperm and ovum, injecting the sperm into the ova, and then implanting the fertilized embryo into the uterus. Approximately half of assisted reproduction treatments (ART) are related to male infertility and this number has more than doubled between 2009-2013 (Human Fertilisation and Embryology Authority 2013). ICSI is effective but has potential health risks for the female partner such as Ovarian Hyperstimulation Syndrome ("The Management of Ovarian Hyperstimulation Syndrome" 2016) and is expensive. It is critical to conduct future research to improve pathways available for couples having difficulty in conceiving.

1.6 OBESITY: A PANDEMIC

1.6.1 Definitions and epidemiology

Body Mass Index (BMI) is used as an index to classify obesity and it is widely acceptable that BMI >30 kg/m^2 defines obesity (WHO 2004). Obesity rates have grown dramatically in England from 1993 to 2015. As a result, 27% of adults in England are obese with half of obese adults being male as rates between male and female obesity are equal (Baker 2017). Obesity not only affects cardiovascular and metabolic health but also influences reproductive potential. Expectedly, epidemiological data from Punab et al. 2016 in Estonia confirm that almost a quarter (22%) of adult males attending tertiary fertility centres are obese. Although a 20% of Esthonian men are obese, obese male partners attending tertiary fertility services are 1.8 times (95% CI: 1.28-2.53) as likely to be infertile than non-obese male partners of pregnant women accessing relevant tertiary care services (Punab et al. 2017). Male fertility treatments are scarce hence developing management pathways for obese men could make a substantial difference for infertile couples.

1.6.2 Evidence of obesity-induced male infertility

Obesity has a significant negative correlation with ejaculate volume, semen concentration, sperm motility and morphology in subfertile men (Bieniek et al. 2016). Data from the Longitudinal Investigation of Fertility and the Environment (LIFE) study in 501 couples from Texas showed that there is a linear association between higher (BMI) and higher incidence of low sperm count as well as low normal morphology (Eisenberg et al. 2014). Additionally, a meta-analysis based on 13,453 men demonstrated an inverse relationship between BMI and abnormal sperm count. Obese men compared with normal weight men (BMI 18.5 – 24.9 kg/m2) have increased risk of oligospermia (sperm concentration <15M/ml) or azoospermia (absence of sperm in semen) with OR 1.97 (95% CI: 1.27-

3.07) (N. Sermondade et al. 2013). The Agricultural Health Study in North America also highlighted that men with BMI above 30 kg/m^2 are twice as likely to have infertility compared to men with BMI 20-22 kg/m^2 (Sallmén et al. 2006). Obese men are therefore more likely to have low semen parameters and subfertility related to low semen parameters.

Obese men are not only more likely to experience infertility due to oligospermia but also more likely to have low pregnancy rates via assisted reproduction. It has been observed that paternal BMI is associated with decreased blastocyst development and reduced live birth rates following assisted reproduction therapies (Hassan W. Bakos et al. 2011). Retrospective analysis of 239,127 of fresh in vitro fertilization (IVF) cycles from the 'Society for Assisted Reproductive Technology' registry showed that IVF outcomes decline with in couples with high BMI, even though results did not reach statistical significance. Implantation and live birth rates were not significantly lower for couples with male BMI of 35-39.9 kg/m^2 compared to couples with male BMI 18.5-24.9 kg/m^2 (Provost et al. 2016). However, recent meta-analyses demonstrate that pregnancy rates and lives births are reduced following assisted reproduction in couples with obese male partners (Campbell et al. 2015; Mushtaq et al. 2018).

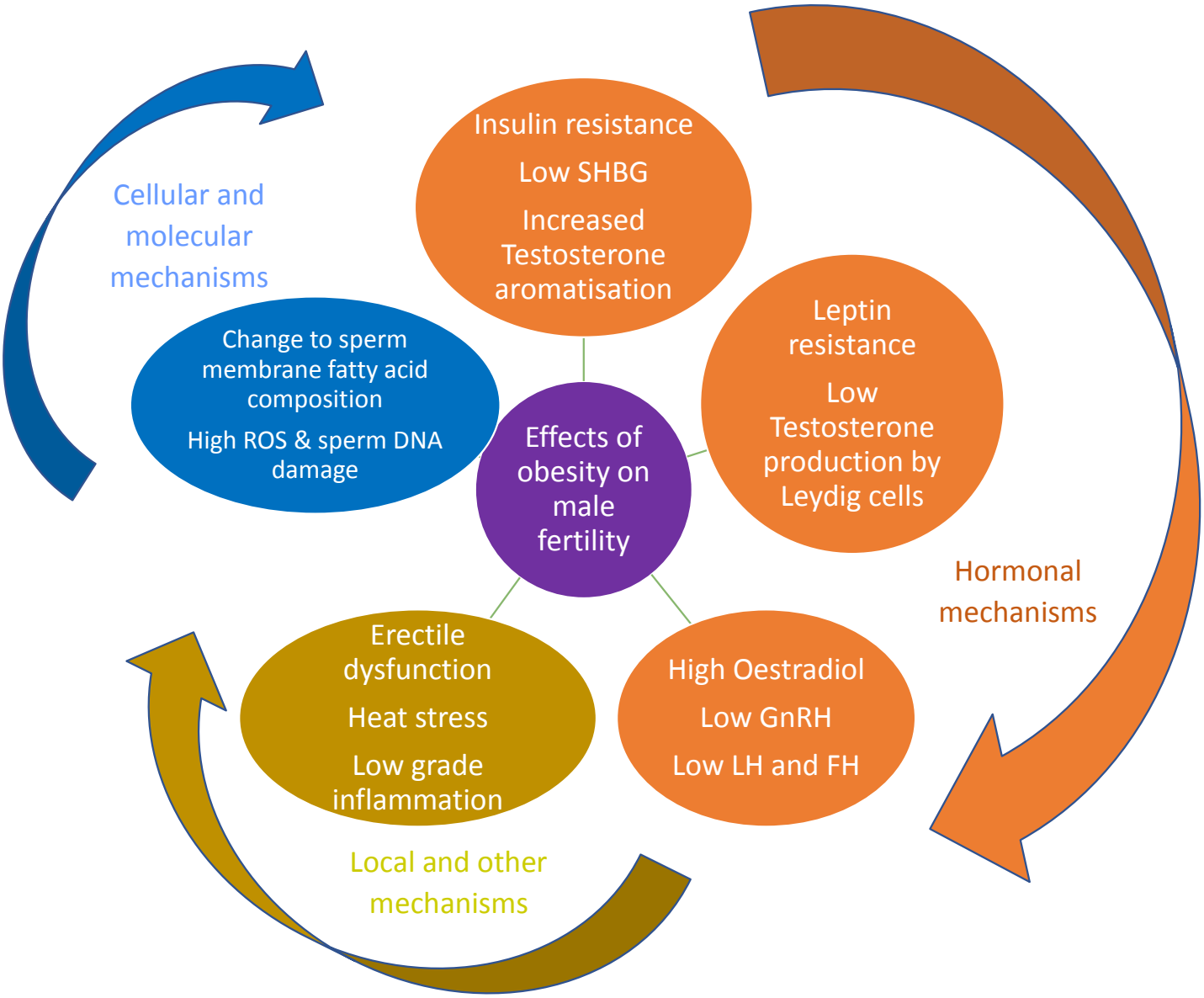
In conclusion, increased BMI has a negative impact on semen quality (Thomsen et al. 2014). Hypogonadotrophic hypogonadism has a synergistic effect with high BMI and poor sperm DNA integrity in obesity (Macdonald et al. 2010) leading to 10% risk absolute risk of pregnancy non viability (Cambel et al. 2015). ROS is also considered significantly higher in men with BMI >28 kg/m^2 ($P<0.01$)(Q. Yang et al. 2016) and could have a negative impact on sperm function when produced in excess. Sperm DNA damage in protein coding regions (exons) most likely results in defective placentation and non-viable pregnancies (Bakos et al. 2011).

1.7 MECHANISM OF OBESITY-INDUCED MALE INFERTILITY

1.7.1 Effects of obesity on reproduction

The effect of male obesity on reproduction is exercised via different pathways yet, it is not fully understood. Obesity alters the balance of HPG axis, modifies the micro-testicular environment with impaired testosterone as well sperm production and causes oxidative stress with increased sperm DNA damage (Figure 1.6). Subsequently sperm quality declines, leading to infertility and health problems transmitted to the offspring via epigenetic mechanisms (Y. Liu and Ding 2017).

Figure 1.6 Obesity and male infertility There are several mechanisms involved in obesity-induced male infertility. Firstly, hormonal mechanisms include hypogonadotrophic hypogonadism, high oestradiol levels and leptin as well as ghrelin resistance. Secondly, there are local as well as other mechanisms implicated such as inflammatory response, heat stress, erectile dysfunction and obstructive sleep apnoea related to obesity. Finally, obesity-induced infertility has been linked to seminal oxidative stress, sperm DNA damage and epigenetic changes [adapted from (Craig et al. 2017)].



1.7.2 The role of hormonal mechanisms in obesity-induced male infertility

Hypogonadotrophic Hypogonadism and hyperinsulinemia

Testosterone is synthetized in the testicle and diffuses out of the Leydig cells to exert systemic actions in male tissues. It is mainly bound by Sex Hormone Binding (SHBG) and is then metabolised to oestrogen by aromatase. In obese men aromatase activity is high therefore, oestrogens levels rise inappropriately. Peripheral insulin resistance additionally inhibits SHBG production by hepatocytes and delivery of testosterone to peripheral tissues is reduced. Leydig cells also reduce their secretory activity in the presence of excessive insulin and testosterone levels drop further (Pitteloud et al. 2005). Biologically active oestradiol along with circulating free testosterone unbound to SHBG (Pasquali 2006; Hawksworth and Burnett 2019) exert negative feedback to the HPG axis and result in hypogonadotrophic hypogonadism (Davidson et al. 2015).

Leptin

Adipokines such as leptin are also disrupted in obesity and contribute to HPG dysfunction. Leptin is increased in overfeeding states due to resistance. Leptin resistance leads to defective hypothalamic signalling, reduced gonadotrophin secretion and subsequent hypogonadotrophic hypogonadism (G. Alves et al. 2016). Leptin resistance with increased aromatase activity are the main mechanisms causing defective hypothalamic function and low testosterone in obesity.

Ghrelin

Ghrelin offers additional links between hormonal imbalance and defective testicular metabolism in obesity. Low ghrelin acts synergistically to leptin resistance to impair glucose and lipid metabolism (Ana Dias Martins, Majzoub, and Agawal 2018; Ana D. Martins et al. 2015) . In this context, Leydig cell proliferation is inhibited (G. Alves et al. 2016) and there is further production of immature spermatids. Defective Sertoli cell function with poor Leydig cell secretory activity result to immature sperm, prone to oxidation and local inflammatory response.

1.7.3 Testicular dysfunction and other effects of obesity on male fertility

Testicular dysfunction in the context of inflammation, apoptosis and leptin resistance

Obesity is also characterised by a chronic inflammatory state. High calorie diets increase body weight, glucose and lipid levels with subsequent rise in the metabolic rate to sustain the body energy expenditure (Oliveira et al. 2017). Production of cytokines such as tumour necrosis factor-α (TNF-α) and interleukins (IL-1, IL-6 and IL-18) increases in parallel to the metabolic rate in obesity and induces oxidative stress. Cytokines and interleukins have a negative impact not only on the HPG axis but also on male gonads. The consequence is a low-grade systemic as well as testicular inflammatory response with high levels of oxidative stress.

High fat diet induced obesity in C57BL6 mice results in programmed death of testicular germ cells within the seminiferous tubules via autophagy. Autophagy is the selective degradation of cellular components. Autophagy cross-regulates with apoptosis to maintain sperm production in the testicle.

However, in obese mice autophagy is hyperactivated causing oxidative stress and disrupting the architecture of seminiferous tubules. As a result, germ cell function becomes defective and the weight of the testes declines (Mu et al. 2017).

Leptin resistance in obesity results in high seminal leptin concentrations and altered cytoplasmic lipid droplet consistency. Accumulation of lipid droplets triggers lipid peroxidation and germ cell apoptosis (Ana D. Martins et al. 2015). Sperm DNA damage and oxidative stress are also accentuated in the presence of leptin or insulin resistance (Wang et al. 2018). Apart from seminal oxidative stress, leptin resistance has deleterious effects on sperm motility (Elfassy et al. 2018). In experimental mouse models (C57BL/6), exogenous leptin reduces the expression of tight junction proteins crucial to the organisation of the blood-testis barrier. The blood-testis barrier divides seminiferous tubules into compartments to allow maturation of spermatozoa. Damage in this barrier causes germ cell loss, low sperm count and motility (Wang et al. 2018). Hyperleptinaemia inhibits testosterone production by the testicle via direct action on the Leydig cells (Shukla et al. 2014). In obese leptin-deficient animals (ob/ob) lower gene expressions for LH and FSH receptors were observed along with fewer testicular germ cells due to apoptosis (F. F. Martins, Aguila, and Mandarim-de-Lacerda 2017). As a result, obese mice fail to maintain a specialized microenvironment for germ cell development and spermatozoa demonstrate poor progressive motility (Ghanayem et al. 2010).

Heat stress, erectile dysfunction and obstructive sleep apnoea related to obesity

Apart from inflammation and oxidative stress, other detrimental effects of obesity on reproduction include defective thermoregulation, poor endothelial function and low testosterone production due to poor quality sleep (Ana Dias Martins, Majzoub, and Agawal 2018). The increase in lower abdominal

fat disrupts scrotal thermoregulatory mechanisms in male, resulting in high testicular temperature and heat stress, with diminished sperm DNA quality and germ cell apoptosis (Gharagozloo et al. 2016). Endothelial dysfunction in obesity reflects a decrease in nitric oxide (NO), which is associated with high cholesterol, insulin resistance and oxidative stress. NO is the primary neurotransmitter responsible for the relaxation of penile smooth muscles and when released at suboptimal levels, erections are achieved with difficulty (Shamloul and Ghanem 2013). Finally, sleep fragmentation due to obstructive sleep apnoea decreases LH production and further reduces circulating testosterone levels contributing to the general inflammatory status (Craig et al. 2017). Animal studies involving obese rodents demonstrate low systemic levels of testosterone, reduced testicular mass and lack of sexual behaviour with low mating rates (Yadav et al. 2018; Crean and Senior 2019).

1.7.4 Cellular and molecular mechanisms implicating obesity- induced male infertility

Reactive oxygen species and sperm DNA damage

Oxidative stress is elevated in obesity at systemic as well as testicular level. Excessive aromatase activity converts testosterone to oestradiol and inhibin B levels consequently drop. Low inhibin B reflects a reduction in Sertoli cell numbers and impaired phagocytosis of immature spermatozoa. Spermatozoa with disproportionate cytoplasm or arrest at any point during spermiogenesis generate ROS that further attack sperm DNA and cause loss of integrity (Ashok Agarwal et al. 2018). Sperm DNA fragmentation in obesity is attributed to high ROS production which surpasses seminal antioxidant capacity and impairs sperm quality leading to infertility (Lewis et al. 2013).

The seminiferous and epididymal epithelium become disrupted by high oxidative stress in obesity (Y. Liu and Ding 2017). Chronic inflammatory response at testicular level is associated with production of

cytokines that interfere with both Sertoli and Leydig cell function. For example, TNF-a and IL-1 cause direct damage to the assembly of junctional proteins supporting the network of Sertoli cells. Thereby the niche of the seminiferous epithelium becomes defective with significant impairments in spermatogenesis. Pro-inflammatory cytokines inhibit LH function leading to additionally low testosterone and poor sperm quality (Y. Liu and Ding 2017).

Epigenetics

Epigenetics is the study of acquired but heritable modifications in gene function at the mitotic or meiotic level, without causing any changes in the DNA sequence. Epigenetic modifications are heritable and influence the health of offspring (Gunes et al. 2016). Therefore, the epigenetic status of spermatozoa is crucial to understand obesity-induced male infertility. Sperm of obese men has been shown to undergo epigenetic changes, such as the following:

I. DNA methylation. DNA methyltransferace proteins establish methylation imprinting in sperm genes. Sperm DNA methylation has been associated with impaired sperm concentration, motility and morphology (Navarro-Costa et al. 2010). A cross-sectional study comparing 13 lean and 10 obese men presented the first epigenetic mapping of spermatozoa and found changes in DNA methylation patterns in genes. Weight loss after Roux-en-Y gastric bypass surgery showed changes in DNA methylation patterns in genes at one-week and one-year post surgery (Donkin et al. 2016).
II. Histone acetylation. Histone acetylation plays a critical role in protecting from DNA damage. High fat diets induce acetylation of late spermatids, resulting in increased levels of DNA damage (Davidson et al. 2015).
III. Differences in non-coding RNA expression levels. There are significant differences in mRNA levels between lean and obese, possibly transmitted to the embryo and

consequently affecting development (Davidson et al. 2015). In addition, noncoding RNA and mRNA in the testes of rats were analysed with microarray-based gene expression analysis. Microarray analysis is a method that uses microscopic RNA spots attached to a solid surface to measure the expression of large regions of a genome. It was demonstrated that noncoding RNA and mRNA of obese rats interact to downregulate the expression of proteins with antioxidant action. Obese animals therefore had poor retinol metabolism that led to immature spermatozoa with defective acrosome and decreased spermatozoal numbers. Obesity induced by high fat diet alters protein expression in the testes, activates pathways related to dysplastic cytoskeleton and causes testicular remodelling with ischaemia due to low oxygen levels (X.-Y. Yang et al. 2018).

The relationship between epigenetics is an area of increasing interest. Epigenetic studies could offer a better understanding of sperm dysfunction in obesity and explain how sperm function changes with weight loss does, since epigenetic modifications are potentially reversible.

1.8 EFFECT OF WEIGHT LOSS ON HUMAN MALE REPRODUCTION

1.8.1 Weight loss for male infertility: first administration to humans

The concept of weight reduction to treat infertility in obese men is not new since the first case report was published in 1959 by Alexandre in France. Carbohydrate restriction, diuretics and multivitamins were offered to an infertile man with BMI of 31.4 kg/m2 and sperm count of 25 M/cm^3 at baseline. He successfully lost 8 kg in 5 months and his sperm count increased to 125 M/cm^3 (Alexandre 1959). Low energy diet or bariatric surgery are both acceptable strategies for weight reduction and improvement of metabolic profile in cases of male infertility, but there is not enough evidence to support their routine use in the context of infertility due to obesity.

1.8.2 Weight loss via diet

Diet prescription with an energy deficit below the estimated daily energy requirements is an established method to achieve weight loss. Very low-energy diets in the 1970s were associated with multiple nutrient deficiencies and inadequate amount of protein. Nonetheless most recent food-based formula diets close to 800kcal/day or more are effective for weight loss in the short term (Mulholland et al. 2012). For example, in 1991 Frost et al prescribed 1100–1600 kcal/day in obese patients and achieved mean weight loss of 2.9-3.3 kg over a period of 12 weeks (Frost et al. 2007). A review confirmed that low energy diets from 25 days to 9 months are associated with a mean weight loss of 13.6kg (±5.5) and significant improvement in total cholesterol as well as fasting glucose (Mulholland et al. 2011). Very low-calorie diet for nine weeks is sufficient to increase SHBG and free testosterone at statistically significant levels (Niskanen et al. 2004). In addition, mild intensity of aerobic activity for 150 min/weekly and energy deficit of 170-250kcal/day to achieve 10% weight loss increases testosterone, normalises oestradiol levels and reverses obesity related hypogonadism

without having to resort to bariatric surgery (de Lorenzo et al. 2018). The effects of low energy diets on metabolic parameters and hypogonadism are well described in the literature, but data on weight loss via caloric restriction and its effects on semen parameters are limited with no previous randomised control studies performed so far (Table 1.4).

Lifestyle changes, such as weight loss in obese couples, are associated with increased pregnancy rates (Faure et al. 2014). A nested Canadian pilot study randomised male partners of obese infertile women to lifestyle advice or no advice at all. Couples who made lifestyle modifications increased the odds for their couple to conceive. It appears that male partners who conceived lost more weight (0.83 kg ± 4.58 vs. +2.54 kg ± 4.25, p=0.009), ate more fruits per day (+0.41 ± 0.60 vs. -0.06 ± 0.79, p= 0.018) and spent less time watching TV compared to the non-exposed group (Belan, M. Duval, K. Farrah, J. Youssef, A. Carranza-Mamane, B. Pesant, M. Langlois, M. Baillargeon 2015). The possible reason underlying the increase odds for conception was demonstrated by a prospective study in Denmark, which involved 27 obese men participating in a 14-week weight loss program. These men were supported to lose weight with diet and exercise and were categorised in three groups depending on percentage weight loss: (I) 3.5-12.1%, (II) 12.2-17.1% and (III) 17.2- 25.4%. Group III with greatest percentage weight loss had statistically significant (P=0.02) increase in total sperm count [193 million (95% CI: 45; 341)] whilst group II with 12.2-17.1% percentage weight loss had non-significant (P=0.96) reduction in their DNA Fragmentation Index [-1 (95% CI:-11, 9)] (Berger Håkonsen et al. 2011). A cohort of infertile obese men from Bangalore reduced their BMI by 7.9% and achieved significant improvement in their sperm DNA fragmentation index as well as sperm morphology (Mir et al. 2018). In summary, weight loss via diet in obese infertile men can improve their male hormonal profile and possibly their sperm count or sperm DNA quality.

Table 1.4 Studies investigating the effects of weight loss via diet on semen parameters in men.

Study	Male, n	Study population	Results/ Conclusion	Comments
Berger Håkonsen et al. 2011	43	Initial cross- sectional study during a residential weight loss program and subsequent longitudinal study of 27 men in a 14-week diet programme	15% median weight loss with increase in total sperm count, T, SHBG with no improvement in DFI	No control group
Faure et al. 2014	6	Case series on 3-8 weeks diet and exercise. Sub-cohort from the ALIFERT study	3.9% BMI reduction, significant improvement in DFI, TGL, T/E ratio, 1 live birth per couple	Case series with control group (n=7) that had no hormonal or seminal parameters examined
Belan 2015	52	Controlled prospective cohort study nested in a randomized-controlled study for couples attending a Canadian Fertility Academic Clinic. Follow up over 12.7 months	Male partners with improved weight and lifestyle increase the odds of their couple to conceive	Nested study, flawed control selection
Mir et al. 2018	105	Prospective study cohort from the Infertility Department or weight loss centres in Bangalore, on 12-	7.9% BMI reduction, significant improvement in mean	No control group

		week diet. Follow up 6 to 12 months	DFI and sperm morphology	

T; Testosterone, DFI; DNA fragmentation index, BMI; body mass index, TGL; triglycerides, T/E ratio; Testosterone over oestradiol ratio.

1.8.3 Weight loss via bariatric surgery

Although weight loss via diet can improve lipid profile, glycaemic control, increase Testosterone and SHBG levels, bariatric surgery, is more effective on improving male reproductive hormones. Weight loss via bariatric surgery, such as Roux-en-y-Gastric-Bypass (RYGB), has positive metabolic outcomes. RYGB is shown to achieve a mean weight loss reduction from baseline of −46.8 kg (95% CI: −48.0 to −45.5; mean percent change, −35.0) at 2 years of follow up. Consequently, significant reductions are observed in glucose (95% CI: −0.86 to −0.03; mean percent change, −0.44 mmol/l) and LDL cholesterol (95% CI: −0.47 to −0.10; mean percent change, −0.25 mmol/l) (Adams et al. 2017). According to recent meta-analysis though, most significant improvements after bariatric surgery are observed in total testosterone levels (95% CI: 6.51 to 10.95; mean change, 8.73 nmol/l). It is interesting that total testosterone levels post bariatric surgery improve at a greater level compared to total testosterone improvement post low-calorie diet (95% CI: 1.68 to 4.07; mean change, 2.87 nmol/l) (Corona et al. 2013). Greater testosterone improvements are observed in men with greatest weight reduction and this improvement is accompanied by increase in gonadotrophins and reduction in oestradiol. However, the improvement in testosterone post bariatric surgery is faster than the change in seminal parameters (Samavat et al. 2018).

Changes in semen parameters in men losing weight after bariatric surgery have not been thoroughly investigated in the literature so far (Table 1.5). It has been reported that assisted reproduction outcomes are impaired by deteriorating sperm features at 12-18 months post bariatric surgery

(Lazaros et al. 2012). The deterioration in semen parameters post bariatric surgery is managed with intracytoplasmic sperm injection (ICSI) of fresh spermatozoa to achieve pregnancy in severe cases of infertility (Nathalie Sermondade et al. 2012). A possible explanation is that bariatric surgery causes deterioration of semen parameters in men shortly after surgery. Men losing an average of 70kg after RYGB, demonstrate azoospermia on spermiograms and spermatogenic arrest at testicular biopsies up to 15 months following RYBG. It is possible that excessive weight loss causes dramatic impairment in male reproductive potential in morbidly obese men undergoing bariatric surgery (di Frega et al. 2005). Impairment in semen parameters post bariatric surgery has been attributed to inadequate replacement, progressive depletion or malabsorption of micronutrients such as zinc, copper and selenium (Rosenblatt, Faintuch, and Cecconello 2017). However, the full pathophysiological mechanisms underlying spermatogenic arrest in the acute phase post bariatric surgery are not well understood.

According to most recent studies, morbidly obese men submitted to RYGB are not shown to have significant differences in their semen parameters 24 months post-surgery compared to obese men undergoing simple observations over the same period of time (Reis et al. 2012). Legro et al. demonstrated that bariatric surgery acutely suppresses spermatogenesis and reduces sperm concentration (95% CI: -92 to 8; mean change, -42mil/ml) within the first month post-surgery. This acute weight loss period is followed by a subsequent increase in sperm concentration up until a year after surgery (95% CI: -88 to 154; mean change, 33mil/ml) (Legro et al. 2015). Notably, a recent meta-analysis concluded that men undergoing bariatric surgery have no change in their sperm concentration or motility after an overall follow-up period of 6-24 months (Wei, Chen, and Qian 2018).

Table 1.5. Effects of bariatric surgery on semen parameters.

Study	Male, (n)	Study population	Results/ Conclusion	Comments
Calderón et al. 2019	15	Prospective cohort study of men undergoing RYGB or sleeve gastrectomy and 24 months follow-up	Serum inhibin B, kisspeptin increased whilst fasting insulin and leptin decreased. Sperm concentration, motility and morphology remain unchanged.	No control group available and relative short follow-up period
Samavat et al. 2018	23	Prospective case-control study of men undergoing RYGB and 6 months follow-up	No statistically significant improvement in sperm count, motility and volume. Reduction in sperm DNA fragmentation and seminal IL-8	Small study, no information on pregnancy rates
El Bardisi et al. 2016	46	Prospective cohort study of men undergoing sleeve gastrectomy and 12 months follow-up	Patients with severe oligospermia at baseline have statistical improvement in sperm concentration	No control group available

Study	Male, (n)	Study population	Results/ Conclusion	Comments
Legro et al. 2015	6	Prospective cohort study of men undergoing RYGB and 12-months follow up	No significant change in seminal concentration, motility or volume 12 months post-surgery, suggestive of a possible threshold of weight loss to improve male reproductive function	Small sample size, no control group available
Reis et al. 2012	10	Prospective case-control study of men undergoing RYGB and 24 months follow up	No significant differences in semen parameters 24 months post-surgery compared to obese men undergoing simple observations	No power calculation
Sermondade et al. 2012	3	Case series of men undergoing sleeve gastrectomy and 6-24 months follow up	Deterioration in all sperm features at 12-18 months post bariatric surgery	Small sample size

Massive weight loss from bariatric surgery most likely has a neutral effect on semen parameters over at least a 2 year follow up period (Wei, Chen, and Qian 2018). Longer and possibly more intense follow up of men undergoing bariatric surgery is required, with frequent time points for follow up of metabolic, reproductive as well as seminal profile. Despite limitations on studies concerning bariatric surgery and male fertility, available data demonstrate that bariatric surgery may not improve dramatically male reproductive potential for the following reasons:

i) Rapid weight loss is linked to a relative undernutrition status, even if supplements are commonly provided after surgery. Bariatric surgery such as RYGB reduces iron levels, red blood cell folate, selenium, vitamin A, C, riboflavin, B6, B12, thiamine, B9 and vitamin D levels. Supplements are provided before and after surgery particularly for vitamins B12, D and folate but optimal dietary quality or full adherence to supplements provided is not always guaranteed (Rosenblatt, Faintuch, and Cecconello 2017). As a result, men after bariatric surgery are at risk of nutritional deficiencies. The risk of low trace elements has a negative impact on regulation of spermatogenesis and germ cell division. In addition, the risk of low vitamin D has negative impact on sperm motility (Rosenblatt, Faintuch, and Cecconello 2017). Both examples highlight how nutritional deficiencies after bariatric surgery, adversely affect male reproductive potential.

ii) Massive weight loss after bariatric surgery mobilizes fat and oestrogens from adipose tissue. For example, excessive adipose tissue in obese men undergoing bariatric surgery could hypothetically release lipophilic contaminants previously accumulated via the food chain. The release of lipophilic contaminants could disrupt male fertility, as these contaminants have been associated with decreased semen quality (Du Plessis et al. 2010). Also, oestrogen metabolising enzymes become saturated and persistently high oestrogens despite weight loss exert negative feedback on the HPG axis (Calderón et al. 2019). The negative feedback on HPG axis pre-exists as obese men before bariatric surgery have low gonadotrophins (Davidson et al. 2015; de Lorenzo et al. 2018). Men with low gonadotrophins are usually given gonadotrophin analogues to achieve spermatogenesis and subsequently conceive for a

median period of 28.2 months (P. Y. Liu et al. 2009). Therefore, the investigation of male gonadal function after bariatric surgery may require more than 24 months follow-up to provide meaningful results on seminal parameters and pregnancy outcomes.

Diet is an alternative method to achieve weight loss in obese men. There are no available data in the literature comparing the effects of bariatric surgery to the effects of weight loss via diet on male fertility. Even though men undergoing bariatric surgery have greater weight loss and a steeper increase in their testosterone levels (Corona et al. 2013; de Lorenzo et al. 2018), effects on semen parameters are controversial. Therefore, weight loss via diet provides a flexible model to investigate seminal parameters and how they change during different levels of caloric restriction or percentage weight loss. For instance, 4-8% BMI reduction results in significant improvement in sperm DNA fragmentation index (Faure et al. 2014, Mir et al. 2018). Also 17.2- 25.4% weight reduction results in significant improvement in sperm count (Berger Håkonsen et al. 2011).

1.9 RATIONALE FOR STUDY

The regulation of male fertility constitutes a promising area for ground-breaking research. It is important to further develop and supplement conventional semen analysis. Animal studies could demonstrate key areas for focus in the research for male fertility medical therapies. More research on male subfertility due to obesity could offer better understanding on how weight loss affects metabolic parameters, reproductive hormones and sperm function. Since diagnostic tools and treatment options for male infertility remain limited, future research could reduce reliance on ART, with behavioural solutions being particularly attractive from the economic perspective and, potentially also for the couple themselves in terms of reduced instrumentation and with health benefits carrying forward into future life beyond conception.

1.10 SYNOPSIS OF THESIS CHAPTERS

In the second chapter I explored the role of seminal reactive oxygen species (ROS) as a novel marker of sperm function in male partners of women with RPL. Recurrent pregnancy loss (RPL), also termed recurrent miscarriage, is defined as loss of three or more consecutive pregnancies prior to 24 weeks from the last menstrual period. Although female factors in RPL are well known, male factors require further investigation. It is known that dysfunctional sperm can impede fertilisation of the oocyte, cause defective placentation, poor embryo development or even lead to pregnancy loss (Esteves 2016). My study showed that male partners of women with idiopathic RPL have significant abnormalities in their metabolic as well as reproductive profile, which result in impaired sperm function.

In the third chapter, I examined whether AZD5904 a potent irreversible myeloperoxidase inhibitor improves reproductive function in obese male mice. Obesity is a chronic inflammatory state, where production of cytokines and interleukins is increased at systemic as well as seminal level (Oliveira et al. 2017). Oxidative stress probably mediates defective sperm function due to high fat diet induced obesity. I hypothesized that high fat diet in mice would result in impaired semen parameters, increased seminal oxidative stress and sperm DNA damage. My study also investigated the effects of AZD5904 on sperm function in male mice with fat diet induced obesity and suggested that longer studies on appropriate animal models would be required to make definitive conclusions.

Finally, I examined if weight loss via low energy diet could affect the reproductive function of obese men. Obesity is associated with male factor infertility. Interestingly, pregnancy rates via assisted reproduction are reduced amongst couples with obese male partners. Male obesity is linked to hypogonadotrophic hypogonadism, erectile dysfunction and impaired spermatogenesis, with no currently available effective treatment. I hypothesised that weight lost via low energy diet in obese men would improve metabolic and reproductive hormonal function as well as become a starting point for treating male factor infertility associated with obesity.

Chapter 2

Elevated semen oxidative stress in male partners as a novel marker of recurrent miscarriage

2.1 INTRODUCTION

2.1.1 Recurrent pregnancy loss

Recurrent pregnancy loss (RPL; recurrent miscarriage), affects 1-5% of couples seeking fertility (El Hachem et al. 2017). Pregnancy loss or miscarriage is the spontaneous termination of pregnancy before the 24th week of gestation, when the foetus reaches viability. RPL was defined as the loss of three or more consecutive pregnancies before 24 weeks of gestation (Royal College Obstetricians and Gynaecologists (RCOG) 2011). The definition was updated on 2017, shortly after recruitment was completed recruitment for the current study, to include two rather than three consecutive miscarriages (RPL ESHRE 2017). Regardless of its definition, multiple pregnancy losses can have a negative psychological impact on couples trying to conceive. Therefore, research is essential as half of the cases of RPL remain unexplained and the time required to achieve successful pregnancy remains needlessly prolonged.

2.1.2 Management practices for couples with RPL

Women with RPL are routinely screened for aetiological factors such as chronic or recurrent infections (Byrn and Gibson 1986), anti-phospholipid syndrome, thrombophilia, endocrine, immune and genetic factors (Stirrat 1990), as well as anatomical abnormalities (Devi Wold, Pham, and Arici 2006). The Royal College of Obstetrics and Gynaecologists in 2011 recommended detailed diagnostic screening for women experiencing RPL (RCOG 2011). However, fifty per cent of RPL cases have been reported as idiopathic (Stephenson 1996) and it is plausible that male factors could account for some cases of idiopathic RPL. Notably, most recent guidelines from the European society of human reproduction & embryology Society (ESHRE), sperm quality and male lifestyle factors should be investigated in couples with RPL (PRL ESHRE 2017).

2.2.3 The role of sperm DNA fragmentation in miscarriage

Over the last thirty years a significant decline has been observed in sperm concentration (Levine et al. 2017). Also, previous research suggests that male partners affected by RPL have impaired sperm quality with reduced total motility, low normal morphology and increased sperm DNA damage (Jayasena et al. 2019; Imam et al. 2011). Sperm DNA fragmentation has previously been found to be a reasonable predictor of pregnancy outcome and male infertility (Luke Simon et al. 2011). Meta-analyses have suggested the role of sperm DNA fragmentation on miscarriage after spontaneous pregnancy or assisted reproduction treatment (Robinson et al. 2012; Zhao et al. 2014).

Men with high sperm DNA fragmentation undergoing assisted reproduction treatment have 13% - 16% less pregnancies with their partners compared to men having low sperm DNA (R. Henkel et al. 2004). Pregnancy outcomes may be suboptimal in men with high sperm DNA fragmentation due to impaired placentation and embryonic development resulting in pregnancy loss. Paternally imprinted genes play an important role of placentation, which is critical to embryo viability particularly in the context of assisted reproduction (Tesarik, Greco, and Mendoza 2004). The role of paternally imprinted genes in placentation is mainly illustrated by animal studies, observing that mouse embryos from two paternal genomes (androgenotes) have deficient embryo formation but relatively preserved placental formation. Conversely, mouse embryos from two maternal genomes (parthenogenotes) have deficient placental formation with relative sparing of embryo formation (Brevini TAL, 2013).

Semen reactive oxygen species (ROS) are a natural by-product of oxygen metabolism and are physiologically required for capacitation and acrosome reaction in sperm. In cases of poor sperm function ROS is produced in excess causing oxidative stress and potentially sperm DNA fragmentation

(R. R. Henkel 2011). It is therefore clinically important to investigate whether novel diagnostic markers of sperm function as ROS may be related to miscarriage. Further studies may enable an improved understanding of how the paternal genome regulates placentation and what can be done to improve pregnancy outcomes for couples with RPL.

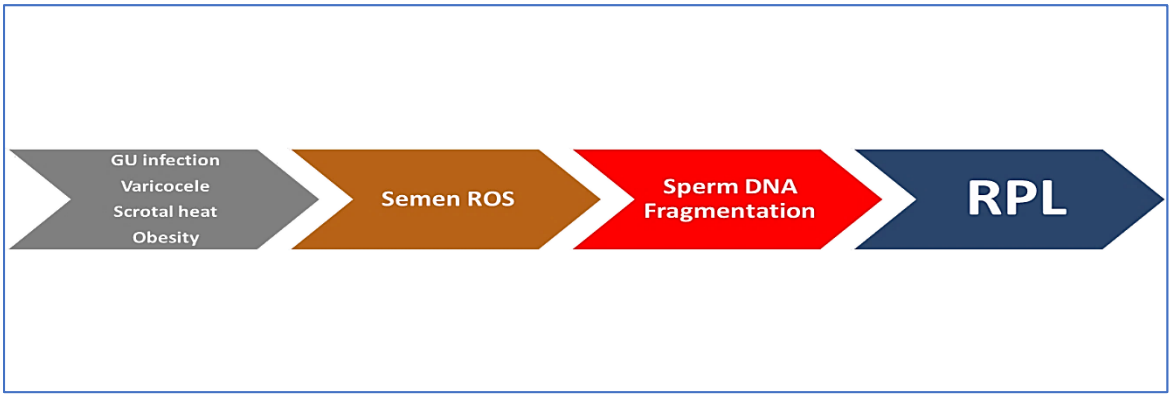
2.2 STUDY RATIONALE, HYPOTHESIS AND AIMS

Half of RPL cases remain idiopathic despite detailed clinical and laboratory investigation of the female partner. It is therefore important to investigate potential contribution of the male partner. Dysfunctional sperm can impede fertilisation of the oocyte, cause defective placentation and eventually lead to pregnancy loss (Esteves 2016).

Hypothesis

Male partners of women with idiopathic RPL have significant abnormalities in metabolic, reproductive endocrine and sperm function (Figure 2.1).

Figure 2.1 Risks factors for male infertility and a hypothetical model in the relationship between semen ROS, DNA fragmentation and recurrent pregnancy loss (RPL).



Aims

To investigate if serum levels of reproductive hormones, semen ROS and sperm DNA fragmentation are different between male partners of women with RPL and healthy, unselected age-matched men from the general population.

2.3 METHODS

2.3.1 Participant Recruitment

Male partners of women with RPL were recruited from the recurrent miscarriage clinic at St. Mary's Hospital, London between September 2016 and May 2017 (Jayasena et al. 2019). Couples referred to the recurrent miscarriage clinic included in the study experienced three or more pregnancy losses before the 24th week of gestation (RCOG 2011). Healthy male controls were recruited through local adverts. Ethical approval was granted by the West London & GTAC Local Research Ethics Committee (Ref 14/LO/1038), and the study was performed in accordance with the Declaration of Helsinki. Each participant attended the Andrology Department, Hammersmith Hospital for a single appointment, where he produced semen, had blood testing and completed a reproductive history questionnaire.

2.3.2 Semen Analysis

Semen samples were produced on site in private rooms within the Department of Andrology, Hammersmith Hospital, UK. Samples were produced following 2-7 days of sexual abstinence and incubated at 36 $\pm$ 1°C for liquefaction up to 60 minutes prior to analysis. Semen analysis was performed according to World Health Organisation (WHO) 2010 guidelines and UKNEQAS (UK National External Quality Assessment Service) accreditation. Sperm morphology was examined on Papanicolaou pre-stained slides, using Kruger strict criteria ("WHO Laboratory Manual for the Examination and Processing of Human Semen" 2010).

2.3.3 Measurement of seminal ROS levels

ROS was measured using an established in-house chemiluminescence assay that was based on measurements of light emission via luminol (5-amino-2,3-dihydro-1,4-phtalazinedione) chemiluminescence (Vessey et al. 2014). Luminol stock solution was made every 3 months and was stored in room temperature at 20-25°C in the dark. For the purposes of the current study, 50μl luminol stock solution mixed with 950μl DMSO was prepared to make up a total of 1000μl of working solution for daily use. In addition to the luminol stock solution, the following three solutions were also made up daily:

I. Negative control solution; this was made after aliquoting 400μl of phosphate-buffered saline (PBS) to an eppendorf and adding 100μl of luminol working solution. Negative control mean value had to be <120RLU/sec to allow reliable measurements.
II. Positive control solution; this was made after aliquoting 395μl of PBS to an eppendorf and adding 5μl of 30% hydrogen peroxide (H_20_2). Finally, 100μl of luminol working solution was added to complete the preparation. Positive control mean value had to be >100,000 RLU/sec to allow reliable measurements.
III. Specimen assay was made of 400μL neat (native) semen mixed with 100μL working solution containing luminol.

Each sample was gently mixed immediately before taking luminometer readings (GloMax; Promega Corporation; Madison, WI, USA). Negative controls were placed into the luminometer immediately after preparation, so that readings could be taken every minute for ten minutes. Once all ten readings were taken, the mean value was calculated. Chemiluminescence was expressed as mean relative light units per second (RLU/sec), as measured over 10 minutes at minute intervals. Following negative control solutions, chemiluminescence was measured for positive controls at least 20 minutes before

semen sample production by the participant. Finally, chemiluminescence was measured for seminal specimens. ROS value was calculated via the following formula:

$$\text{ROS} = \frac{\text{Mean seminal sample chemiluminescence} - \text{Negative control chemiluminescence}}{\text{Sperm concentration}}$$

In-house validation was performed to ensure consistent positive and negative calibration daily. Prior to commencing the study, the assay had been run daily in the Andrology Department, Hammersmith Hospital for over a year. All analysis runs contained negative and positive control samples. The reference range for semen ROS was <3.8 RLU/sec/million sperm (Vessey et al. 2014).

Luminol chemiluminescence assay is the most commonly used technique to detect oxidized end products. Lucigenin chemiluminescence is a very similar technique using lucigenin but has the disadvantage that it can only detect extracellular free radicals, primarily superoxide. In contrast to lucigenin, luminol can detect both intracellular and extracellular deoxygenation, including hydrogen peroxide, superoxide, and hydroxyl ions (Vessey et al. 2014). We therefore used luminol chemiluminescence to detect intracellular and extracellular free radicals in the semen.

2.3.4 DNA Fragmentation Analysis

DNA Fragmentation was measured using the Halosperm G2 kit (Halotech DNA SL, Madrid, Spain) according to the method described by Fernández et al. (José Luis Fernández et al. 2005; Jose Luis Fernández et al. 2003). In brief, semen samples were mixed with heated inert agarose and cooled on pre-treated glass slides. A denaturant agent was added to lysis solution, followed by staining with eosin and thiazine. Slides were subsequently viewed under bright-field light microscopy to assess sperm chromatin dispersion (SCD). Using the method, a large halo was seen around sperm without substantial DNA breakage, due to spreading DNA loops emerging from a central core. However, no halo or a minimal halo is seen around sperm containing fragmented DNA. The Halosperm test kit was internally validated in the Andrology department Hammersmith Hospital. Samples with a DNA fragmentation index (DFI) <15% were considered normal as directed by the kit (Evenson et al. 1999).

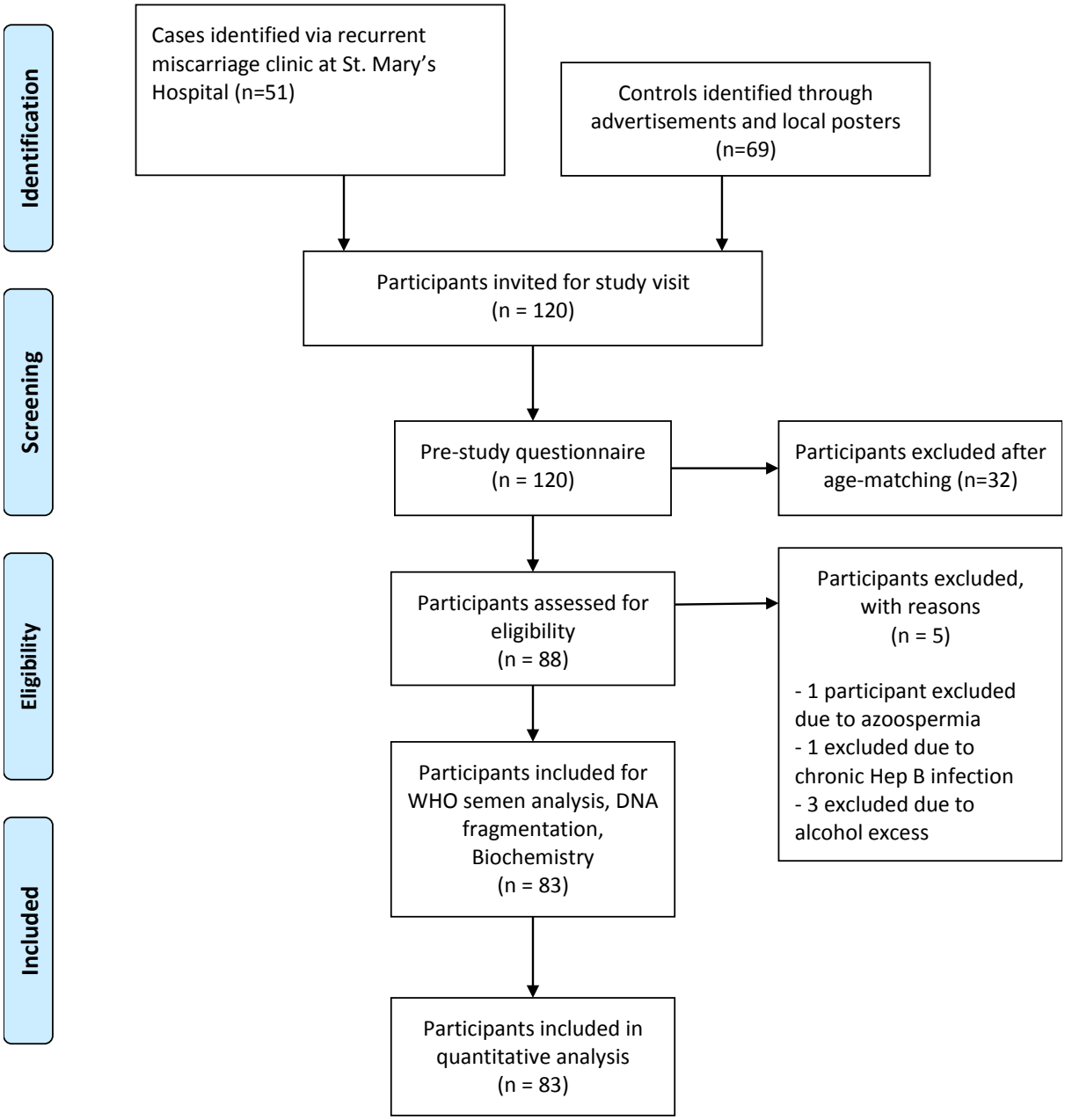
2.3.5 Endocrine Biochemistry

Morning blood samples were analysed for serum luteinizing hormone, (LH), follicle-stimulating-hormone (FSH), oestradiol, testosterone and sex-hormone binding-globulin (SHBG) in the clinical biochemistry department of Charing Cross Hospital, using Abbott ARCHITECT an automated immunoassay platform under UKNEQAS accreditation.

2.3.5 Protocol

Informed consent was obtained from all participants, including cases and controls. Participants completed a comprehensive questionnaire to check baseline clinical characteristics. The questionnaire included questions regarding their date of birth, smoking status, alcohol intake per week, previous diagnosis of varicocele, sexually transmitted disease (STI) and use of regular medication. The questionnaire also allowed screening for clinical conditions directly linked to impaired fertility such as testicular tumours or torsion, cancer therapy or use of cytotoxic medication, systemic autoimmune disease, chronic or acute systemic illness. Two participants with male infertility due to chronic hepatitis B virus infection were excluded. Three study participants were excluded after the first study visit due to having excess alcohol intake above 21 units/week (Figure 2.2). Thirty-two age matched participants were selected for inclusion in this study. Following completion of the questionnaire, subjects attended a single study visit as outlined in the study protocol. Participants with semen parameters outside the reference range, including volunteers, were contacted by a study investigator and asked to attend an additional hospital visit. During this visit, participants were informed about their results by a research doctor or a specialist nurse in a private consultation room. A confidential letter was then sent to their named general practitioner, in case participants would like to have further counselling or referral to specialist services.

Figure 2.2 Flow diagram outlining the selection process for the recurrent pregnancy loss (RPL) study



2.3.6 Statistical Analysis

Data analysis was performed using GraphPad Prism v.5. Quantitative data was assessed for normality using D'Agostino-Pearson normality test, followed by appropriate parametric (Unpaired t-test) or non-parametric (Wilcoxon-Rank-Sum test) analysis. Group comparisons with respect to categorical variables were performed using Fisher's-exact test. All hypothesis testing was two-tailed; $p<0.05$ was considered statistically significant. Data are presented as either mean + standard error of mean (SEM).

2.4 RESULTS

A. Clinical characteristics

The two groups self-reported via the study questionnaire past medical conditions that could potentially be linked to poor sperm quality. Control and case subjects had similar characteristics regarding smoking and alcohol intake (Table 2.1). Sexually transmitted infection, orchidopexy and varicocele, which are known sources of ROS (Agarwal et al. 2018) were similar in frequency between groups. Also use of steroids and antihypertensives, such as calcium channel blockers, which can negatively affect spermatogenesis, was homogenous between groups (Mortimer et al. 2013). Five participants from the control group and eighteen from the RPL group had previously fathered children.

Table 2.1. Clinical Characteristics: Data for age, ethnicity, body mass index (BMI), weekly alcohol intake and other clinical characteristics are presented as mean ± SEM; * P<0.05

Parameter	Controls (n=34)	Partners of women with RPL (N=49)
Age (years)	36.7 ± 0.9	37.6 ± 0.6
White ethnicity	23	34
Asian Indian ethnicity	3	5
Asian other than Indian ethnicity	4	3
Afro-Caribbean ethnicity	2	3
Other ethnicity	2	4
BMI (kg/m^2)	26.0 ± 0.8	27.6 ± 0.7 *
Smokers (%)	2 (6%)	5 (10%)
Alcohol units/week	9.4 ± 1.7	13.6 ± 1.4
Varicocele	1	2
Orchidopexy	1	0
Previous sexually transmitted infection	3	4
Immunosuppressant or steroids	3	4
Other regular medications; antihypertensive, analgesics	2	3

B. Reproductive hormone profile

Serum reproductive hormone levels in both groups are shown in Table 2.2. Levels of non-fasting serum morning testosterone were slightly lower in the RPL group when compared with control group (mean serum testosterone in nmol/L: 16.0 + 0.8, RPL; 17.3 + 1.4, control, P>0.05). Furthermore, levels of serum oestradiol were non-significantly lower in the RPL group when compared with the control group (mean serum oestradiol in pmol/L: 86.5 + 3.4, RPL; 91.4 + 4.8, control, P>0.05). Serum levels of luteinizing hormone (LH) were also non-significantly lower in the RPL group when compared with controls (mean serum LH in iU/L: 2.7 + 0.2, RPL; 4.4 + 1.1, control, P>0.05). Serum FSH, sex hormone binding globulin (SHBG), Total cholesterol, LDL and HDL levels were similar between men with RPL and healthy controls.

Table 2.2 Endocrine parameters & lipid profile of age-matched subjects. Data presented as mean ± SEM

Parameter	Partners of women with RPL (n=49)	Age-matched controls(n=34)
LH (2-12 iu/L)	**2.7 + 0.2**	**4.4 + 1.1**
FSH (1.7-8 iu/L)	**3.1 + 0.2**	**3.4 + 0.3**
Oestradiol (<190 pmol/L)	**86.5 + 3.4**	**91.4 + 4.8**
SHBG (15-55 nmol/L)	**29.4 + 1.3**	**33.4 + 2.4**
Testosterone (10-30 nmol/L)	**16.0 + 0.8**	**17.3 + 1.4**
Total Cholesterol (<5 mmol/l)	**5.3 ± 0.6**	**5.1 ± 0.2**
LDL (<3 mmol/l)	**3.33 ± 0.2**	**3.14 ± 0.2**
HDL (>1 mmol/l)	**1.27 ± 0.1**	**1.32 ± 0.1**

C. Semen parameters

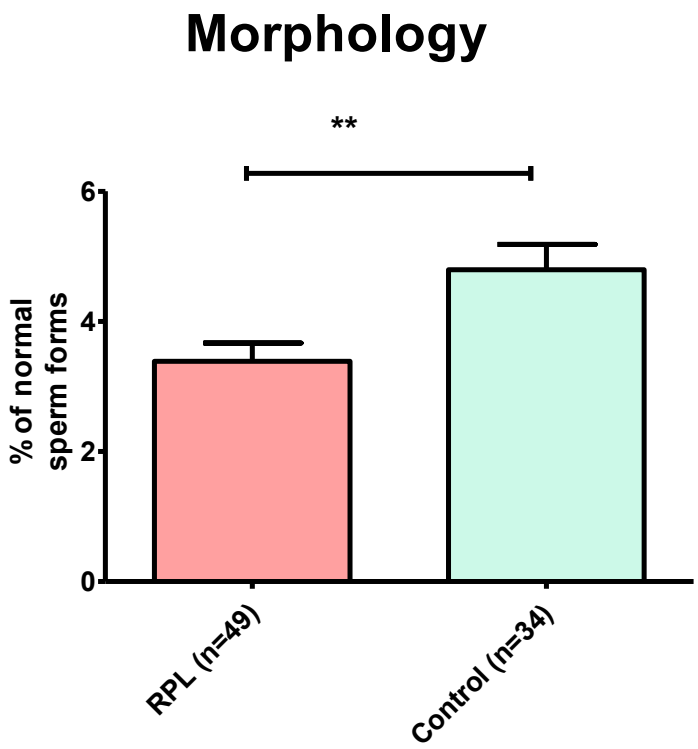
Semen analysis results for the RPL and control groups are summarised in Table 2.3. No significant differences were found in conventional semen parameters between the two groups other than sperm morphology.

Table 2.3 Semen parameters of subjects. Data presented as mean + SEM; ** P<0.01 vs. controls

Parameter	Partners of women with RPL (n=34)	Controls (n=49)
Volume (>1.5 mL)	**3.8 + 0.2**	**3.6 + 0.2**
Sperm Concentration (>15 million/mL)	**59.7 + 8.7**	**55.9 + 2.5**
Progressive Motility (≥32%)	**49.9 + 2.0**	**54 + 3**
Total Motility (≥40%)	**64.1 + 1.9**	**67.1 + 2.7**
Total Motile Count (≥ 20 million motile sperm/ejaculate)	**105.8 + 15.7**	**122.9 +18.1**
Morphology (≥4 % normal)	**3.4 + 0.3 ****	**4.8 + 0.4**

Total motile count is the proportion of motile sperm within the ejaculate and is calculated by multiplying the ejaculate volume (ml) by the concentration (million sperm/ml) by the total motility (percentage % of total motile sperm). Men in the RPL group had non-significantly lower total motile sperm when compared with controls (mean total motile count: 105.8 + 15.7, RPL; 122.9 +18.1, control, P>0.05). The RPL group had significantly lower percentage of morphologically normal sperm using WHO criteria (% sperm with normal morphology: 3.4 ±0.3, RPL; 4.8±0.4, control, P<0.01) when compared with controls (Figure 2.3).

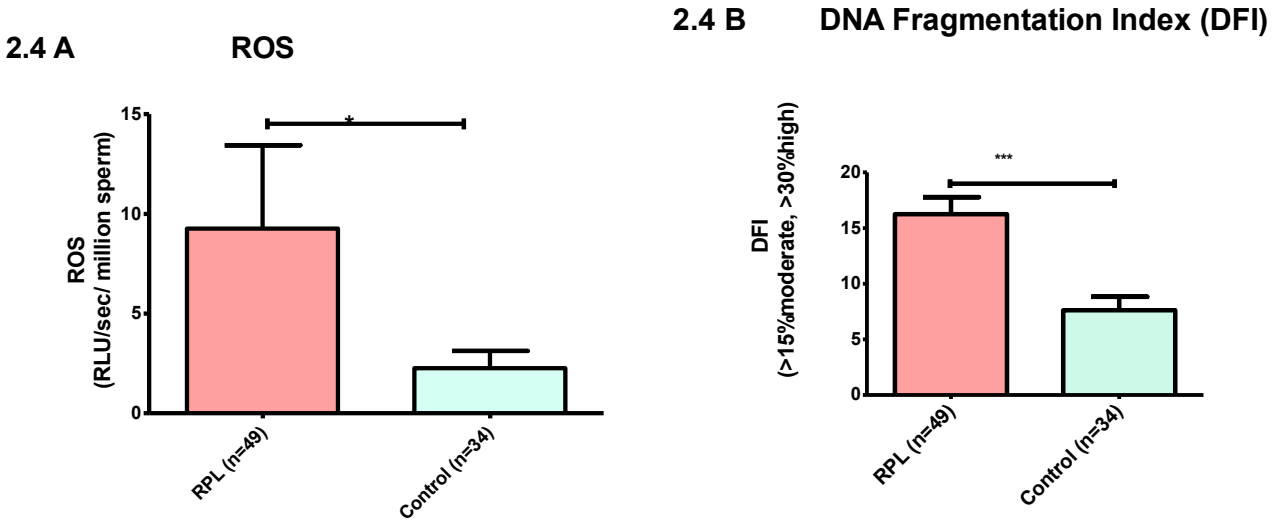
Figure 2.3 Conventional sperm characteristics of male partners of women with recurrent pregnancy loss when compared the control group. Bar graphs compare normal sperm morphology, in recurrent pregnancy loss (RPL) group versus control group. Data presented as mean ± SEM; **P<0.01.



D. Molecular sperm characteristics

Male partners of women with RPL had considerably high levels of semen oxidative stress and sperm DNA fragmentation, which are known to impair sperm function. Men from the RPL group had a 4-fold increase of semen ROS when compared with age-matched controls (mean ROS: 9.3 + 4.2, PRL; 2.3 + 0.9, control, P<0.05; Figure 2.4 A). Mean levels of sperm DNA fragmentation were also 2-fold higher in the RPL group when compared with controls (mean DFI: 16.3 + 1.5, PRL; 7.6 + 1.2, control, P<0.0001; Figure 2.4 B).

Figure 2.4 Molecular sperm characteristics of male partners of women with recurrent pregnancy loss when compared with the control group. Bar graphs compare reactive oxygen species (ROS) (A) and DNA fragmentation index (DFI) (B), in recurrent pregnancy loss (RPL) group versus control group. Data presented as mean ± SEM; *P<0.05; ***P<0.001.



E. Correlation between DNA fragmentation index and semen morphology or seminal ROS

Despite normal morphology being significantly lower in the RPL group compared to the control, no significant correlation was observed with molecular sperm characteristics. Specifically, DNA fragmentation did not correlate with sperm morphology or seminal ROS in the RPL or control group (Table 2.4).

Table 2.4 Association of subject DNA fragmentation index with sperm morphology and seminal ROS. R^2; coefficient of determination

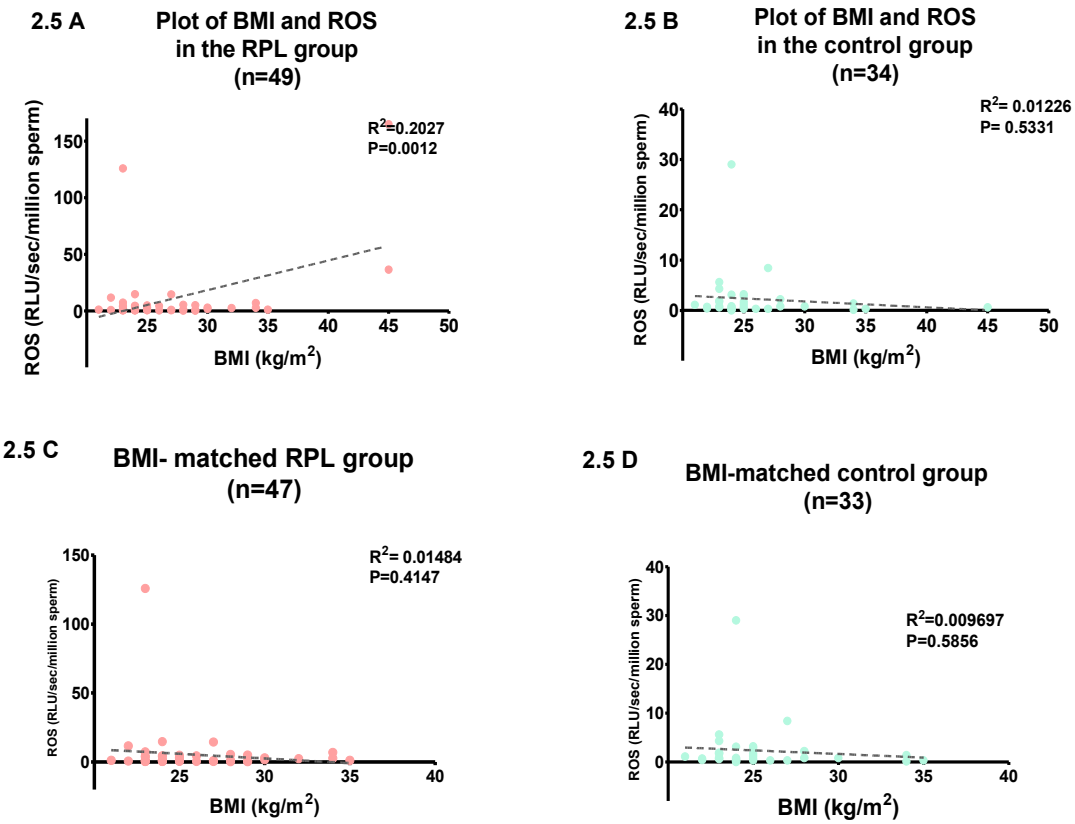
Parameter	Group	DNA fragmentation index
Morphology	RPL	R^2 = 0.005; P = 0.63
	Control	R^2 = 0.002; P = 0.81
ROS (RLU/sec/10^6)	RPL	R^2 = 0.021; P = 0.35
	Control	R^2 = 0.120; P = 0.05

F. Correlation between subject BMI with seminal ROS

Having observed that mean body mass index (BMI) was higher in the RPL group when compared with the control group (Table 2.1), I investigated the association between BMI and semen ROS. There is probably a meaningless correlation of BMI with ROS levels in the RPL group (R^2=0.2027, P=0.35) (Figure 2.5 A). However, for BMI-matched subjects there is no correlation of BMI with seminal ROS between groups (Figure 2.5 C, Figure 2.5 D). BMI and semen ROS appear to have little to no collinearity in my study, but this could be attributed to small sample size and further research with a larger sample size would be required to investigate any possible association. In practice, BMI and semen ROS could be

related as shown by previous studies (Tunc, Bakos, and Tremellen 2011). It is notable that the RPL group had only one-unit higher BMI compared to the control group. Hence, men in the RPL group may be have been exposed to other factors linked with obesity and oxidative stress, such as sleep apnoea, high fat diet or psychosocial stress (Agarwal et al. 2018), which would have to be investigated in future studies.

Figure 2.5 Semen oxidative stress in male partners of women with recurrent pregnancy loss when compared with age-matched subjects. Scatterplots present levels of semen reactive oxygen species (ROS) in recurrent pregnancy loss (RPL) group and control group when plotted against subject body mass index (BMI) (A, B) as well as BMI for BMI-matched subjects (C, D). R^2, coefficient of determination.



In addition, BMI in the control as well as RPL group, is not associated with sperm DNA fragmentation (Table 2.5).

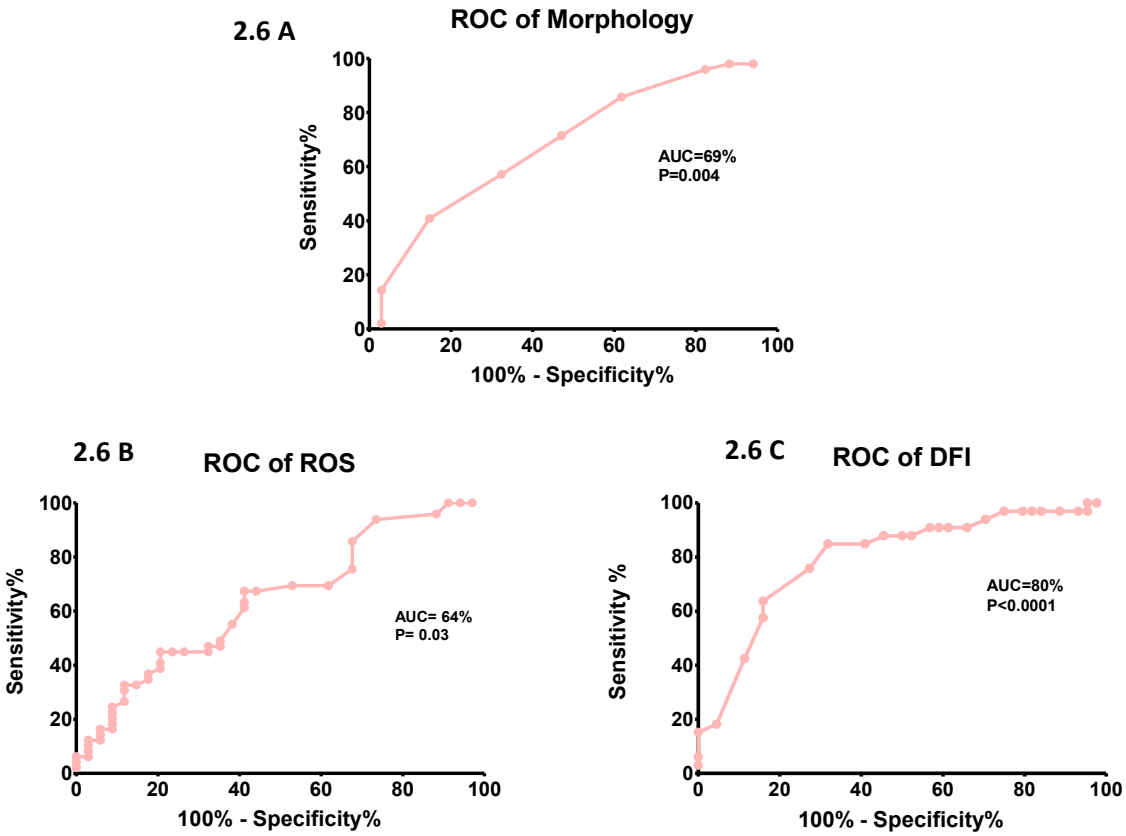
Table 2.5 Association of subject body mass index (BMI) with ROS and sperm DNA fragmentation index. R^2; coefficient of determination

Parameter	Group	BMI (kg/m^2)
ROS ($RLU/sec/10^6$)	RPL	R^2 = 0.020; P = 0.44
	Control	R^2 = 0.012; P = 0.46
DNA fragmentation index (Halo G2 >15% moderate)	RPL	R^2 = 0.014; P = 0.93
	Control	R^2 = 0.015; P = 0.51

G. Comparing reproductive parameters between controls and men with RPL

Sperm morphology, semen ROS and sperm DNA fragmentation were significantly different between RPL cases and controls. We therefore investigated the potential of these factors as screening tests to distinguish men with RPL from controls in the study using Receive Operator Characteristic (ROC) analyses. ROC curves plot the sensitivity of a screening test on the y axis against the false discovery rate (1-specificity) on the x axis. The area under the ROC curve (ROC AUC) measures the two-dimensional area under the ROC curve which is an aggregate measure of performance. ROC analysis suggested that sperm morphology, semen ROS, and sperm DNA fragmentation each discriminated significantly between controls and men with ROS (Figure 2.6). The greatest discriminator between control and ROS groups was sperm DNA fragmentation, which had a ROC AUC value of 80% (P<0.0001 vs. line of non-discrimination).

Figure 2.6 Receiver operation characteristics of reproductive parameters in male partners of women with recurrent pregnancy loss when compared with subgroup of control subjects aged >30 years. Receive operator characteristics (ROC) analyses for normal sperm morphology (A), semen reactive oxygen species (ROS; B) and sperm DNA fragmentation (c). Area under curve (AUC) values are presented for each parameter.



2.5 DISCUSSION

The reason for recurrent pregnancy loss remains undiagnosed in half of all cases (Ford and Schust 2009). Female partners undergo systematic screening for recurrent pregnancy loss but there is no recommended routine screening for male partners. It is known that sperm DNA plays a role in placentation. It is therefore plausible that male partners of women with PRL have high risk of sperm DNA fragmentation (Robinson et al. 2012). Increased sperm DNA fragmentation may be caused by genitourinary infection, varicocele, scrotal heat and obesity (Wagner, Cheng, and Ko 2018) but the mechanism which links sperm DNA fragmentation and RPL is unclear. The current study is the first study evaluating endocrine parameters and sperm function in male partners of women with RPL. Endocrine parameters and sperm function of male partners of women with RPL were compared with age-matched control subjects from the general population. The current study reports markedly elevated levels of semen ROS, high sperm DNA fragmentation and reduced sperm morphology in men affected by RPL when compared with control subjects (Jayasena et al. 2019).

Metabolic and endocrine parameters in male partners of couples with RPL

No significant differences were reported between male reproductive hormones and lipid profile of the two groups. Total cholesterol was higher by 4% in the RPL group, whilst testosterone and oestradiol were lower by 8% and 7% respectively. Abnormal lipid profile is associated with abnormal lipid consistency of the sperm membrane and may lead to increased oxidative stress (Andersen et al. 2016; Ashok Agarwal et al. 2018). Intratesticular testosterone acting in paracrine manner at high concentration on Sertoli cells is also critical to spermatogenesis (Fauser et al. 1986). Reduced testosterone production with suboptimal lipid profile could be a possible explanation for defective

spermatogenesis and suboptimal semen parameters leading to unsuccessful conception or pregnancy loss.

Semen parameters in male partners of couples with RPL

Male partners of women with RPL had significantly lower normal sperm morphology versus controls. The cause of poor sperm morphology is not well understood. Previous studies hypothesised that oxidative stress from free radicals could lead to sperm DNA breaks and affect chromatin compaction, resulting in abnormal sperm morphology (Ashok Agarwal, Tvrda, and Sharma 2014; Oumaima et al. 2018). We did not observe a significant correlation between sperm DNA fragmentation and sperm morphology or seminal ROS, although the absence of correlation may due to small number of participants.

Role of seminal oxidative stress in RPL

ROS can be generated on background of smoking, excess alcohol, previous testicular surgery such as orchidopexy, varicocele, genitourinary infection and immunosuppressive medication (Agarwal et al. 2018). Elevated ROS represent a common mediator between lifestyle factors, testicular pathologies, toxin exposure and reduced reproductive potential. Oxidative stress with high ROS could consequently induce placental dysfunction via DNA fragmentation and implantation failure (Gupta et al. 2007), but the mechanism is not yet determined. We observed that mean levels of ROS were significantly higher in men with RPL when compared with controls, using a previously described and validated luminol chemiluminescent assay (Jayasena et al. 2019).

The only clinical characteristic that was significantly different between the two groups was BMI. Men with female partners experiencing RPL were significantly heavier compared to controls. A low positive correlation (R^2=0.23, P=0.0039) has previously been reported between ROS and BMI, as ROS levels increased above the normal weight range of 25kg/m^2 (Tunc, Bakos, and Tremellen 2011). In the current study, men from the RPL group had slightly higher levels of seminal ROS with increased BMI. However, this association disappeared after a cut-off BMI below 40 kg/m^2 was used for a BMI-matched analysis between groups. Seminal ROS reflect broader sperm function and obesity could potentially lead to elevated ROS, but future studies are required to investigate the precise nature of the association with recurrent pregnancy loss.

A previous study performed within an Indian population suggested that male partners of women with RPL had almost 4-fold higher ROS levels compared to male controls with proven fatherhood within the last twelve months (Imam et al. 2011). This study involved young men with RPL in their third decade of life compared to fertile controls. My study supports the findings of this previous study in a more ethnically diverse population, including white or Afro-Caribbean men. Men from the general population with no proven fatherhood were also included in the control arm of my study as they offered a more rigorous group for comparison. In case male partners of women with RPL were compared to established fathers, the clinical value of the current study findings may have been overestimated. The choice of control group with unproven fertility could indicate that the conclusions of my study revealed genuine abnormalities in the reproductive physiology of men affected by RPL when compared with men from the general male population, rather than just fathers.

Excessive semen ROS induce sperm mitochondrial damage, immature cell apoptosis and sperm DNA fragmentation (Wagner, Cheng, and Ko 2018). ROS additionally lead to sperm dysfunction by lipid peroxidation of the sperm cell membrane and subsequent DNA damage. Therefore, ROS testing

cannot be interpreted independently of sperm DNA fragmentation testing. Semen ROS values depend upon individual male parameters, whilst sperm DNA fragmentation reflects the quality of sperm DNA contents mostly referring to the genetic health of the offspring (A. Agarwal et al. 2017).

Role of sperm DNA fragmentation in RPL

Multiple studies have reported that sperm DNA fragmentation is elevated in male partners affected by RPL when compared with unaffected men (R. Henkel et al. 2004; Robinson et al. 2012). Sperm DNA fragmentation has been shown to have a negative impact on natural conception or assisted reproductive technology outcomes (Zhao et al. 2014). Absence of a relationship between sperm DNA fragmentation and miscarriage has also been reported (Coughlan et al. 2015). Different cut-off values for sperm DNA fragmentation were considered relevant by different authors, which may explain why previous studies have been contradictory (Aitken et al. 1998). My results support the view that men with RPL have significantly elevated sperm DNA fragmentation when compared with controls (Jayasena et al. 2019).

Sperm DNA damage in male partners of women with RPL occurs in the context of elevated semen oxidative stress produced by leukocytes and immature spermatozoa (Jayasena et al. 2019). Sperm membrane rich in lipids could be the starting point for ROS production, which subsequently lead to lipid peroxidation and saturation of free radical scavengers. The amount of ROS produced cannot be counterbalanced by the endogenous antioxidant capacity and the result is loss of membrane fluidity, impaired motility and high membrane permeability causing sperm DNA fragmentation (Ko, Sabanegh, and Agarwal 2014). Fertilization of the oocyte by sperm with fragmented DNA is possible. However, if

the proportion of fragmented sperm DNA is high, embryo development could arrest and cause miscarriage or birth defects (Gupta et al. 2007).

Sperm DNA fragmentation and seminal ROS have recently been studied in in male partners with idiopathic recurrent miscarriage, attending an Iranian institute for assisted reproduction (Kamkar, Ramezanali, and Sabbaghian 2018). The percentage of sperm with DNA defects was two-fold higher in RPL patients compared to controls. My study similarly showed that male partners with idiopathic recurrent miscarriage not only have significantly higher ROS and sperm DNA fragmentation, but also significantly lower normal sperm morphology compared to controls (Jayasena et al. 2019). In a recent study couples with infertility due to isolated poor sperm morphology, ROS and abnormal sperm morphology were positively correlated with DNA fragmentation (Oumaima et al. 2018). Similar significant correlations were not observed in my study.

Taking into consideration that the RPL group was significantly overweight compared to the control group, I investigated the association of subject BMI with ROS as well as with DNA fragmentation. I did not observe a significant association of BMI with ROS levels or DNA fragmentation. In contrast to my results, an observational study involving healthy young men showed significantly higher sperm DNA fragmentation in overweight men compared to men with BMI below 25 kg/m^2 (Kort et al. 2006). However, Eisenberg et al. have shown that overweight men attempting to conceive had lower incidence of sperm DNA fragmentation with higher BMI (Eisenberg et al. 2014). These data suggest that several factors other than BMI may influence sperm DNA fragmentation and further research is required.

Diagnostic performance

Finally, this is the first study investigating the performance of sperm morphology, semen ROS and sperm DNA fragmentation to distinguish men with RPL from controls using ROC curve analysis. These three potential screening factors had reasonable diagnostic performance as ROC AUC was significantly different from the line of non-discrimination. Sperm DNA fragmentation was the best performing test to distinguish men with RPL from controls.

Limitations

It is important to consider limitations of the study. Since commencement of the current study, the definition of RPL changed to include two rather than three consecutive miscarriages (RPL ESHRE 2017). A few men were excluded from my study before this change came into effect as they experienced fewer than three pregnancy losses. It is possible that my results could have been slightly different if these men with less than 3 pregnancy losses were included in the RPL group.

Several methods of sperm DNA fragmentation measurement are available. The Halosperm method used for my study is an index of abnormal chromatin packaging rather than a direct assessment of DNA damage itself (Jose Luis Fernández et al. 2003). It would be interesting to compare results from the current study with other measurement methods of sperm DNA fragmentation, such as sperm chromatin structure assay, TUNEL and COMET (see section 1.4.3 Sperm DNA fragmentation index, Chapter 1), which measure sperm DNA damage directly and have higher reported sensitivities (L Simon et al. 2014). It is also important to consider that some of the included participants may have suffered genitourinary infections, which may occur in the absence of symptoms. It would be important to

investigate if participants had an undiagnosed infection with further seminal microbial analysis and whether these infections were significantly more frequent in men with RPL. These studies are currently undergoing in my research laboratory.

Furthermore, reference values of sperm DNA fragmentation and seminal ROS associated with male infertility are variably different between study groups worldwide (Agarwal, Ahmad, and Sharma 2015; Robinson et al. 2012). Future meta-analyses could be of great importance to quantify heterogeneity across studies and determine if the observed abnormalities of sperm function in RPL translate to pathogenic changes leading to pregnancy loss.

Conclusion

In summary, my study reports that male partners of women with RPL have abnormalities in reproductive and sperm function, including seminal oxidative stress and sperm DNA damage. My data could have important implications for the management of couples with RPL, as male partners of women with idiopathic RPL require detailed investigation of their reproductive function. There is no experimental model for sperm DNA damage causing RPL. My study suggests that endocrine, metabolic and molecular sperm profiling could potentially offer crucial diagnostic information for couples with recurrent miscarriage risk. Seminal ROS with DNA damage could direct future research to novel therapeutic targets.

2.6 FUTURE WORK

We investigated the metabolic and endocrine profile of men whose partners experienced RPL but due to small study numbers we did not detect significant differences between cases and controls. A larger study could clarify if testosterone and cholesterol levels play a role in oxidative stress, DNA fragmentation and recurrent miscarriage. Despite the unknown fertility status of the control group, I observed impairments of reproductive function in men affected by RPL when compared to the control group.

Sources of ROS include varicocele, tobacco usage, alcohol, obesity, genitourinary infections, microorganism mutations and viral infections (Agarwal et al. 2018). I have observed similar frequency of varicocele, smoking, alcohol and treated infection between the two groups. It is probable that a few men may have had asymptomatic infections that were not taken into consideration. Future studies could play an essential role to identify these infections and potentially describe which microorganisms are present in men with RPL compared to microorganisms commonly present in men with proven fertility. It would also be interesting to investigate if treatment of varicocele and genitourinary infection in male partners of women with RPL, could reduce the risk of future miscarriage.

Interestingly, male partners of women with RPL were significantly overweight compared to controls. Future studies could investigate obesity and dietary interventions to establish if weight loss and healthy eating could improve sperm function. Several ongoing studies are investigating whether the administration of dietary or pharmaceutical anti-oxidants in subfertile men with completed clinical investigations excluding infectious or surgical causes of increased ROS and DNA damage, could improve pregnancy outcomes (Showell et al. 2014). Anti-oxidants protect from ROS cellular damage

(Ko, Sabanegh, and Agarwal 2014) and animal studies with novel drugs could open new treatment avenues for couples with unexplained male factor infertility.

In summary, my work suggests that male partners of women with RPL have increased risk of potentially treatable abnormalities in sperm function, sperm DNA fragmentation and elevated semen ROS. It strengthens the case for routine testing of men affected by RPL and has the potential to improve the managements of couples with RPL in the future.

Chapter 3

Effect of pharmacological inhibition of myeloperoxidase on reproductive function in obese male mice

3.1 INTRODUCTION

3.1.1 The effect of obesity on sperm function

Emerging research suggests that obesity is a risk factor for male infertility. Twenty-two per cent of severely infertile men are obese. Severe male infertility refers to men with severe oligospermia, which is a term used for sperm counts between 1-10 million/ejaculate or azoospermia, which is the term used to describe the complete absence of sperm in the semen (Punab et al. 2017). A large meta-analysis demonstrates that obesity is strongly associated with poor sperm quality (N. Sermondade et al. 2013). Unfortunately, there is currently no therapy recommended to improve poor sperm quality. Couples with male factor infertility therefore require assisted reproduction therapy to conceive, which is usually effective but can also cause life threatening complications, such as ovarian hyperstimulation syndrome (Alper, Smith, and Sills 2009).

3.1.2 Mechanisms of infertility in male obesity

Obesity is associated with peripheral insulin resistance, decreased liver SHBG synthesis and enhanced testosterone aromatisation (Pasquali 2006). The increased aromatisation of androgens to oestrogens exerts negative feedback on the HPG axis and the consequence is mild hypogonadotrophic hypogonadism (Davidson et al. 2015). Reduced synthesis and release of intratesticular testosterone may impair spermatogenesis. However, effects of obesity on male reproductive potential are multifactorial and not fully explained by hypogonadotrophic hypogonadism. Obesity may also cause testicular dysfunction through unidentified molecular mechanisms, which reduce sperm function resulting in impaired sperm quality (Craig et al. 2017).

3.1.3 Evidence of a potential role for semen ROS and sperm DNA fragmentation in male infertility

Oxidative metabolism leads to the generation of unstable molecules called reactive oxygen species (ROS). It is known that low levels of semen ROS generation in sperm are essential for fertilization, acrosome reaction, hyperactivation, movement and capacitation of the spermatozoa to prepare for interaction with the oocyte (Makker, Agarwal, and Sharma 2009). However, increased semen ROS are seen in 25%-80% of infertile men. Excessive ROS may cause oxidative stress, the state of imbalance between ROS and the antioxidant mechanisms present to counteract ROS (Agarwal et al. 2018).

ROS production may be increased by obesity, smoking, excessive alcohol, air pollution or radiation (Agarwal et al. 2018). Endogenous ROS production is also increased by the presence of semen leukocytes, immature spermatozoa or varicocele (Dutta, Majzoub, and Agarwal 2019). High levels of seminal ROS may cause spermatozoal damage. Data suggest that high fat diet for 9-18 weeks increases sperm mitochondria ROS measured by fluorescence microscopy, as well as sperm DNA damage measured by TUNEL in mice (Palmer et al. 2012; H W Bakos et al. 2010). Similarly, obese men attending dietetic clinics had moderately elevated sperm DNA fragmentation from 10% to 18% (Berger Håkonsen et al. 2011) whilst obese infertile men have been found to have sperm DNA fragmentation from to 20% to 40% (Mir et al. 2018). In both studies, sperm DNA fragmentation was measured by the sperm chromatin structure assay. Reduction of semen ROS production could therefore provide a novel therapeutic approach for male infertility.

3.1.4 AZD5904 as a potential drug to reduce semen ROS

AZD5904 is a novel irreversible human myeloperoxidase inhibitor that could play a role in male infertility associated with oxidative stress and sperm DNA fragmentation. Myeloperoxidase is primarily expressed in neutrophil granulocytes (Dutta, Majzoub, and Agarwal 2019) and produces hypochlorous acids (HClO/HOCl) to provide anti-microbial action for semen. The products of these reactions, mainly hypochlorous acids and oxygen radicals, cause sperm damage due to unregulated ROS production (Pullar et al. 2017). Myeloperoxidase inhibitors have been previously developed by AstraZeneca, London, UK to treat multiple sclerosis and chronic obstructive pulmonary disease (COPD), but their development has not been advanced by the manufacturer despite them being well tolerated in clinical studies. Since myeloperoxidase has been shown to generate ROS, a drug inhibiting myeloperoxidase might reduce seminal oxidative stress and improve sperm quality as well as sperm function.

3.2 STUDY RATIONALE, HYPOTHESIS AND AIMS

With the exception of gonadotrophin treatment for HH, surgical sperm retrieval and assisted reproduction, there is no effective treatment for male infertility. Myeloperoxidase is hyperactivated in chronic inflammatory states, such as high fat diet induced obesity and produces ROS in semen leukocytes. The rationale of this study is to test a novel myeloperoxidase inhibitor in mice with diet-induced obesity, which has potential to improve male reproductive function.

Hypothesis

Administering AZD5904 a highly selective myeloperoxidase inhibitor would improve semen parameters in mice during a high fat diet period.

Aims

1. Investigate the effects of obesity on reproductive function in male mice.
2. Investigate the effects of AZD5904, a myeloperoxidase inhibitor on sperm quality in obese male mice.

3.3 METHODS

3.3.1 Study 1

Diet induced obesity in mice from week 1 to week 6

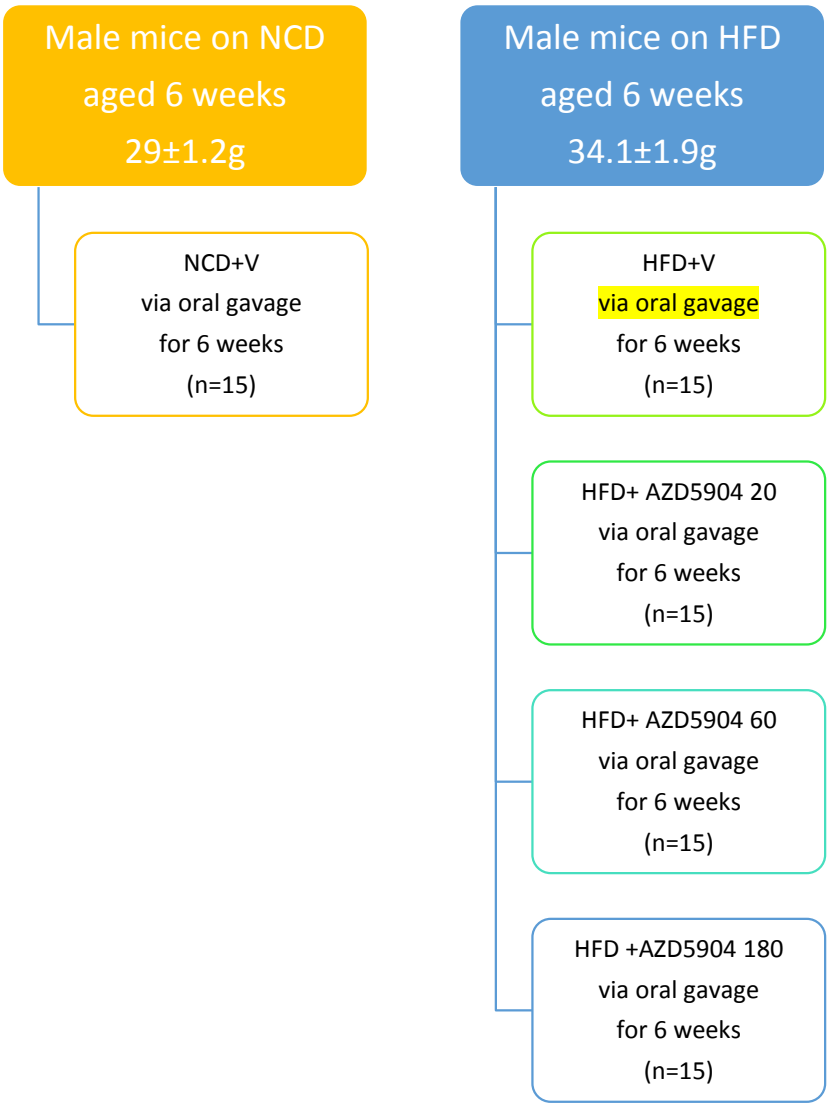
Study 1 was completed in Imperial College Centre for Biomedical Services (CBS). The experiments were performed under home office license project 70/8068, held by Professor Kevin Murphy, Endocrinology and metabolism, Faculty of Medicine. All animal procedures were performed by staff members holding home office personal license. A total of 75 mice (n= 75) of the strain C57BL/6 were studied. The starting weight for all mice was 20 g on average and their age was 6 weeks. The weight of the mice was within the normative value range for male C57BL/6 mice, 6-week-old (Reed, Bachmanov, and Tordoff 2007). Mice are considered juvenile at 6 weeks as their average lifespan is approximately 24 months (Dutta and Sengupta 2016). Fifteen mice were used as control and were fed with normal chow diet (NCD; 3% fat), while the remaining 60 mice were fed with high fat diet (HFD; 60% fat). All

mice were given their respective diet for a 6-week period and were weighed every week. The mice were housed in groups of 5 in ventilated cages and exposed to 12-hour light/dark cycle.

Drug or control administration in mice for week 7 to week 12

All 75 mice were then randomised to one of five groups containing 15 mice. Mice were given either a solution of AZD5904 or a vehicle solution twice daily each for another 6 weeks, from week 7 to week 12 Figure 3.1). The mice admitted AZD5904 by means of oral gavage. AZD5904 was administered by experienced CBS staff. A size 22-gauge tube was placed down the throat and into the stomach. This tube was used to administer the respective solutions. A volume of 0.3 ml of AZD5904 solution or a vehicle solution was administered, which consisted of normal saline.

Figure 3.1 Summary of the protocol for study 1. Effect of AZD5904 on male reproductive function in male mice fed with high fat diet [Group 1 – normal chow diet (NCD) + vehicle (V) only (n= 15) (NCD + V), Group 2 – high fat diet (HFD) + vehicle only (n=15) (HFD + V), Group 3 – HFD + AZD5904 of concentration 20 micromol/kg/day (n=15) (HFD + 20), Group 4 – HFD + AZD5904 of concentration 60 micromol/kg/day (n=15) (HFD + 60), Group 5 - HFD + AZD5904 of concentration 180 micromol/kg/day (n=15) (HFD + 180)].



Sample collection at the end of 12 weeks study period

After the 12-week period, all mice were terminated by cervical dislocation by qualified personnel. After termination, the testes were dissected by Dr Bryn Owen (Non-Clinical Lecturer in Endocrinology and Investigative Medicine. The caudal part of the epididymis was selected for sperm harvesting for two reasons. Firstly, the caudal part of the epididymis is relatively easy to identify during dissection. Secondly, epididymal spermatozoa appear to possess all the necessary functionality of mature sperm (Chauvin et al. 2012). The caudal part of the epididymis was mashed with small dissecting scissors inside an eppendorf tube to release stored sperm and then put in 500µl of sperm washout media. Irvine solution with 7% Human Albumin Solution (HAS) was used for sperm micromanipulation.

Excluded mice

One mouse on normal chow diet was excluded due to behaviour suggestive of ill-health requiring sacrifice mid-way through the study. Weight below 30 g was in the non-obese range for C57BL/6 mice. Sixteen mice on high fat diet were excluded from the analysis as they did not reach a weight of 30 g. Two mice were also excluded because an error happened when handling their samples making a total of 19 mice excluded from the analysis.

3.3.2 Study 2

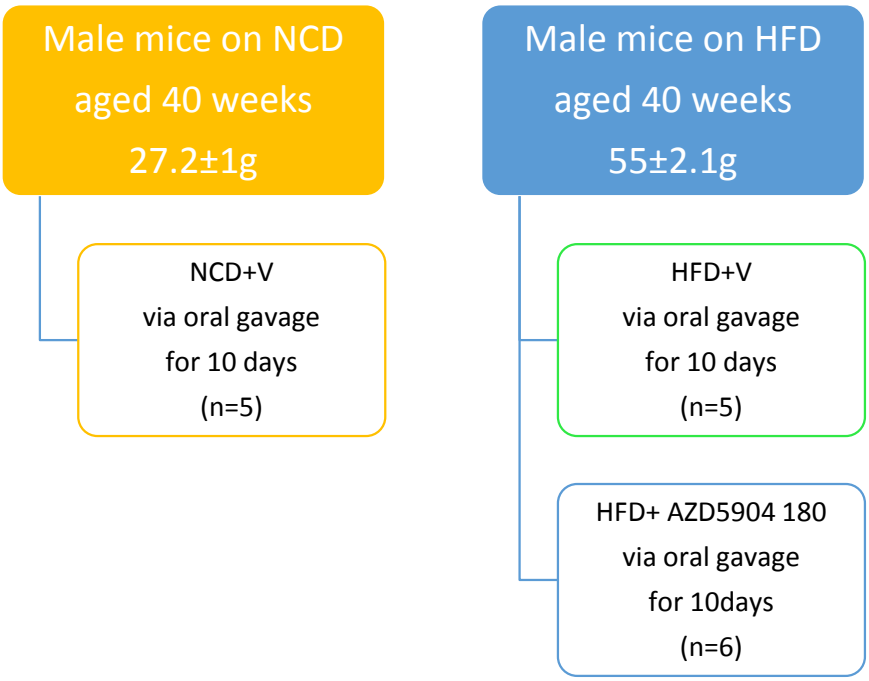
Diet induced severe obesity in mice

Study 2 was completed using the methods of study 1 with the following exceptions. Three groups were studied: i) mice aged 40 weeks on NCD given vehicle solution (NCD +V, n=5), ii) male mice aged 40 weeks on HFD given vehicle (HFD +V, n = 5), iii) male mice on HFD aged 40 weeks given AZD5904 180 micromol/kg/day (HFD + AZD5904 180, n=6). Mean weight of mice in NCD was 27.2 ± 1.0 g. Mean weight of mice on HFD was 55. ± 2.1 g. The average mouse lifespan is 24 moths (Dutta S et al, Life Sciences 2016) and mice in study 2 were aged 40 weeks.

Drug or control administration in severely obese mice

Mice were administered drug or vehicle solutions by means of an oral gavage twice daily for a period of 10 days. Group allocations are summarised in Figure 3.2. A size 22-gauge tube was placed down the throat and into the stomach be experienced CBS staff. The tube was used to administer the respective solutions. A volume of 0.3 ml of AZD5904 solution or a vehicle solution was administered, which consisted of normal saline. Pharmacokinetic parameters for AZD5904, as developed by AstraZeneca, allow a maximum oral dose of 180 micromol/kg/day. Mice in study 2 were obese and weighed more than 40g therefore, high dose of 1.8mg/kg/day of AZD5904 solution was used for ten days. All mice completed the study and full measurements were performed.

Figure 3.2 Summary of the protocol for study 2. Effects of AZD5904 on reproductive function in male mice with established obesity Group 1 – Chow diet + vehicle (v) only (n = 5) (NCD +V), Group 2 – High fat diet (HFD) + vehicle (n = 5) (HFD + V), Group 3 – HFD + highest dose of AZD5904 180 micromol/kg/day (n = 6)(HFD +AZD5904 180)].



3.3.3 Semen Analysis

Suspension of caudal epididymal sperm was placed in an incubator at 37°C for 5 minutes before analysis, to separate sperm from seminal proteins (liquefaction). After incubation, part of the sperm solution was used to determine sperm count and motility by an NHS biomedical scientist experienced at performing semen analysis. The analysis was carried out following the WHO 2010 guidelines for the examination of human semen (WHO Edition 2010) and according to previous animal studies (Chauvin et al. 2012) (Table 3.1).

Table 3.1 WHO human semen parameters reference limits

Parameter	**Lower reference limit (95% CI)**
Sperm concentration (10^6 per ml or M/ml)	15 (12–16) M/ml
Total motility (%)	40 (38–42) %

3.3.4 ROS measurement

ROS was measured using an established in-house chemiluminescence assay that was based on measurements of light emission via luminol (5-amino-2,3-dihydro-1,4-phtalazinedione) chemiluminescence (Vessey et al. 2014). Luminol stock solution was stored in room temperature at 20-25˚C in the dark. In the current study, 50µl luminol stock solution mixed with 950µl DMSO were prepared to make up a total of 1000µl of working solution for daily use. In addition to the luminol stock solution, the following two solutions were also made up:

I. Negative control solution; this was made after aliquoting 400µl of phosphate-buffered saline (PBS) to an eppendorf and adding 100µl of luminol working solution. Negative control mean value had to be <120RLU/sec to allow reliable measurements.

II. Specimen assay was made of 100µL sperm solution mixed with 100µL working solution containing luminol.

Each sample was gently mixed immediately before taking luminometer readings (GloMax; Promega Corporation; Madison, WI, USA). Negative control solution was placed into the luminometer immediately after preparation, so that readings could be taken every minute for ten minutes. Once all ten readings were taken, we calculated the mean value. Chemiluminescence was expressed as mean relative light units per second (RLU/sec), as measured over 10 minutes at minute intervals. Following negative control solutions, we measured chemiluminescence for the sperm solutions. Final ROS value was calculated via the following formula:

ROS = Mean sperm solution chemiluminescence – Mean negative control chemiluminescence

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Sperm concentration

The reference range for semen ROS was <3.8 RLU/sec/million sperm. Luminol chemiluminescence can detect intracellular and extracellular free radicals in the semen.

3.3.5 DNA fragmentation

The sperm DNA fragmentation analysis was done by Sperm Comet Ltd, at the Institute of Pathology, Belfast, Northern Ireland. Sperm Comet assay for sperm DNA fragmentation analysis was selected over Halosperm (see section 2.3.4 DNA Fragmentation Analysis, Chapter 2) due to its ability to assess

sperm DNA fragmentation with only a few thousand sperm. In contrast, Halosperm requires a minimum of one million sperm per ml for quantification of sperm DNA fragmentation. In single-cell gel electrophoresis (SCGE), also known as the Comet assay analysis sperm cells were initially embedded in agarose gel. High concentration of salts and detergents were used to lyse the cells resulting in formation of deproteinized nuclei (nucleoids). Electrophoresis was then carried out on the nucleoids whereby the broken DNA strands migrate towards the anode to form a comet tail (Lewis et al. 2013). The larger the comet tail, the higher the degree of DNA damage in the sample (Maria and Lorences 2009). DNA fragmentation was reported as average comet score (ACS) with a higher percentage comet score representing a higher degree of DNA fragmentation.

3.3.6 Data analysis

All data analysis was performed using GraphPad Prism 5.0 software, including graphs and statistical testing. ANOVA with post-hoc Tukey's test calculator for comparing multiple treatments was used for data analysis. A P value of <0.05 was considered to be statistically significant.

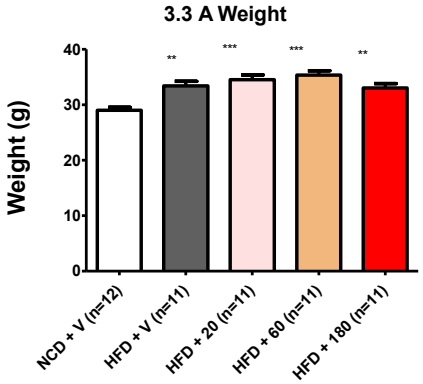
3.4 RESULTS

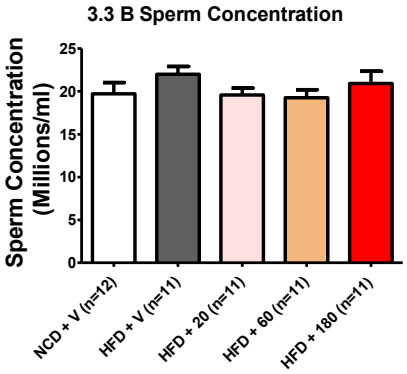
3.4.1 Study 1

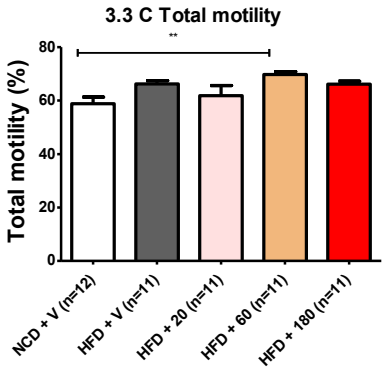
A total of 75 mice were fed with NCD or HFD and subsequently given either vehicle solution or a solution of AZD5904 twice daily at three different doses of 20, 60 or 80 micromol/kg/day. At the end of the 12-week study period, there was a small but significant increase in the weight of the mice in the high fat diet groups compared to the controls (Weight in g: 29.0 ± 1.2, NCD + V; 33.4 ± 1.9, HFD + V, P≤ 0.01 vs NCD + V; 34.6 ± 1.9, HFD + 20, P≤ 0.001 vs NCD + V; 35.4 ± 1.8, HFD + 60, P≤0.001 vs NCD + V; 33.1 ± 1.8, HFD + 180, P≤0.01 vs NCD + V). The high fat diet mice had an average weight increase of approximately 4-6 g of when compared to the NCD group (Figure 3.3 A).

The sperm concentration was not significantly different between the 5 groups at the end of the 12-week study period (sperm concentration in million/ml: 19.7 ± 2.9, NCD + V; 22.0 ± 2.1, HFD + V; 19.6 ± 1.8, HFD + 20; 19.3 ± 2.1, HFD + 60; 20.9 ± 3.2, HFD + 180) (Figure 3. 3 B). A statistically significant higher sperm total motility was observed in the HFD + 60 group when compared to the control NCD + V group (Total motility in %: 58.8 ± 5.4, NCD + V; 66.2 ± 2.7, HFD + V; 61.8 ± 8.4, HFD + 20; 69.7 ± 2.4, HFD + 60, P≤ 0.01 vs NCD + V; 66.1 ± 2.7, HFD + 180). However, there was no significant difference when comparing the control NCD group to the other groups (Figure 3.3 C). In summary, we failed to observe any significant reductions in sperm function during HFD during study 1. Although previous murine studies showed decreased sperm concentration and motility in obese male mice, murine spermatozoa were found more resistant to oxidative damage with unaffected reproductive capacity compared to human spermatozoa exposed to oxidative damage (Aly and Polotsky 2017).

Figure 3.3 Changes in weight, sperm concentration and total motility between the 5 groups of mice in study 1. (A) weight change between the 5 groups over the 6-week period of vehicle or drug administration. (B) sperm concentration changes between the 5 groups over the 6-week period of vehicle or drug administration. (C) change in sperm total motility between the 5 groups over the 6-week period of vehicle or drug administration. NCD – normal chow diet, V – vehicle, HFD – high fat diet, 20 – 20 micromol/kg/day of AZD5904, 60 – 60 micromol/kg/day of AZD5904, 180 – 180 micromol/kg/day of AZD5904. NCD + V (n=14), HFD + V (n=10), HFD + 20 (n=11), HFD + 60 (n=11), HFD + 180 (n=10). Data are expressed as mean ± SEM with 95% CI; **P≤ 0.01; ***P≤ 0.001







3.4.2 Study 2

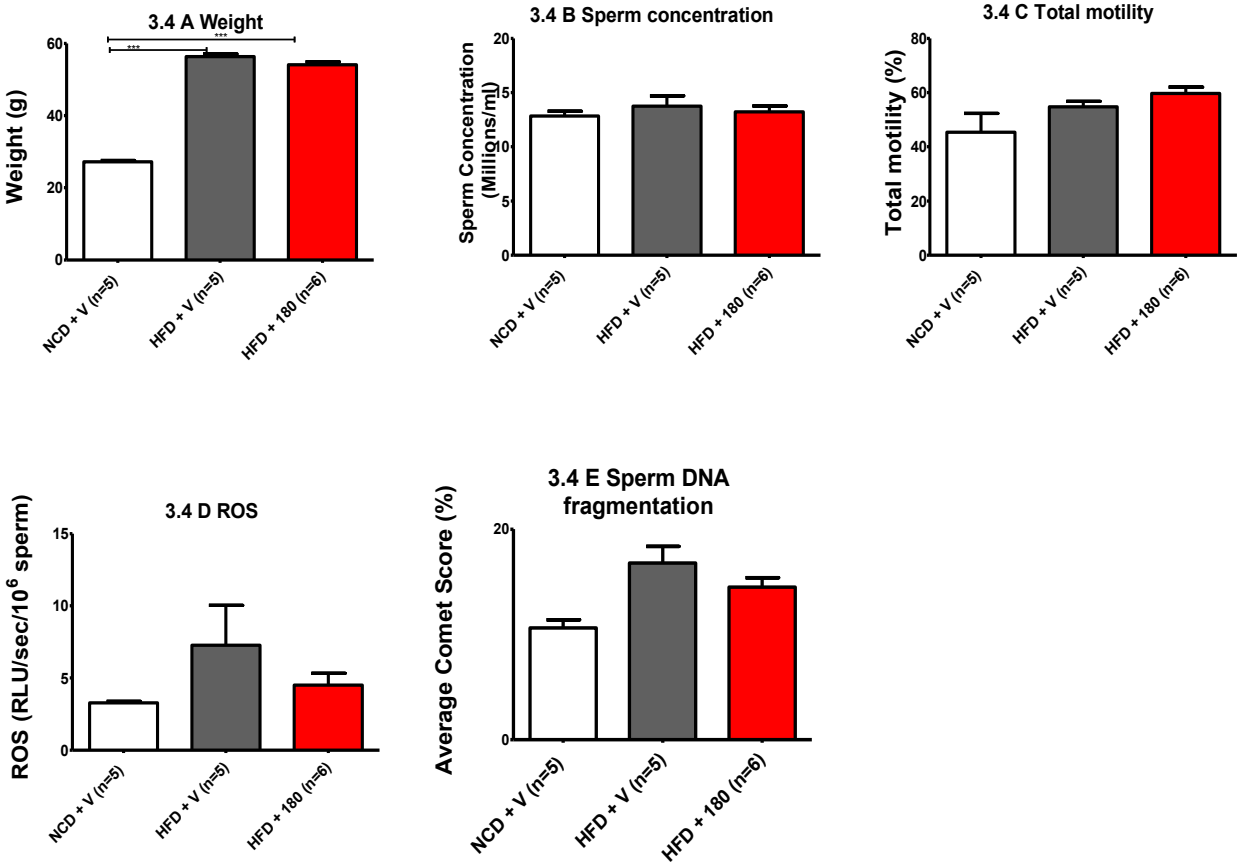
Mice in study 1 had a comparatively modest weight gain, owing to the short duration of HFD. In study 2, I therefore investigated the effects of AZD5904 in male mice with established obesity. Sixteen mice were divided into 3 groups, were fed and subsequently given drug or vehicle solutions for a period of 10 days. Mice fed HFD had 2-fold greater weight compared to mice fed NCD (weight in g: 27.2 ± 1.0, NCD + V; 56.3 ± 2.1, HFD + V, P≤ 0.001 vs NCD + V; 54.1 ± 2.1, HFD + 180, P≤ 0.001 vs NCD + V). The increase in weight during study 2 was achieved over a 9 -month period of high fat diet (Figure 3.4 A).

Sperm concentration was similar among the 3 groups (sperm concentration in million/ml: 12.8 ± 1.3, NCD + V; 13.8 ± 2.6, HFD + V, P>0.05 vs NCD +V; 13.2 ± 1.5, HFD + 180, P>0.05 vs NCD + V) (Figure 3.4 B). Sperm concentration in study 2 was substantially lower than study 1 which included younger mice having shorter exposure to high fat diet. Total motility was similar between groups. However, total motility was non-significantly higher in HFD +180 group when compared to the NCD + V group (total motility in %: 45.4 ± 19.4, NCD + V; 54.8 ± 5.4, HFD + V, 59.7 ± 6.2, HFD + 180, P= 0.07 vs NCD + V) (Figure 3.4 C).

ROS was non-significantly higher in the HFD + V group compared to the other groups (ROS in RLU/sec/10^6: 3.3 ± 0.4, NCD + V; 7.3 ± 7.7, HFD + V, P>0.05 vs NCD + V; 4.5 ± 2.2, HFD + 180, P>0.05 vs HFD+V) (Figure 3.4 D). Also, analysis of the sperm DNA fragmentation in study 2 suggested that it was slightly higher in the HFD + V group compared to other groups (DNA fragmentation in average comet score (ACS) %: 7.0 ± 2.2, NCD + V; 10.9 ± 4.9, HFD + V, P>0.05; 8.1 ± 1.5, HFD + 180, P>0.05 vs HFD + V) (Figure 3.4 E). In summary, obesity led to non-significant increase in semen ROS and sperm DNA

fragmentation, which may have been ameliorated by AZD5904. However, AZD5904 had no significant effect on sperm function during the study.

Figure 3.4 Changes in weight, sperm concentration, total motility, ROS and DNA fragmentation during AZD5904 in obese mice. Effects of high-dose AZD5904 on HFD in male mice fed for 9-months. (A) weight change between the 3 groups. (B) change in sperm concentration between the 3 groups. (C) change in total motility between the 3 groups (D) ROS change between the 3 groups. (E) Change in sperm DNA fragmentation between the 3 groups. NCD – normal chow diet, V – vehicle, HFD – high fat diet, 180 – 180 micromol/kg/day of AZD5904. NCD + V (n=5), HFD + V (n=5), HFD + 180 (n=6). Data are expressed as mean ± SEM with 95% CI; ***P≤ 0.001.



3.5 DISCUSION

There is currently no available treatment for primary idiopathic male infertility. Almost a quarter of male partners presenting to infertility centres are obese (Punab et al. 2017) and the commonest treatment option offered is assisted reproductive technology (ART). These techniques are often accompanied by excessive psychological and financial burden as couples might require repeated attempts before successful fertilization. We used high fat diet to induce obesity in male mice and investigate if semen parameters, ROS and DNA fragmentation could be improved by using AZD5904, a myeloperoxidase inhibitor. Surprisingly, HFD failed to decrease sperm concentration or motility in mice fed HFD compared to controls. A possible explanation would be that HFD has different effects on sperm function in mice compared to humans. Larger studies are required to see if reductions in semen ROS as well as sperm DNA fragmentation levels following AZD5904 are significant in adult mice fed a long-term high fat diet.

Weight gain

Mice in study 1 had a mean weight gain of 5g and did not reduce their sperm function significantly. Even though mice were fed high fat diet, they were kept in cages of five over a relatively short period of time with unclear activity levels. They were also given AZD5904 via oral gavage, a common experimental technique for accurate dosing with potential negative impact on weight gain. It is likely that oral gavage in study 1 mice prevented weight gain due to possible oesophageal trauma, aspirational episodes and subsequent weight loss (Jones, Boyd, and Wallace 2016). Previous studies investigating the effect of obesity on semen parameters reported that mean weight gain of 14g was followed by significant reduction in semen parameters (Palmer et al. 2012; H W Bakos et al. 2010). Study 1 data in the context of insufficient weight gain were therefore difficult to interpret. Longer

exposure to HFD prior to AZD5904 administration in study 2 led to a mean weight gain of 28g, which was followed by an upward trend to ROS and sperm DNA fragmentation in the HFD group compared to the control.

Changes in sperm concentration

I did not observe any change in sperm concentration in adult mice following high fat diet. This observation is consistent with two previous mice studies (Ghanayem et al. 2010; H W Bakos et al. 2010). However, two other studies have observed a statistically significant decrease in both sperm concentration and motility in mice following high fat diet (Yan et al. 2015; Mu et al. 2017). It is important to note that a recent study including young mice on high fat diet for 4 weeks reported significant increase in circulating testosterone as well as sperm concentration compared to mice fed normal chow diet over the same time period (Zhang et al. 2017). Both testosterone levels and sperm concentration decreased non-significantly at 12 weeks following HFD compared to the control group. A possible explanation for this paradoxical effect of high fat diet on sperm concentration in young mice may be that high fat diet exerts an initial stimulatory effect on reproductive function, which probably attenuates as obesity advances with age (Zhang et al. 2017). In conclusion, sperm concentration in study 1 might have decreased after a longer period of exposure to high fat diet. Also, large numbers of mice in study 2 with advanced obesity and older age could have been associated with significant changes in sperm concentration.

Changes in sperm total motility

Both study 1 and 2 showed non-significantly increased sperm motility with high fat diet, which contrasts with observations from previous studies. Four previous mice studies and one study with Sprague-Dawley rats have observed significant decrease in sperm motility in animals fed high fat diet compared to animals fed control diet (Palmer et al. 2012; H W Bakos et al. 2010; Fan et al. 2015; Mu et al. 2017). Mice were kept in open cages in groups of five and probably had increased cage activity. It is possible that cage activity counteracted the effects of high fat diet on sperm motility as exercise reduces inflammation related to obesity and leads to increased progressive sperm motility (Hajizadeh Maleki and Tartibian 2018). Interestingly, the addition of AZD5904 at 60 micromol/kg resulted in significantly increased sperm motility in young mice with high fat diet induced obesity compared to the control group. Future studies with standardised animal age and activity could be helpful to confirm this result.

Changes in ROS

Previous studies reported that mice fed high fat diet for more than 9 weeks had significantly higher mitochondrial ROS compared to mice fed control diet over the same time period (H W Bakos et al. 2010; Palmer et al. 2012). Sperm cells membranes contain high quantities of polyunsaturated fatty acids which contribute to membrane fluidity. However, excessive levels of polyunsaturated fatty acids make spermatozoa deficient in antioxidants and susceptible to ROS damage (Ashok Agarwal, Saleh, and Bedaiwy 2003). My data agreed with previous studies by showing mild increase in semen ROS levels in mice fed high fat diet for 40 weeks compared to the control group. Results from this current study suggest that high dose AZD5904 for ten days in severely obese older mice may reduce semen ROS. It therefore remains possible that inhibition of semen ROS production with AZD5904 is a

potential novel therapy for male infertility. Future studies are required to develop an appropriate murine model of male infertility to test the effect of AZD5904.

DNA fragmentation levels

Previous studies reported that sperm DNA fragmentation levels were significantly higher in obese mice fed high fat diet mice compared to mice fed control diet (H W Bakos et al 2010; Palmer et al. 2012). Increased oxidative stress leads to sperm DNA damage and fragmentation, which in turn decreases the chances of fetus development in mice and impairs the long-term health of the offspring (Aly and Polotsky 2017). Results from study 2 showed a non-significant increase in sperm DNA fragmentation levels in the high fat diet group compared to the control group. Developing an appropriate murine model to examine the effectiveness of AZD5904 in reducing sperm DNA fragmentation could clarify future therapeutic implications for AZD5904 in this context.

Limitations

It is important to consider constrictions of study design imposed by ethical governance and resources. Feeding mice in study 1 for a longer time period may have achieved adequate exposure to high fat diet to observe a significant decline in semen parameters. Mice in study 2 were also administered AZD5904 for only 10 days due to constraints on the duration of the study imposed by the animal project licence. Given that mice in study 2 had prolonged exposure to high fat diet, the effect of AZD5904 could have been significant on semen oxidative stress if given for a longer time period. Mice in both studies were kept in groups of 5 per cage and since the cage was open, it was not known whether all mice ate equal amounts of food or had similar activity levels.

Conclusions

No previous study has investigated the effects of a myeloperoxidase inhibitor in reducing obesity-induced damage to sperm. The current study investigated the effects of the myeloperoxidase inhibitor AZD5904 on fertility in male mice with high fat diet induced obesity. Overall, we observed no significant effects of AZD5904 on sperm function, DNA fragmentation or semen ROS. Our data do not support the hypothesis that myeloperoxidase inhibition is an effective therapy for male infertility.

3.6 FUTURE STUDIES

Future studies could be designed to study the effects of different doses of AZD5904 given to older adult male mice with high fat diet induced obesity. Human sperm is not as resistant to DNA damage as is mouse sperm. Although a human study on the effects of AZD5904 might require time to complete, it could reveal more applicable and clinically relevant results. Performing hormonal assays to test for FSH, LH, oestradiol and testosterone would be useful to identify the link between semen parameters and endocrine function.

Chapter 4

The physiological effect of weight loss on male fertility

4.1 INTRODUCTION

4.1.1 Weight loss via bariatric surgery in the context of male fertility

Obesity impairs fertility through multiple mechanisms, including insulin resistance, increased conversion of testosterone to oestrogen and potential cellular damage due to oxidative stress (Craig et al. 2017). The most effective treatment for obesity is bariatric surgery, but the acute starvation-like state induced by surgery interrupts sperm function, making this an inappropriate treatment to male fertility. Assisted reproduction outcomes have been reported to be abnormally low 18 months post bariatric surgery (Lazaros et al. 2012). Legro et al also observed that bariatric surgery reduces sperm concentration by 65% within the first month after bariatric surgery. This reduction in sperm concentration was followed by a mild increase from baseline, twelve months after surgery (Legro et al. 2016). However, a recent meta-analysis suggested that men undergoing bariatric surgery have no change in their sperm concentration or motility even 24 months post-surgery (Wei, Chen, and Qian 2018). Rodent models exhibit different response to excessive weight loss after bariatric surgery compared to human semen parameters, which deteriorate dramatically after surgery (di Frega et al. 2005). Remarkably Sprague–Dawley rats with high-fat-diet-induced obesity increase their sperm motility 8 weeks after sleeve gastrectomy (Xiang et al. 2018).

4.1.2 Weight loss via diet in the context of male fertility

Diet prescription with an energy deficit below the estimated daily energy requirements is an established method to achieve weight loss (Frost et al. 2007). Caloric restriction via low energy formula diets offering 800 – 1500kcal per day were previously used to achieve rapid weight loss and reduce insulin requirements in obese patients with type 2 diabetes (Brown et al. 2019). Recent evidence suggests that mild weight loss following diet programmes could improve male fertility. For example, high fat diet is associated with disorganisation of the seminiferous tubules, increased apoptotic index within testicular cells and reduced spermatocytes in mice (Zhang et al. 2017). In male mice undergoing high fat diet, exercise or a combination of diet and exercise increase sperm motility, reduce sperm mitochondrial ROS and improve sperm DNA fragmentation in comparison to mice continuously fed with high fat diet. Similarly, two of four human studies investigating the effect of weight loss via diet on semen parameters report improvement on sperm DNA fragmentation (Faure et al. 2014) and morphology (Mir et al. 2018) after weight loss as well as increase in the likelihood to conceive (Faure et al. 2014, Belan 2015). However, no previous randomised controlled trials (RCTs) have been performed to investigate the effect of dieting on sperm function in men.

4.2 STUDY RATIONALE, HYPOTHESIS AND AIMS

At present, there is no therapy established to improve sperm function in male factor infertility. Affected couples undergo complex and expensive forms of assisted reproduction therapies, which are frequently associated with health risks. Although evidence suggests that modest weight loss is linked with increased likelihood to conceive, there are no previous randomised controlled studies on caloric restriction and male fertility. In addition, the level of caloric restriction or weight loss required to achieve significant improvements in sperm function has not been investigated yet. Targeted caloric restriction could therefore provide a simple, non-pharmacological therapy for men with obesity-related infertility.

Hypothesis

Weight loss via diet will improve sperm function in obese men

Aims

1. To determine the optimum level of weight loss via caloric restriction to improve semen parameters in obese men.

2. To investigate the effects of different levels caloric restriction and weight loss on reproductive hormones and metabolic parameters in obese men.

4.3 METHODS

4.3.1 Participant Recruitment

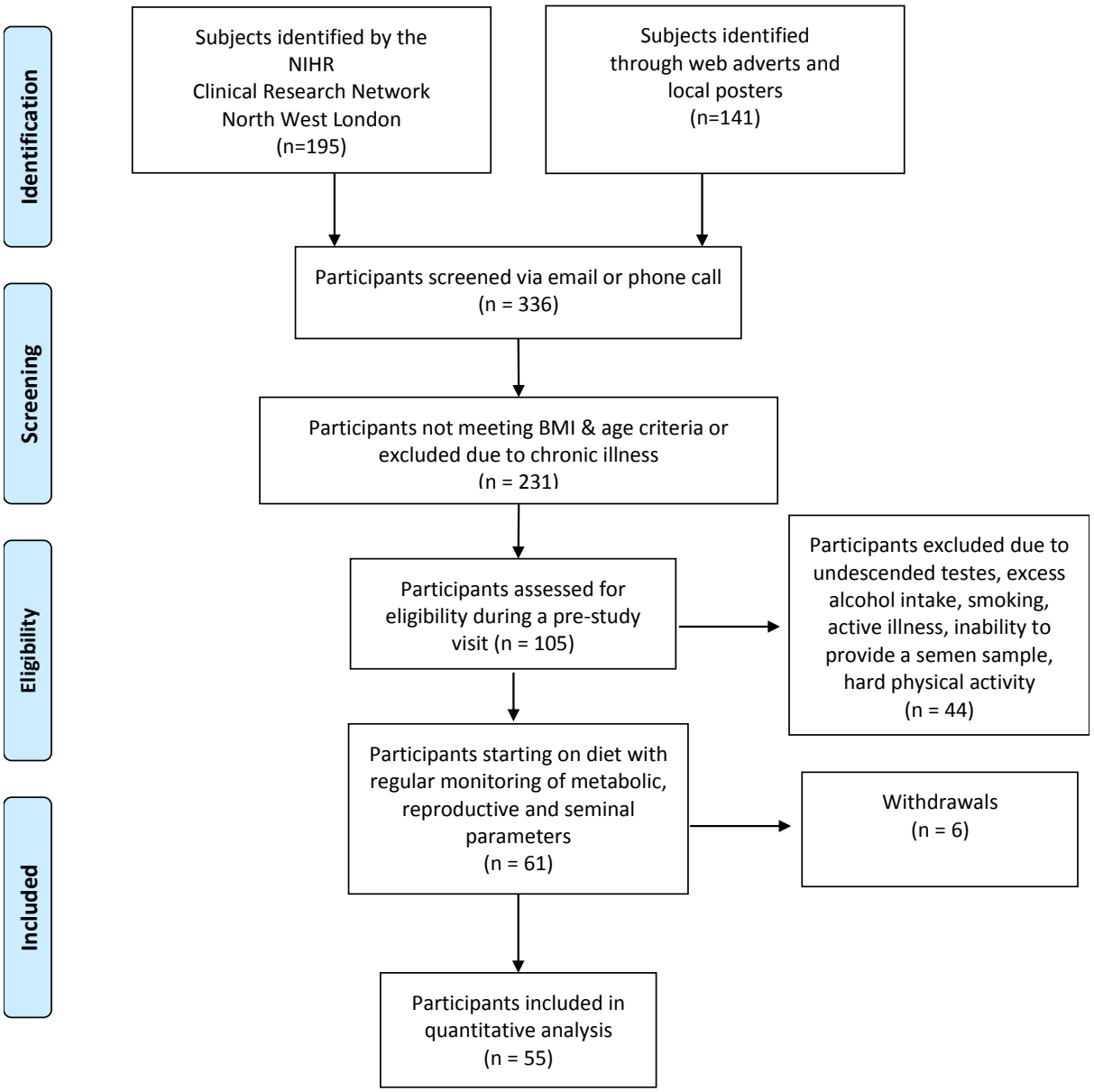
Ethical approval was granted by the London-Queen Square Research Ethics Committee (Registration number 18/LO/0376). Written informed consent was obtained from all participants. The study was performed in accordance with the Declaration of Helsinki. Participants were recruited through local posters or web adverts on the Imperial College website. Recruitment was additionally supported by the National institute for health research Clinical research network (NIHR CRN) at North West London. The CRN supports patients, the public and health care organisations to participate in research and improve care (https://www.nihr.ac.uk/explore-nihr/support/clinical-research-network.htm).

Each participant attended the Imperial College Research Facility for an initial pre-study visit to ensure study criteria were met. Full medical history was obtained, including previous cancer diagnosis or use of cytotoxic medication, systemic immunological disease, chronic or acute systemic illness, smoking status, alcohol intake per week, sexually transmitted disease (STI) and use of regular medication such as anabolic steroids, opiate analgesia and calcium channel blockers which are likely to affect sperm function (Mortimer et al. 2013). Additional clinical examination allowed screening for clinical conditions directly linked to impaired fertility such as undescended testicles, testicular tumours or varicocele. Baseline measurements of serum luteinizing hormone, (LH), follicle-stimulating-hormone (FSH), oestradiol, testosterone, sex-hormone binding-globulin (SHBG) fasting glucose, HbA1c and total cholesterol were performed. Participants asked to provide a semen sample in a designated private room in the Andrology Department. Semen samples were produced on site following 2-7 days of sexual abstinence (WHO 2010).

Forty-four participants were excluded due to undescended testicles, excess alcohol intake >30 units per week, active smoking status, inability to provide a semen sample, hard physical activity or chronic illness with use of medications likely to affect sperm function (Figure 4.1). The upper limit of weekly alcohol intake likely to cause negative effects on sperm parameters has not been determined so far. An observational study showed that consumption of more than 25 units of alcohol per week could be associated with significant reduction in sperm concentration and morphology (Bendayan et al. 2018). Two study participants consumed more than 21 units of alcohol per week with no clinical or biochemical evidence of chronic liver disease. Although their semen analyses were within range, they started the diet after forty days of alcohol abstinence to allow completion of a full spermatogenesis cycle and agreed to abstain from alcohol throughout the study period. Sixty-one age matched participants were selected for inclusion in this study. Six withdrew due to acute illness likely to affect the results of the study or difficulty to commit to the study schedule. Fifty-five participants completed the study.

Power study calculation was performed in collaboration with Dr. Les Huson, Senior Statistician, NIHR Imperial Clinical Research Facility. A pilot study was performed in five men with obesity attending Hammersmith Hospital andrology clinic due to poor sperm quality, who were not taking any hormonal therapies. During a 4-week formula LED with 800kcal daily energy intake, a 6-fold increase in median sperm concentration was observed. Sample size calculation was based on the methodology of Pinheiro et al. (Pinheiro, Bornkamp, and Bretz 2006) and these pilot data. It was estimated that 12 subjects would be required in each of dietary group for greater than 90% power to detect a statistically significant linear trend in increased sperm concentration across the groups (alpha=0.05, two-sided).

Figure 4.1 Flow diagram outlining the selection process for study investigating the effect of weight loss on male fertility (NIHR; National institute for health research)



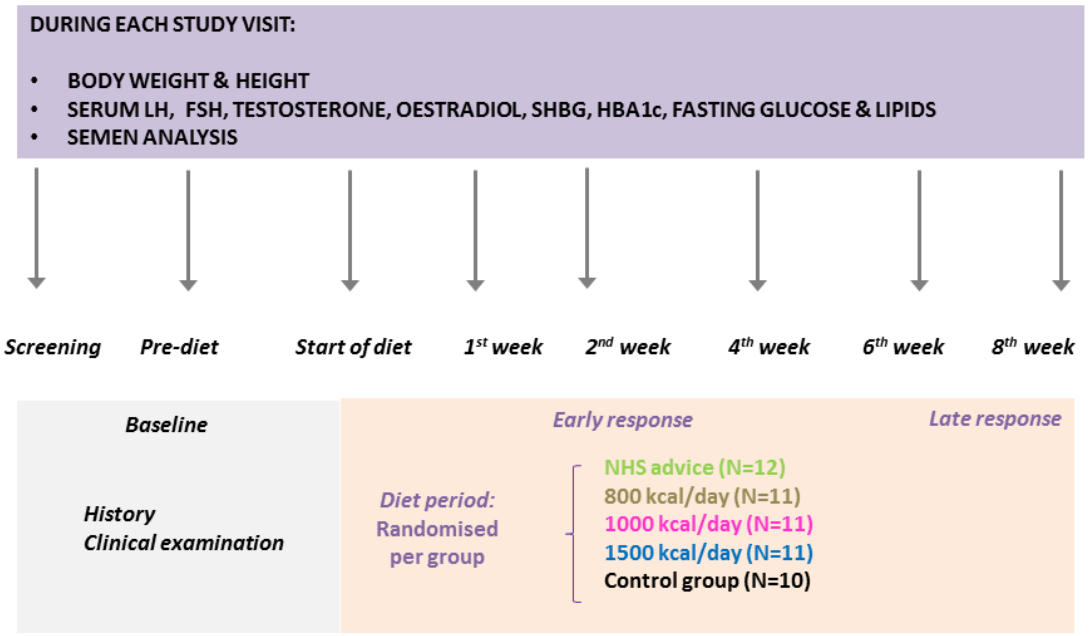
4.3.2 Protocol

A physiological, randomized, controlled design study was performed using a behavioural intervention. Fifty-five participants who were randomised to receive one of four different dietary regimens, completed the study (Figure 4.2 Protocol summary). Participants were allocated in each of five study groups, so 11 participants received 800 kcal/day, 11 participants 1000 kcal/day, 11 participants 1500kcal/day, 12 participants received 1800-2200kcal/day as per NHS recommendations and 10 participants no dietary intervention but observation only.

Baseline period: This initial two-week prior commencement of the eight-week diet period allowed the measurement of baseline values of metabolic and reproductive hormones. During screening, pre-diet and start of diet visits, all participants provided semen samples.

Diet period: During week 1-8 of the protocol, participants allocated in each of five study groups underwent diet via caloric restriction or simple observation. The 800, 1000 and 1500 kcal/day groups used Cambridge weight plan products (CWP) of 200kcal per product alone or in combination with normal meals to achieve the desired level of caloric intake. This period was subcategorised to the early response diet period and refers to the 1st, 2nd and 4th week of the diet as well as the late response diet period, which refers to the 6th and 8th week of the diet. During each study visit, body weight was measured, participants had their metabolic as well as reproductive hormone profile monitored and produced a semen sample (Figure 4.2).

Figure 4.2 Protocol summary. Participants meeting the study criteria (N=55) were invited for a pre-diet study visit and were subsequently randomised into one of the study groups.



4.3.3 Caloric restriction via Cambridge weight plan products or standard NHS recommendations

Low energy diet was offered to participants in a combination of bars, shakes or soups of 200kcal per CWP product along with 2 litres of fluids such as black tea, coffee and water. CWP products contained an array of macronutrients (Table 4.1). Participants were asked to take four CWP products if they were randomised to take 800 kcal/day, four products and a healthy meal of 200kcal if randomised to 1000 kcal/day or one product and 3 healthy meals if randomised to 1500kcal/day.

Table 4.1. Nutritional information for CPW products. Ingredient and nutritional information [adapted from (Hockey 2014)].

	Energy (Kcal)	Saturated fats (g)	Mono-un saturated fats (g)	Poly- un saturated fats (g)	Sugars (g)	Polyols (g)	Starch (g)	Fibre (g)	Protein (g)
Bar	203	2.8	1.8	1.6	17.7	5	0.8	3.1	12.7
Shake	200	0.5	0.3	1.2	18.9	0	7.9	2.8	15.8
Soup	200	0.6	0.5	1.6	9.5	0	13.1	2.7	16.6

Participants following standard NHS recommendations were asked to make daily decisions on their energy intake to achieve 1800- 2200kcal/day. Energy intake for weight loss was based on the Miflin-St. Joer equations (Mifflin et al. 1990). It was calculated by multiplying the participant's basic metabolic rate with his physical activity level and 800kcal were finally subtracted from this figure. Participants were advised to reduce their portion size and take mainly vegetables and fruits along with smaller portions of bread or rice and healthy protein sources, such as fish, poultry and beans (The British Dietetic Association, Controlling your portions 2015).

4.3.4 Semen Analysis

Semen samples were produced on site in private rooms within the Department of Andrology, Hammersmith Hospital, UK. Samples were incubated at 36±1°C for liquefaction up to 60 minutes prior to analysis. Semen analysis was performed according to World Health Organisation 2010 guidelines (WHO 2010) and UKNEQAS (UK National External Quality Assessment Service) accreditation. Reference ranges for semen analyses were as follows: ≥1.5mL, volume; ≥15million/mL, sperm concentration; ≥40%, total motility; ≥32% progressive motility; ≥4%, normal morphology; ≥20million, total motile count. Total motile sperm count (TMC) was calculated using the formula: sperm concentration (million/ml)* percentage motility (%)* semen volume (ml). Sperm morphology was examined on Papanicolaou pre-stained slides, using Kruger strict criteria.

4.3.5 Endocrine Biochemistry

Morning fasting blood samples were analysed for serum luteinizing hormone, (LH), follicle-stimulating-hormone (FSH), oestradiol, testosterone, total cholesterol, low-density lipoprotein (LDL) and high-density lipoprotein (HDL) in the clinical biochemistry department of Charing Cross Hospital, using Abbott ARCHITECT an automated immunoassay platform under UKNEQAS accreditation. Additionally, hexokinase method for fasting serum glucose analysis was performed and the Tosoh G8 Analyser was used for HbA1c testing by the clinical biochemistry department of Charing Cross Hospital. Reference ranges were as follows: 2-12 iu/L, LH; 1.7-8 iu/L, FSH; <190pmol/L, oestradiol; 10-30nmol/L, 15-55 nmol/L, SHBG; testosterone; <7 mmol/L, fasting glucose; <48mmol/mol, HbA1c; <5 mmol/l, total cholesterol; <3 mmol/l, LDL; >1 mmol/l, HDL.

4.3.6 Statistical Analysis

Data analysis was performed using GraphPad Prism v.8. Quantitative data was assessed for normality using D'Agostino-Pearson normality test, followed by the appropriate analysis of variance (ANOVA). One-way ANOVA was used to compare means of five different groups with Tukey's multiple comparisons test. Time profiles for semen parameters during different study periods were analysed using repeated measures two-way ANOVA with Bonferonni's post-hoc correction. Group comparisons with respect to categorical variables were performed using Chi-Squared test. A $p<0.05$ was considered statistically significant. Data are presented as mean ± standard error of mean (SEM).

4.4 RESULTS

Clinical characteristics of participants

Baseline age, weight, body mass index, waist circumference, testicular volume on clinical examination, metabolic and endocrine profile as well as semen parameters were not significantly different between study groups (Table 4.2). No differences were observed in proportion of white/ non-white participants among the treatment groups.

Table 4.2 Clinical characteristics. Data for age, ethnicity, weight, body mass index (BMI), waist circumference, testicular volume, metabolic and endocrine profile as well as semen parameters. Data presented as mean ± SEM.

Parameter	Control (n=10)	NHS (n=12)	1500 kcal/day (n=11)	1000 kcal/day (n=11)	800 kcal/diet (n=11)
Age (years)	**32.9±2.7**	**40.6±3.5**	**40.6±2.5**	**39.6±2.6**	**40.1±3**
White ethnic group Non-white ethnic groups	**4** **6**	**6** **6**	**4** **7**	**5** **6**	**3** **8**
Weight (kg)	**105.3±3.8**	**110.9±7**	**103.4±3**	**107.8±2.2**	**105.6±3.7**
BMI (kg/m^2)	**35±1.1**	**35.7±1.4**	**33.3±0.9**	**33.8±1**	**34.6±1**
Waist circumference (cm)	**114.9±2.7**	**117.5±4.3**	**117±3.1**	**118.1±4**	**122.5±3.2**
Testicular volume (ml)	**20.3±1.7**	**17.8±1.7**	**19.2±1.3**	**17.4±1.3**	**18.5±1.8**
Fasting blood glucose (mmol/L)	**5.7±0.3**	**6±0.4**	**5.6±0.2**	**5.2±0.1**	**5.9±0.3**
HbA1c (mmol/mol)	**38±2**	**34±2**	**40±2**	**36±2**	**39±2**
Total Cholesterol (mmol/l)	**5.3±0.3**	**4.9±0.3**	**5.1±0.2**	**5.2±0.3**	**5.2±0.2**
LDL (mmol/l)	**3.2±0.3**	**3.1±0.3**	**3.5±0.2**	**3.3±0.3**	**3.4±0.3**
HDL (mmol/l)	**0.9±0**	**1±0.1**	**0.9±0.1**	**1±0.1**	**0.9±0.1**
Triglycerides (mmol/l)	**2.2±0.6**	**1.2±0.2**	**1.2±0.1**	**1.3±0.2**	**1.4±0.2**
LH (iu/L)	**2.8±0.3**	**3.3±0.3**	**3.3±0.2**	**4.3±0.7**	**3.3±0.3**
FSH (iu/L)	**3.7±0.7**	**3.5±0.4**	**4.1±0.6**	**5.4±0.8**	**3.1±0.6**
Testosterone (nmol/L)	**14.1±1.9**	**12.5±1.5**	**14.9±2**	**14.3±1.6**	**11.9±1.4**
SHBG (nmol/L)	**23±4**	**27±3**	**24±2**	**26±3**	**26±3**
Free testosterone (nmol/L)	**0.345±0.03**	**0.281±0.03**	**0.369±0.05**	**0.330±0.03**	**0.273±0.03**
Oestradiol (pmol/L)	**129±16**	**104±2**	**106±3**	**118±9**	**116±6**
Sperm concentration (million/ml)	**72.1±17.2**	**69.1±17**	**71.4±17.7**	**78.8±20.6**	**85±22**
Sperm Total motility (%)	**60.4±1.8**	**59.8±1.5**	**62.4±2.7**	**55.5±2.9**	**58.2±5.6**
Sperm Progressive motility (%)	**55.6±2**	**54.2±2**	**56.1±3.4**	**49.7±2.8**	**54.3±5.1**
Sperm normal Morphology (%)	**6.6±4.1**	**6.7±4**	**8±5.4**	**6.4±3.2**	**3.1±1**
Seminal volume (ml)	**3.1±1**	**3.2±0**	**3.4±0**	**3.4±1**	**3.5±1**
Total motile count (million sperm/ejaculate)	**123.9±48**	**107±27**	**141.4±31**	**121.9±30**	**163.8±49**

Metabolic and reproductive hormone profile throughout the study period

Changes from baseline study period in weight, waist circumference, fasting glucose, HbA1c, total cholesterol, LDL and HDL in all groups are shown in Table 4.3. Weight in the 800kcal/day was significantly reduced by the end of diet period when compared to NHS (weight reduction in kg: 11.0 ± 1.5, 800kcal; 3.1 ± 0.5, NHS, P≤0.0001) and control group (weight reduction in kg: 11.0 ± 1.5, 800kcal; 2.3 ± 0.4, control, P≤0.0001). Similarly, waist circumference in the 800kcal/day group decreased significantly compared to NHS (waist circumference reduction in cm: 11.5 ± 1.9, 800kcal; 4.2 ± 1.5, NHS, P≤0.05) and control group (waist circumference reduction in cm: 11.5 ± 1.9, 800kcal; 2.2 ± 1.0, control, P≤0.05). Weight reduction in the 800kcal/day group was accompanied by significant reduction in fasting glucose in comparison to the 1500kcal/day group (fasting glucose reduction in mmol/L: 1.3 ± 0.3, 800kcal; 0.6 ± 0.1, 1500kcal, P≤0.05). In the 800kcal/day group a significant reduction in total cholesterol was also observed in comparison to NHS group (total cholesterol reduction in mmol/L: 1.2 ± 0.3, 800kcal; 0.5 ± 0.1, NHS, P≤0.05) and control group (total cholesterol reduction in mmol/L: 1.2 ± 0.3, 800kcal; 0.4 ± 0.1, control, P≤0.05).

Table 4.3 Metabolic parameters. Weight, waist circumference, fasting glucose, HbA1c and lipid profile change from baseline to the end of study period (Baseline: study period referring to the mean sperm concentration during screening, pre-diet and start of diet study visits. Data presented as mean ± SEM).

Parameter	Control (n=10)	NHS (n=12)	1500 kcal/day (n=11)	1000 kcal/day (n=11)	800 kcal/diet (n=11)
Weight (kg)	**-2.3± 0.4**	**-3.1 ± 0.5**	**-7.0 ± 0.9** $^{\gamma, \gamma\gamma}$	**-10.1 ± 1.1** $^{\beta, \beta\beta}$	**-11.0 ± 1.5** $^{\alpha, \alpha\alpha, \alpha\alpha\alpha}$
BMI (kg/m^2)	**-0.7 ± 0.1**	**-1.3 ± 0.3**	**-2.3 ± 0.3**$^{\gamma\gamma}$	**-3.1 ± 0.3** $^{\beta\beta\beta, \beta\beta\beta\beta}$	**-3.7 ± 0.5**$^{\alpha, \alpha\alpha, \alpha\alpha\alpha}$
Waist circumference (cm)	**-2.2 ± 1.0**	**-4.2 ± 1.5**	**-9.1 ± 2.2**	**-8.8 ± 2.3**	**-11.5 ± 1.9** $^{\alpha\alpha\alpha\alpha, \alpha\alpha\alpha\alpha\alpha\alpha}$
Fasting glucose (<7mmol/L)	**- 0.5 ± 0.1**	**-0.8 ± 0.1**	**-0.6 ± 0.1**	**-0.7 ± 0.1**	**-1.3 ± 0.3**$^{\alpha\alpha\alpha\alpha\alpha}$
HbA1c (<48mmol/mol)	**-1.5 ± 0.4**	**-1.4 ± 0.3**	**-2.5 ± 0.8**	**-3.4 ± 1.1**	**-3.7 ± 0.1**
Total Cholesterol (<5 mmol/l)	**-0.4 ± 0.1**	**-0.5 ± 0.1**	**-0.8 ± 0.1**	**-1.5 ± 0.2** $^{\beta\beta\beta, \beta\beta\beta\beta}$	**-1.2 ± 0.3**$^{\alpha\alpha\alpha\alpha, \alpha\alpha\alpha\alpha\alpha\alpha}$
LDL (<3 mmol/l)	**-0.43 ± 0.1**	**-0.33 ± 0.1**	**-0.80 ± 0.2**	**-1.09 ± 0.2** $^{\beta\beta\beta\beta\beta}$	**-0.91 ± 0.3**
HDL (>1 mmol/l)	**+0.13 ± 0.0**	**+0.13 ± 0.0**	**+0.03 ± 0.0**	**+0.04 ± 0.0**	**+0.59 ± 0.3**
Triglycerides (1.7mmol/l)	**-0.4 ± 0.1**	**-0.5 ± 0.2**	**-0.4 ± 0.1**	**-0.6 ± 0.1**	**-0.7 ± 0.2**

α= 800kcal vs P≤0.05 1500kcal, αα= 800kcal vs P≤0.0001 NHS, ααα=800kcal vs P≤0.0001 Control, β=1000kcal vs P≤0.0001 NHS, ββ=1000kcal vs P≤0.0001 Control, γ=1500kcal vs P≤0.05 NHS, γγ=1500kcal vs P≤0.01 Control, αααα= 800kcal vs P≤0.05 NHS, ααααα= 800kcal vs P≤0.05 1500kcal, αααααα= 800kcal vs P≤0.05 Control, βββ=1000kcal vs P≤0.01 NHS, ββββ= 1000kcal vs P≤0.01 Control, βββββ=1000kcal vs P≤0.05 NHS

There were no significant changes from baseline study period in serum reproductive hormones between groups as shown in Table 4.4.

Table 4.4 Serum endocrine hormones. Serum LH, FSH, testosterone, SHBG, free testosterone and oestradiol change from baseline to the end of study period (Baseline: study period referring to the mean sperm concentration during screening, pre-diet and start of diet study visits. Data presented as mean ± SEM).

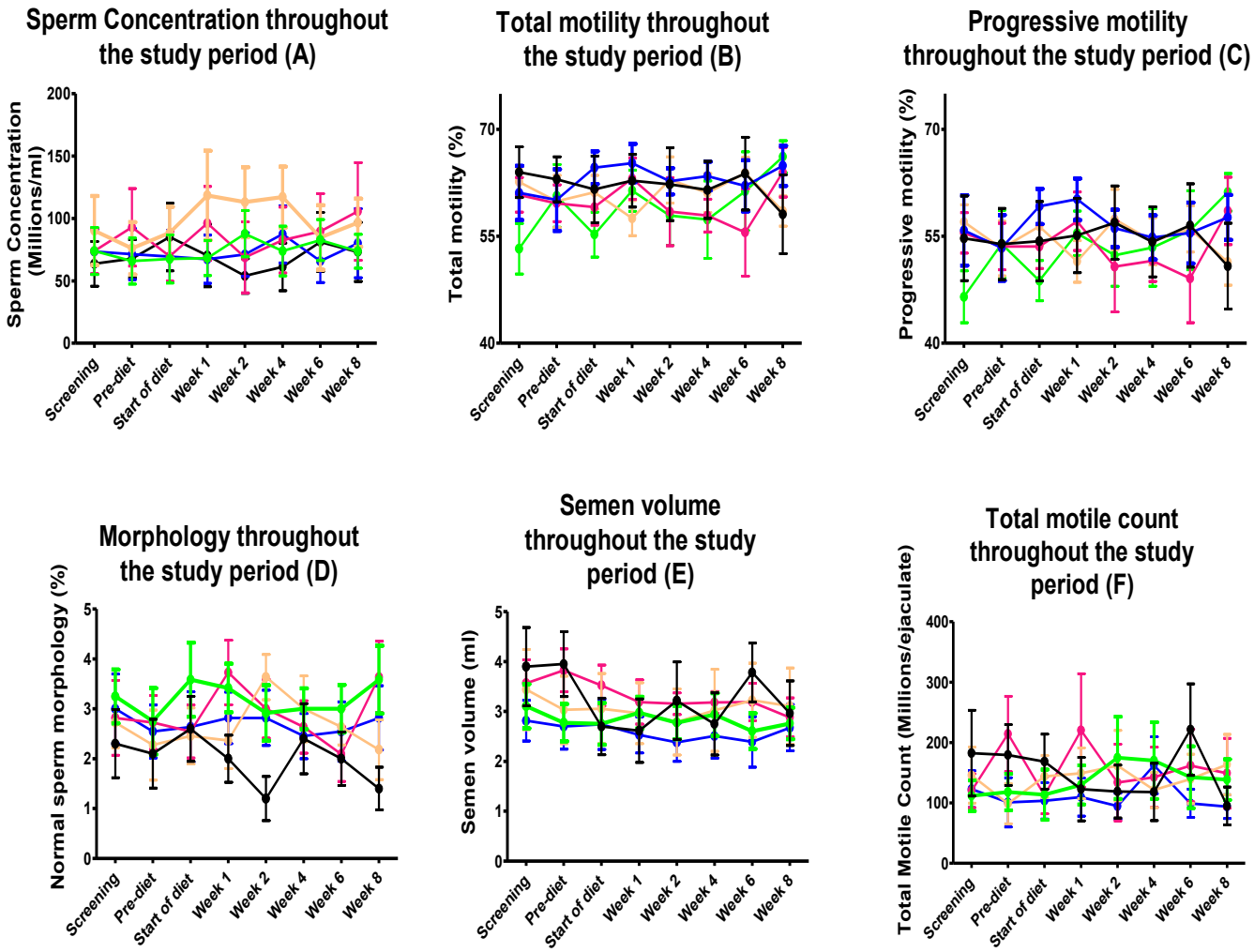
Parameter	Control (n=10)	NHS (n=12)	1500 kcal/day (n=11)	1000 kcal/day (n=11)	800 kcal/diet (n=11)
LH (2-12 iu/L)	**+1.2 ± 0.4**	**+1.2 ± 0.2**	**+0.9 ± 0.3**	**+1.5 ± 0.5**	**+1.3 ± 0.3**
FSH (1.7-8 iu/L)	**+0.4 ± 0.1**	**+0.4 ± 0.1**	**+0.1 ± 0.1**	**+0.2 ± 0.3**	**+0.2 ± 0.2**
Testosterone (10-30 nmol/L)	**+2.5 ± 0.8**	**+2.9 ± 0.9**	**+3.4 ± 0.8**	**+2.8 ± 0.6**	**+4.4 ± 0.7**
SHBG (15-55 nmol/L)	**+4±1**	**+7±2**	**+5±1**	**+9±2**	**+10±2**
Calculated free testosterone (nmol/L) §	**+0.055±0.03**	**+0.073±0.03**	**+0.067±0.02**	**+0.026±0.01**	**+0.076±0.01**
Oestradiol (<190 pmol/L)	**- 8.5 ± 5.3**	**-6.1 ± 1.9**	**-3.1 ± 1.7**	**-13.3 ± 5.5**	**-5.2 ± 4.7**

§ Free testosterone was calculated using the free and bioavailable testosterone calculator based on the Vermeulen formula (http://www.pctag.uk/testosterone-calculator*).*

Time course of caloric restriction effects on semen parameters in obese men

Semen parameters are subject to biological variation within individuals. In a study of this size, it is rarely informative to compare semen parameters during a single time point as biological variation would mash any potential treatment effects. Therefore, repeated measures two-way ANOVA was used to compare the time profiles of semen parameters among treatment groups in obese men. No significant differences in the time profile of any measured semen parameter was observed among the treatment groups (Figure 4.3). On Week 1, Week 2 and Week 4, mean sperm concentration for the 800kcal study group was non-significantly higher compared to the control group (mean increase in sperm concentration in million/ml: Week 1: 48.2 95% confidence interval [CI]=-163.3, 66.9; Week 2: 59.2 95% CI=-174.2, 56.0; Week 4: 56.1 95% CI=-171.1, 59.0, P>0.05 (Figure 4.3 A).

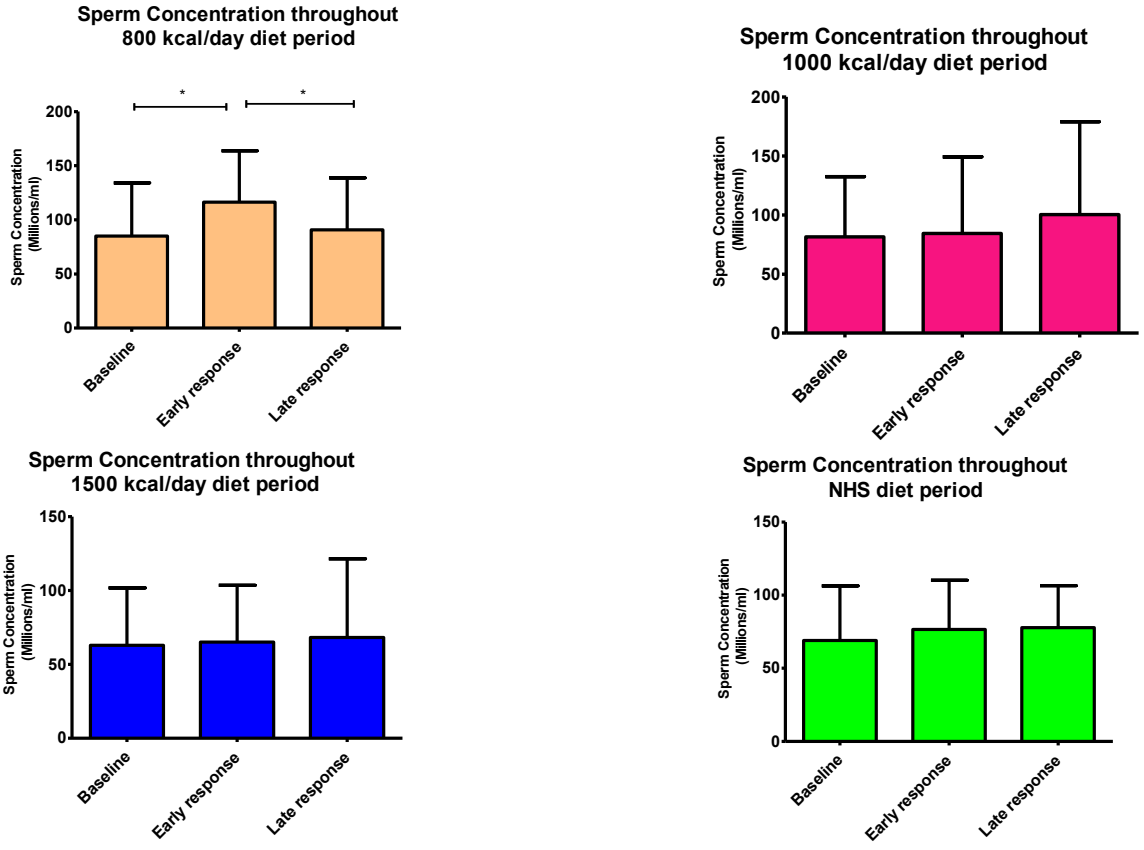
Figure 4.3 Time course of caloric restriction effects on semen parameters of obese men. A-E: Time profiles of sperm concentration (A), total motility (B), progressive motility (C), sperm morphology (D), seminal volume (E), total motile count (F) during study period as measured on screening, pre-diet and start of diet study visits, followed by Week 1, Week 2, Week 4, Week 6, Week 8 of the diet period. Data shown as mean ± SEM for each of the different study groups. Orange: 800kcal, Fuchsia: 1000kcal; Blue: 1500kcal; Green: NHS, Black: Control.



Having observed non-significant increase in sperm concentration during week 1, 2 and 4 of the study period in the 800kcal group, I compared mean sperm function at baseline, early response and late response study period in each treatment group. The increase in mean sperm concentration observed in the 800kcal study group during the early response of the diet period weakened towards the late response of the diet period (Figure 4.3 A). Therefore, the effects of 800kcal diet and every other level of caloric restriction were studied throughout the baseline, early and late response of the study period. On the 800kcal group, early response sperm concentration was significantly higher compared to the baseline (sperm concentration in million/ml: 85.0 ± 22.1, baseline; 116.2 ± 21.3, early response, $P \leq 0.05$) and late response (sperm concentration in million/ml: 90.7 ± 21.2, late response; 116.2 ± 21.3, early response, $P \leq 0.05$) study period (Figure 4.4). Sperm concentration did not change significantly in any other study group (Figure 4.4).

Total motility (Figure 4.5), progressive sperm motility (Figure 4.6) and normal sperm morphology (Figure 4.7) did not change significantly in any other level of caloric restriction.

Figure 4.4 Mean sperm concentration (millions/ml) throughout the study period. (*Baseline*: study period referring to the mean sperm concentration during screening, pre-diet and start of diet study visits, *Early response*: refers to the mean sperm concentration during the 1st, 2nd and 4th week study visits, *Late response*: refers to the mean sperm concentration during the 6th and 8th week study visits). Data shown as mean ± SEM for each of the different study groups; *P≤0.05. Orange: 800kcal, Fuchsia: 1000kcal; Blue: 1500kcal; Green: NHS, Black: Control.



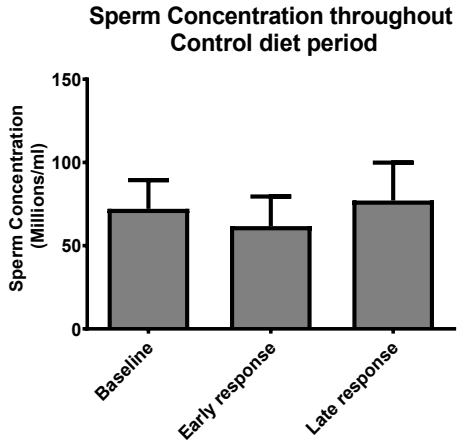
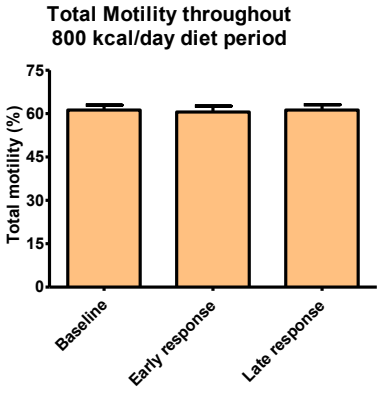
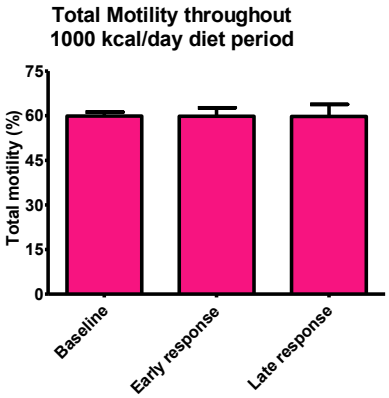
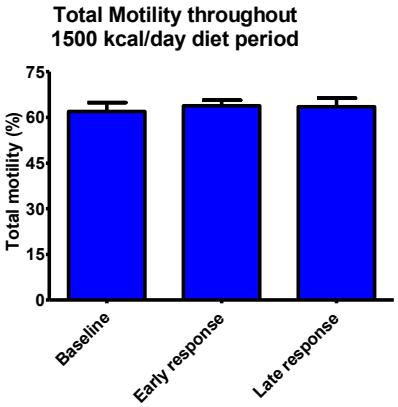
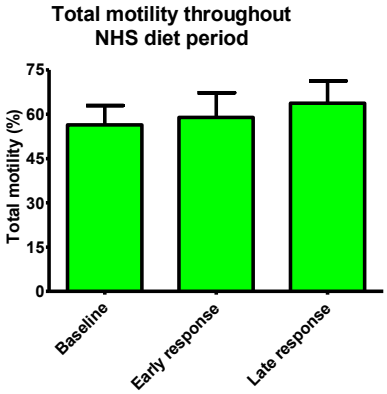

Sperm Concentration throughout
Control diet period
Sperm Concentration
(Millions/ml)
150
100
50
0
Baseline
Early response
Late response

Figure 4.5 Mean total sperm motility (%)throughout the study period (*Baseline:* study period referring to the mean total sperm motility during screening, pre-diet and start of diet study visits, *Early response*: refers to the mean total motility during the 1st, 2nd and 4th week study visits, *Late response:* refers to the mean total motility during the 6th and 8th week study visits). Data shown as mean ± SEM for each of the different study groups; *P≤0.05. Orange: 800kcal, Fuchsia: 1000kcal; Blue: 1500kcal; Green: NHS, Black: Control.


Total Motility throughout
800 kcal/day diet period
Total motility (%)
75
60
45
30
15
0
Baseline
Early response
Late response


Total Motility throughout
1000 kcal/day diet period
Total motility (%)
75
60
45
30
15
0
Baseline
Early response
Late response


Total Motility throughout
1500 kcal/day diet period
Total motility (%)
75
60
45
30
15
0
Baseline
Early response
Late response


Total motility throughout
NHS diet period
Total motility (%)
75
60
45
30
15
0
Baseline
Early response
Late response

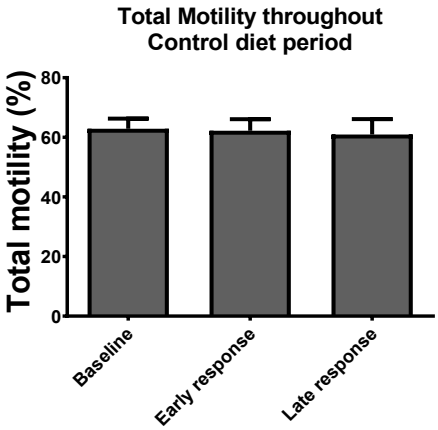
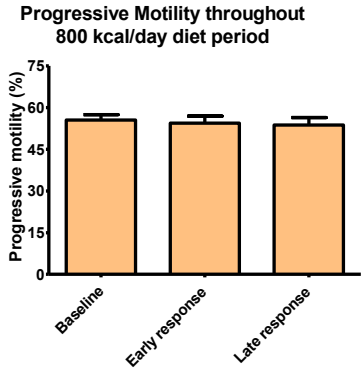
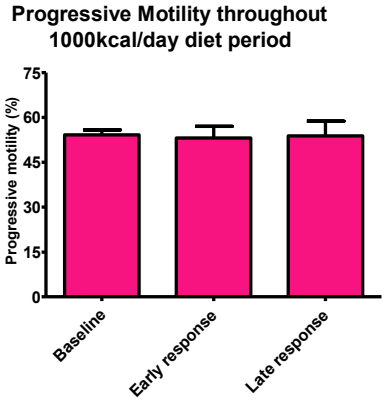
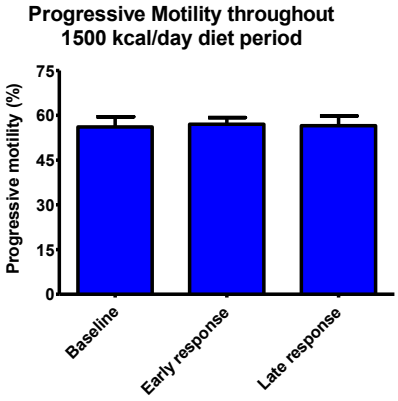
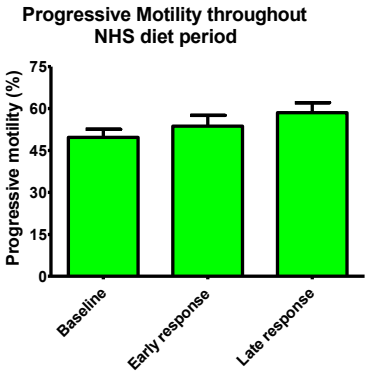

Total Motility throughout
Control diet period
Total motility (%)
80
60
40
20
0
Baseline
Early response
Late response

Figure 4.6 Mean progressive sperm motility (%)throughout the study period (*Baseline:* study period referring to the mean sperm progressive motility during screening, pre-diet and start of diet study visits, *Early response:* refers to the mean progressive motility during the 1st, 2nd and 4th week study visits, *Late response:* refers to the mean progressive motility during the 6th and 8th week study visits). Data shown as mean ± SEM for each of the different study groups; *P≤0.05. Orange: 800kcal, Fuchsia: 1000kcal; Blue: 1500kcal; Green: NHS, Black: Control.









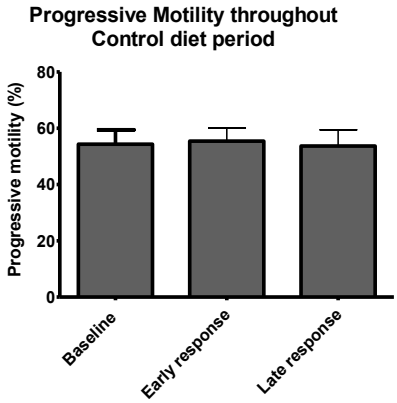
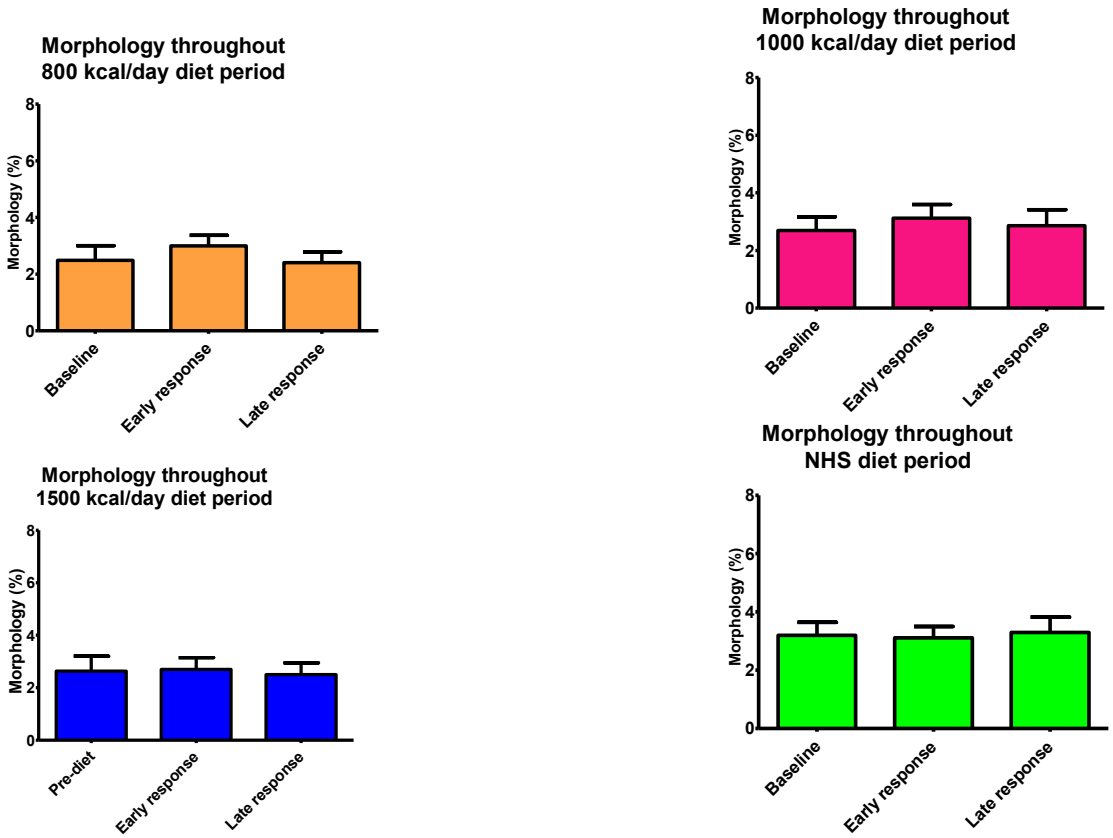
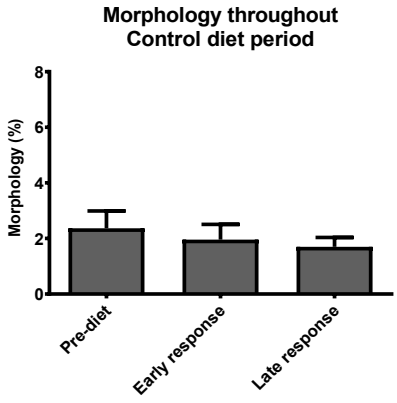


Figure 4.7 Mean normal sperm morphology (%) throughout the study period (*Baseline:* study period referring to the mean sperm morphology during screening, pre-diet and start of diet study visits, *Early response:* refers to the mean sperm morphology during the 1st, 2nd and 4th week study visits, *Late response:* refers to the mean sperm morphology during the 6th and 8th week study visits). Data shown as mean ± SEM for each of the different study groups; *P≤0.05. Orange: 800kcal, Fuchsia: 1000kcal; Blue: 1500kcal; Green: NHS, Black: Control.





I also analysed the cumulative area under the curve (AUC) change in each semen parameter, including concentration, motility, morphology and total motile count per ejaculate, in men with obesity over the eight-week diet period. The AUC represented the two-dimensional area, when calculated between the weeks covering the diet period (from W0 to W8) and the change in each semen parameter from the mean semen parameter levels during the baseline study period (W0=baseline study period or mean of screening, pre-diet and start of diet study visits and W8=end of diet period or week 8). The trapezium rule was used to estimate the overall AUC, which was split into five trapeziums:

AUC= ½ seminal parameter change from baseline at W1 to W0 x first trapezium height +

½ seminal parameter change from baseline at W2 to W1 x second trapezium height +

½ seminal parameter change from baseline at W4 to W2 x third trapezium height +

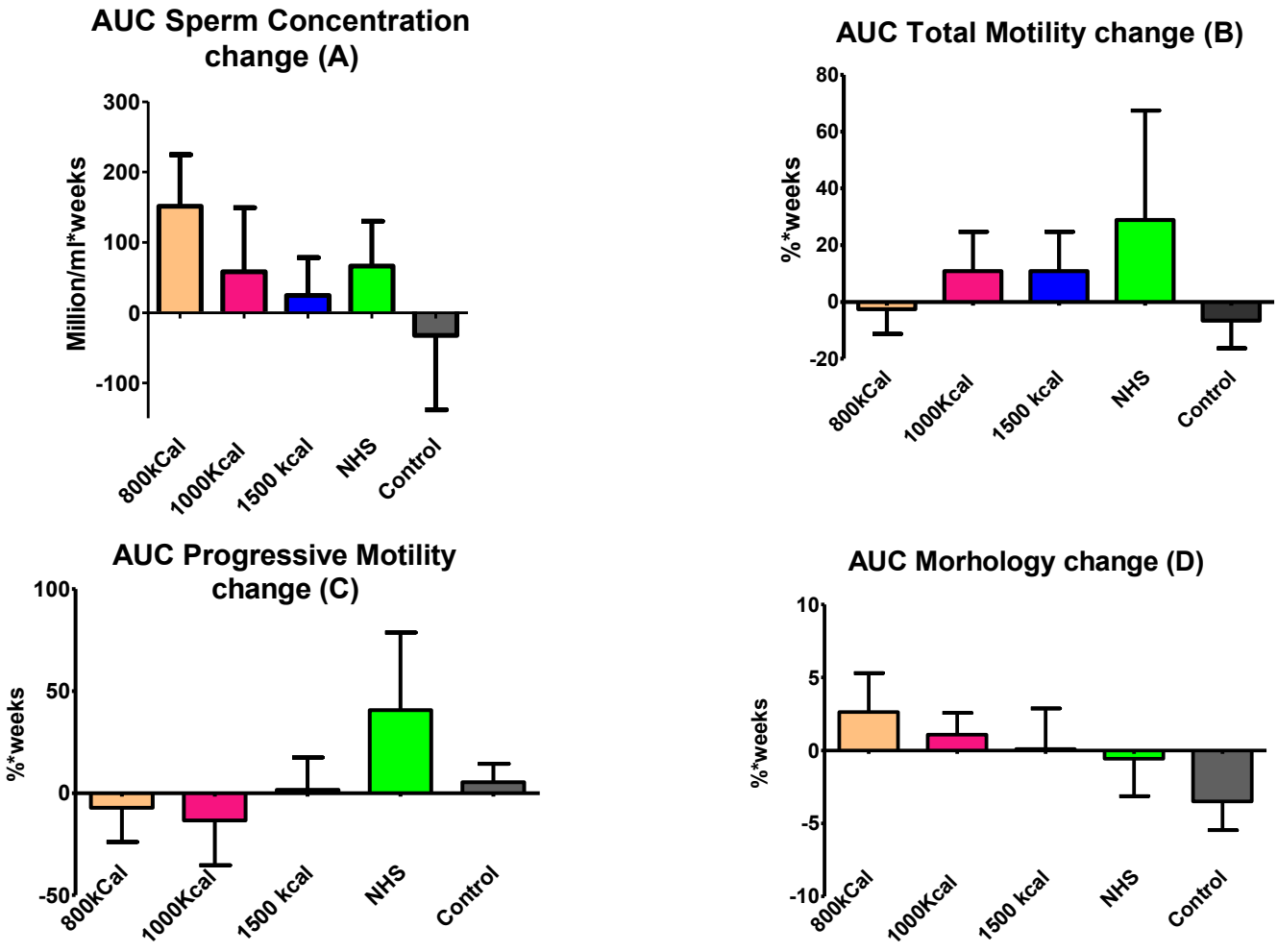
½ seminal parameter change from baseline at W6 to W4 x forth trapezium height +

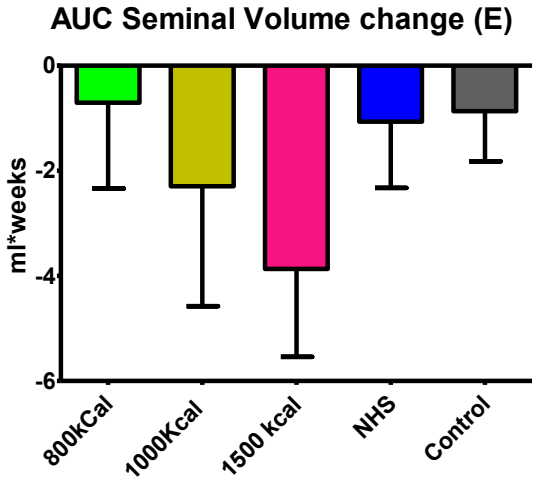
½ seminal parameter change from baseline at W8 to W6 x fifth trapezium height.

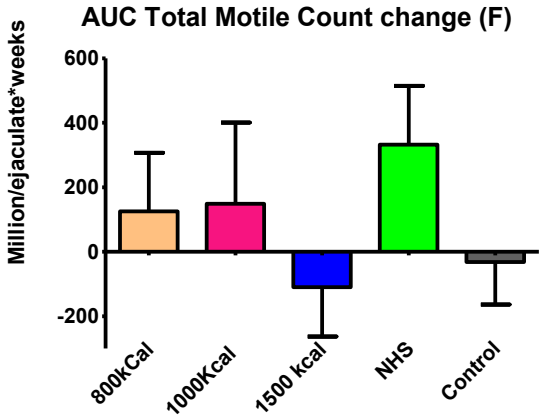
The first trapezium height was calculated between time point W1 and W0 (W1=week 1 after start of diet), the second trapezium height between W2 and W1 (W2=week 2 after start of diet), the third trapezium height between W4 and W2 (W4=week 4 of diet), the fourth trapezium height between W6 and W4 (W6= week 6 after start of diet) and the fifth trapezium height between W8 and W6.

No significant differences in AUC change from baseline of any semen parameter were observed during the study (Figure 4.8).

Figure 4.8 Semen parameters in obese men undergoing four different levels of caloric restriction or simple observations for 8 weeks. A summary of the effects of caloric restriction for each of the different study groups. Orange: 800kcal, Fuchsia: 1000kcal; Blue: 1500kcal; Green: NHS, Black: Control. Data is shown as mean ± SEM (AUC) for sperm concentration (A), total motility (B), progressive motility (C), sperm morphology (D), seminal volume (E) and total motile count (F).



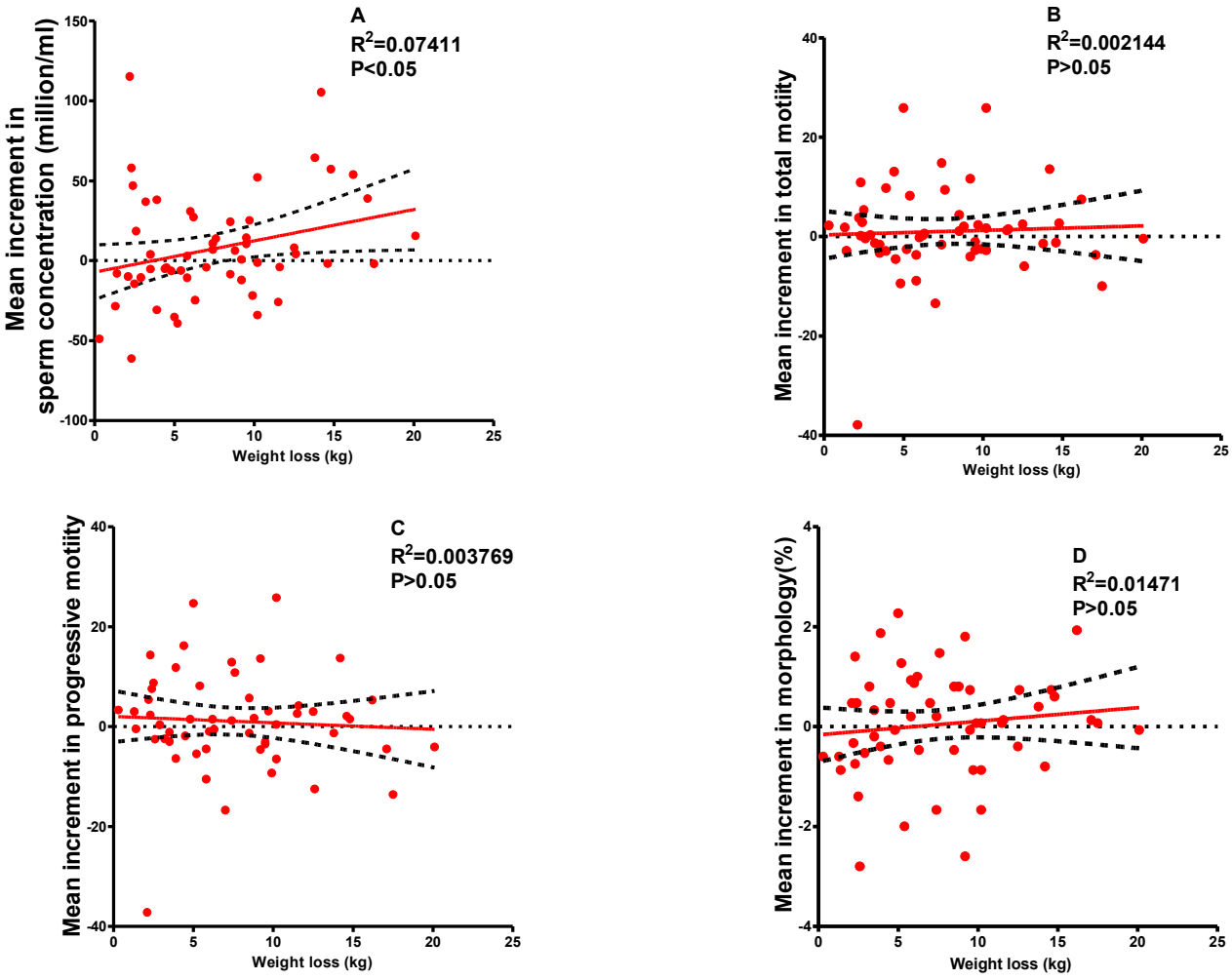

AUC Seminal Volume change (E)
ml*weeks
0
-2
-4
-6
800kCal
1000Kcal
1500 kcal
NHS
Control

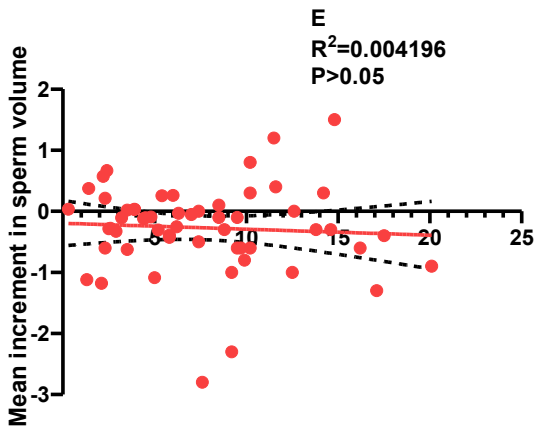

AUC Total Motile Count change (F)
Million/ejaculate*weeks
600
400
200
0
-200
800kCal
1000Kcal
1500 kcal
NHS
Control

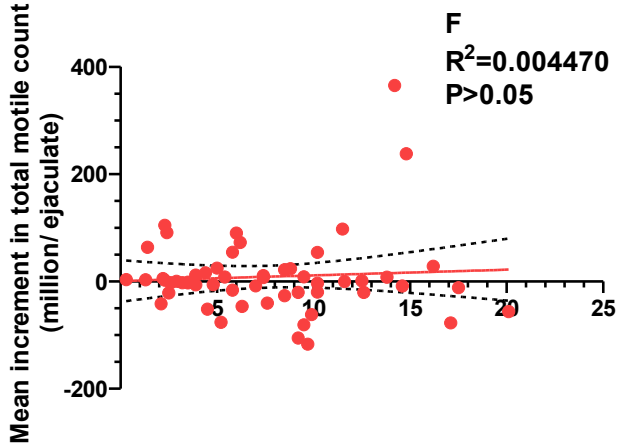
Sperm concentration and correlation with weight loss

Caloric restriction led to significant weight loss in the 800kcal, 1000kcal and 1500kcal group when compared with the NHS diet and control group (Table 4.3). I therefore investigated if changes in weight were correlated with increment from baseline in sperm concentration from baseline in obese men. A scatterplot of sperm concentration mean increment from baseline against weight loss showed a significant correlation although the coefficient of determination was low ($P \leq 0.05$, $R^2=0.07411$) (Figure 4.9 A). No significant correlations were observed between weight loss and increment from baseline in sperm total motility (Figure 4.9 B), progressive motility (Figure 4.9 C), normal morphology (Figure 4.9 D), seminal volume (Figure 4.9 E) as well as total motile count (Figure 4.9 F).

Figure 4.6 Sperm concentration, total motility, progressive motility, normal morphology, seminal volume, total motile count and association with weight loss in obese men undergoing caloric restriction. Scatterplot presents mean increment of sperm concentration (A), total motility (B), progressive motility (C), normal morphology (D), seminal volume (E) and total motile count (F) from baseline plotted against maximum weight loss achieved during the study period (R^2; coefficient of determination).

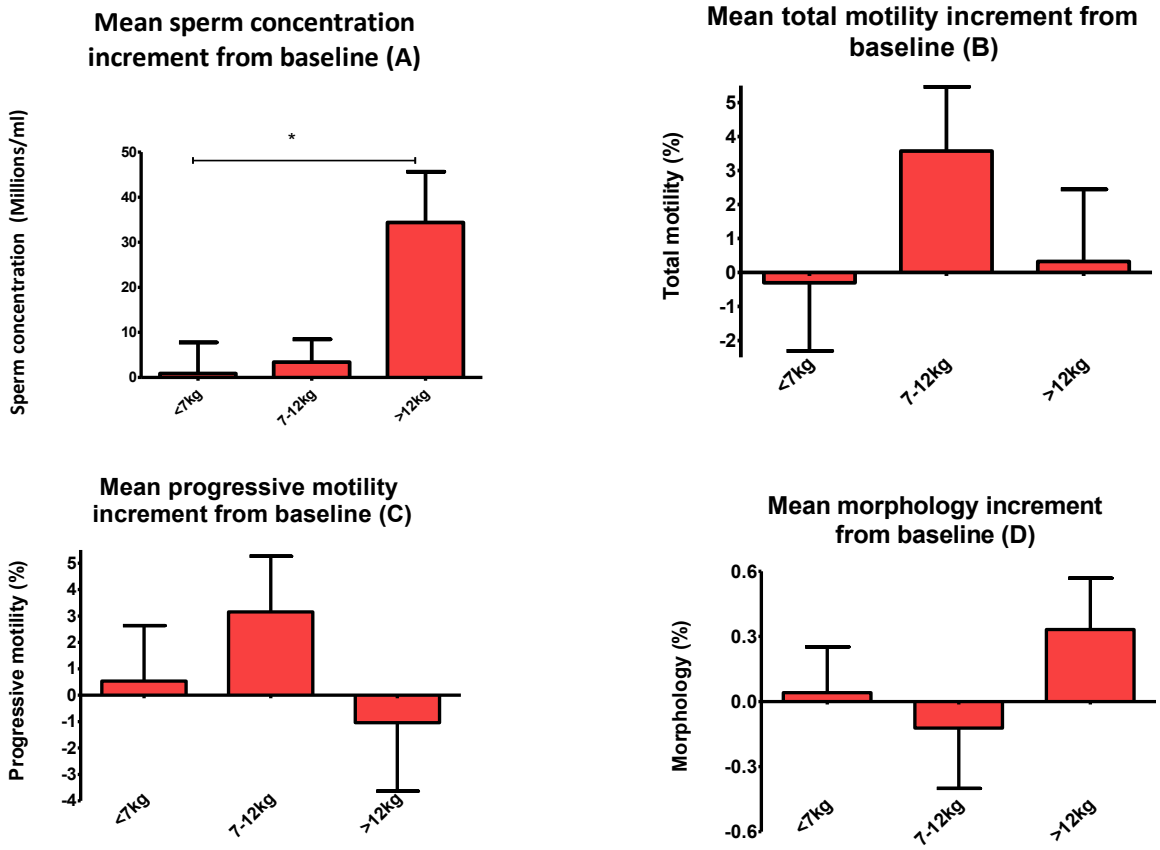


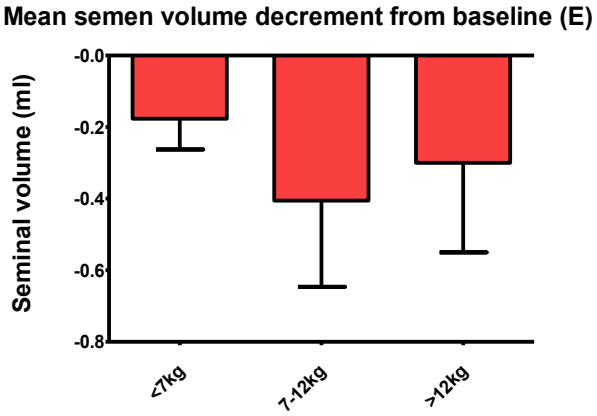

E
R2=0.004196
P>0.05
Mean increment in sperm volume
2
1
0
-1
-2
-3
5
10
15
20
25

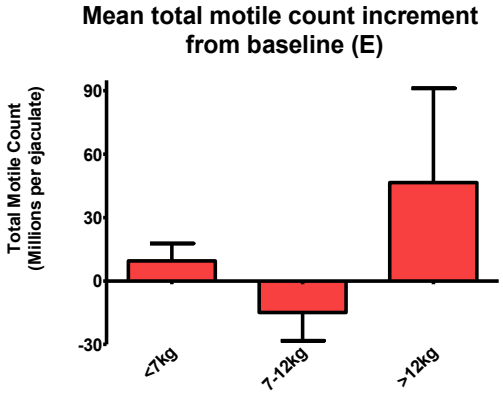

F
R2=0.004470
P>0.05
Mean increment in total motile count (million/ ejaculate)
400
200
0
-200
5
10
15
20
25

Finally, I analysed the mean increment in semen parameters in participants classified by weight loss achieved by the end of study period. Weight loss of more than 12kg resulted in significantly higher mean sperm concentration increment from baseline compared to weight loss of 7kg and below (Figure 4.10 A), but this effect was not observed in other semen parameters (Figure 4.10 B-F).

Figure 4.10 Effect of weight loss on mean increment from baseline study period of sperm concentration (A), total motility (B), progressive motility (C), morphology (D), seminal volume (E) and total motile count (F) after less than 7kg weight loss, 7-12kg weight loss or more than 12kg weight loss. Data presented as pooled mean ± SEM; * P≤0.05




Mean semen volume decrement from baseline (E)
Seminal volume (ml)
-0.0
-0.2
-0.4
-0.6
-0.8
<7kg
7-12kg
>12kg


Mean total motile count increment from baseline (E)
Total Motile Count (Millions per ejaculate)
90
60
30
0
-30
<7kg
7-12kg
>12kg

4.5 DISCUSSION

Obesity has a negative impact on male reproductive potential; however, the effects of weight loss on sperm function of obese men remains unclear. Previous uncontrolled study suggests that 15% median weight loss over 3 months can increase total sperm count (Berger Håkonsen et al. 2011). Ten per cent of BMI reduction has also been reported to increase the percentage of normal sperm morphology in a further uncontrolled study (Mir et al. 2018). No randomised controlled study has been performed previously to investigate the effect of weight loss on male fertility. The current study is the first to report the impact of caloric restriction on semen parameters throughout an eight-week diet period in obese men. It suggests a possible threshold for weight loss leading to significant increase in sperm concentration in obese men.

Effect of caloric restriction in metabolic parameters and hormonal profile

Caloric restriction in the obese population is critical to achieve weight loss and improve metabolic health. A systematic review on formula diets less than 800kcal reported significant weight loss, reduction in waist circumference, fasting glucose and total cholesterol (Mulholland et al. 2012). In my study reduction in weight, waist circumference, fasting glucose and total cholesterol were significantly greater in the 800kcal group compared to the control group. It has been shown that sperm cells rely on glycolysis and Sertoli cells rely on β-oxidation of fatty acids and for their energy requirements (Ana D. Martins et al. 2015; Crisóstomo et al. 2017). Hence optimisation of the metabolic parameters, including total cholesterol and fasting glucose in obese men on 800kcal daily could have led to optimal testicular energy metabolism. This is in keeping with the finding from the current study that the greatest improvement in sperm concentration was observed within the 800kcal group.

Previous studies did not observe significant changes in LDL and HDL-cholesterol with formula diets less than 800 kcal daily (Mulholland et al. 2012). This is consistent with results in my study where LDL and HDL-cholesterol did not differ significantly between groups. However, Paisey et al. observed that participants undertaking regular exercise in combination with formula diets, increased significantly their HDL-cholesterol (Paisey et al. 2002). Participants in my study maintained low or stable activity levels whilst on 800kcal and this probably explains the non-significant change in their HDL-cholesterol. Although the importance of optimal HDL-cholesterol and other lipoproteins on testicular energy metabolism has not been studied, future studies combining caloric restriction and exercise could provide more information.

Obesity is associated with low serum testosterone as well as high serum oestradiol leading to low testosterone: oestradiol ratio (Bieniek et al. 2016). Weight loss with low energy diet has been shown to increase serum testosterone and free testosterone levels in obese men (Niskanen et al. 2004). In the current study there was no significant increase of serum testosterone or calculated free testosterone from baseline between any of the study groups. Interestingly, the 800kcal group achieved the highest increase in testosterone from baseline, although the increase was not significantly higher compared to any other study group. Testosterone increase on this occasion was the highest among any other study group, as the 800kcal group achieved 10% weight reduction, which was the greatest weight reduction between study groups. This observation is also confirmed by Corona et al. who noted that androgen rise is greater in obese men losing 10% to 32% weight by either low calorie diet or bariatric surgery (Corona et al. 2013). It is therefore possible that the non-significant increase in testosterone in the 800kcal group may have played an additional role in the early phase increase in the sperm concentration in obese men. Further studies are needed to explore if

testosterone synthesis is a contributor to improvement in sperm function in obese men undergoing caloric restriction.

A case-series of 6 infertile overweight men showed significant increase of their testosterone: oestradiol ratio over a two-month diet period and 4% BMI reduction (Faure et al. 2014). Serum oestradiol levels were not significantly reduced in obese men undergoing weight loss during my study. It is probable that oestradiol levels remained unchanged due to persistent negative feedback of excessive oestrogens on the reproductive axis which probably requires more than 2 months for reversal (Rosenblatt, Faintuch, and Cecconello 2017; Calderón et al. 2019). A longer study may be required to observe significant change in testosterone as well oestradiol in obese men undergoing weight loss.

Time profiles of semen parameters during the study period

The time profile of sperm concentration in my study indicates slightly higher sperm concentration during week 1, 2 and 4 of the 800kcal (early response) diet period when compared to any other study group. It would therefore appear that 800kcal caloric restriction may have a meaningful impact in the testicular output for this group. Therefore, I examined the effect of 800kcal daily caloric restriction on sperm concentration by comparing the mean sperm concentration during the baseline study period, week 1, 2 and 4 (early response), as well as week 6 and 8 (late response) of the diet period. I observed that sperm concentration in the 800kcal group was significantly higher during the early response than sperm concentration during the baseline study period as well as the late response diet period. A possible explanation for this early phase increase may be that tight caloric restriction to 800kcal daily was accompanied by significant improvements in weight, fasting glucose and total cholesterol.

Nevertheless, sperm concentration decreased during the late response of the diet period suggesting that the eight-week study protocol was too short and did not sufficiently cover waves of spermatogenesis which typically last 42 – 76 days (Misell et al. 2006).

Semen parameters in the 1000kcal, 1500kcal, NHS diet and control groups did not change significantly throughout the study period. Several factors may have accounted for this observation. Firstly, obese men taking 1000kcal and 1500kcal daily did not achieve significant improvements to their glucose and cholesterol profile, despite significant weight loss achieved compared to the NHS diet and control groups. It is possible that participants taking more than 1000kcal and 1500kcal daily did not always choose best balanced meals to make up their total daily energy intake in accordance with recommendations from the British dietetic association. Thirdly, the biological variability of semen parameters is well described in the literature, with sperm concentration representing the most accurate measure of testicular output (Schwartz et al. 1979). It appears that there was a high level of semen parameters variability within subjects in all study groups, which could have obscured important trends in other parameters apart from sperm concentration. Increasing the number of samples per interval (Jarow, Fang, and Hammad 2013) could have reduced variability and produce further significant results in other semen parameters in my study groups but this could be considered in future studies.

Is there a threshold for weight loss to improve sperm concentration in obese men?

The percentage of weight loss required to improve total sperm count was shown to be 15% by Håkonsen et al in a non-randomised controlled study (Berger Håkonsen et al. 2011). Similarly, in the current study 10% weight loss resulted in significant increase in sperm concentration of obese men on

800kcal daily caloric restriction. This suggests that caloric restriction tighter than 1000-2200kcal daily caused significant improvement in sperm concentration, although greater weight loss would be required to improve total sperm count. Results from my study additionally indicate that weight loss of more than 12kg led to higher mean increment of sperm concentration from baseline. Given the suspected association of weight loss with sperm concentration, correlation analysis confirmed a significant association between weight loss and sperm concentration. The strength of this association was weak as other factors may also contribute to sperm concentration increment, such as physical activity (Hajizadeh Maleki and Tartibian 2018).

A non-randomised controlled study by Mir et al. showed that 8% BMI reduction led to significant improvement in sperm morphology after a twelve-week diet period and follow up study visits at six months (Mir et al. 2018). Even though the 1000kcal and 1500kcal group improved their BMI by 9% and 7% respectively, no specific trends were observed in normal sperm morphology in the current study. If a longer than eight-week study was performed, it would be interesting to offer a follow up visit at 6 months to determine significant trends in normal sperm morphology. Notably, I did not observe any significant correlations between weight loss and normal sperm morphology or motility hence it is possible that other factors may stimulate these parameters (Hajizadeh Maleki and Tartibian 2018).

Limitations

It is important to identify potential limitations. Biological variability of semen parameters within individuals is a well-known limitation in male fertility studies and depends on multiple factors such as length of abstinence and waves of spermatogenesis (Schwartz et al. 1979). Participants were asked to

attend multiple study visits with the aim to obtain mean semen parameters of subsequent measurements. Time constraints and study protocol did not allow the collection of more than one sample per study visit to effectively address variability. This may explain why the variability of semen parameters persisted throughout the study period, although it is unlikely that participants would have consented to more frequent study visits or multiple sample productions per visit.

Another interesting consideration is that the current study was performed within an eight-week diet period, while the wave length of spermatogenesis is 42- 76 days. Changes in sperm concentration were detected within 60 days, especially during the early response diet period. However, a longer study period may be indicated to detect changes after several waves of spermatogenesis as well as changes in sperm motility and morphology, which are harder to detect due to the higher degree of biological variability.

Participants were instructed to maintain stable activity levels throughout the diet period, not exceeding 150 minutes a week of exercise as per NHS recommendations. However, most participants maintained a sedentary lifestyle and physical activity remained uncontrolled during the study period. It would have been useful to monitor the effects of physical activity in the context of a structured exercise program. Intense physical activity should not however be offered to participants on 800kcal daily as it would not be easily sustained on such a high level of caloric restriction.

Conclusions

This is the first physiological randomised controlled study designed to investigate sperm function in the context of caloric restriction and how dietary weight loss could improve male reproductive potential in obese men. Tight caloric restriction up to 800kcal daily can lead to 10% weight loss, which is critical to increase sperm concentration in obese men. Weight loss above 12kg in obese men leads to significant increment in sperm concentration in comparison to obese men losing less than 12kg during an eight-week diet period. In addition, significant reductions in waist circumference, fasting glucose and total cholesterol are observed in obese men taking 800kcal daily. Hence obese men on tight caloric restriction achieve significant improvements in their metabolic profile, which are probably associated with meaningful improvement in sperm concentration. Results from this study have important therapeutic implications, as weight loss of 12kg and above via diet may be an effective measure to stimulate testicular output for couples with male factor infertility due to obesity.

4.6 FUTURE STUDIES

Future studies could be designed to observe greater improvements in semen parameters of obese men. For example, the current study did not assess male reproductive potential in obese men undergoing diet over a longer than eight-week diet period and did not provide structured advice on physical activity. A future study could be designed to include a twelve-week diet period combined with exercise and follow up study visits at six or twelve months. Resistance training increases significantly sperm concentration, motility and morphology (Hajizadeh Maleki and Tartibian 2018). Addition of a structured exercise program to diet could therefore be an important lifestyle change to improve sperm function in obese men.

Improvements in the metabolic and hormonal profile of the 1000kcal, 1500kcal and NHS diet groups were less prominent compared to the 800kcal group. It is possible that groups taking extra meals other than CWP products did not always make balanced dietary choices. It has been reported that obese couples attending infertility clinics eat less fruit and vegetable compared to the general population (Belan et al. 2019) and this behaviour probably reflects similar dietary choices with my study groups on combination of CWP products with meals. Observational studies based on dietary questionnaires suggest that omega-3 fatty acids improve sperm count, motility and morphology. Also, consumption of low-fat dairy products has been associated with increased sperm concentration and motility. In contrast, consumption of meat rich in saturated fat or processed food rich in trans-polyunsaturated fat reduces sperm concentration (Hayden, Flannigan, and Schlegel 2018). Therefore, a future study could include a group with restricted energy intake rich in omega-3 fatty acids, low -fat dairy products and absence of processed food or meat containing saturated fat.

Fertility therapies such as weight loss interventions are more likely to be successful when offered to couples rather than individuals. Although couple-based interventions were not included in the current study protocol, they have been related to more efficient weight loss as partner involvement facilitates behaviour change, persistence and cost-effectiveness (Best et al. 2017). It could be useful to design future studies involving obese couples, which could prove to be critical to the development of group clinics that may also be more cost-effective compared to fertility clinics currently offered via the national health service. Education on healthy diet for couples seeking fertility could become a point for long-term lifestyle changes (Best et al. 2017). Finally, future research could also assess the proportion of obese subfertile male achieving a positive pregnancy test after weight loss.

Chapter 5

General Discussion

Male reproductive capacity is increasingly viewed as a marker of general male health (Eisenberg et al. 2016). Contemporary lifestyle factors and most importantly obesity, affect not only semen quality but also male lifespan (Bendayan et al. 2018). It is known that obesity is linked to metabolic disturbances and hypogonadism, which are known risk factors for cardiovascular disease and increased mortality (Muraleedharan and Jones 2014).

A total of four previous studies investigated weight loss via diet to improve male fertility however, none of these studies was randomised controlled (Berger Håkonsen et al. 011, Faure et al. 2014, Belan 2015, Mir et al. 2018). My eight-week study on four different levels of caloric restriction including a control group, showed that sperm concentration was significantly increased in obese men taking 800kcal daily during week 1, 2 and 4 (early response) of the diet period. I observed that participants on 800kcal caloric restriction per day had significant reduction in their weight, waist circumference, fasting glucose and total cholesterol compared to the control group. Notably the 800kcal group had no significant improvement in their hormonal profile compared to other study groups however, testosterone increase was non-significantly higher in the 800kcal group compared to any other group. The significant improvement in sperm concentration in the 800kcal group could be attributed to improvement in the metabolic as well as hormonal profile in the above group, but more work is needed to investigate this further.

The present study is the first randomised controlled study investigating the effects of weight loss on male fertility with important implications for the management of couples with male factor infertility due to obesity. My data suggest that weight loss above 12kg via diet leads to significantly higher sperm concentration form baseline. I observed a weak association between weight loss and increase in sperm concentration, however future studies are required to identify other factors contributing to this increase, such as physical activity. Although my observations were related to sperm concentration,

which is the most representative seminal parameter of testicular output, fertility in the sense of live births after the study period was not assessed. Since the threshold to increase sperm concentration in obese men is identified, future studies in obese men seeking fertility could determine the impact of weight loss on pregnancy rates of obese infertile men.

Semen analysis is a commonly used yet limited test of male reproductive function. In addition to conventional semen analysis, I investigated seminal oxidative stress in male partners of women with idiopathic recurrent pregnancy loss (RPL). RPL is defined as the termination of three or more pregnancies before the 24th week of gestation and is idiopathic in 50% of couples despite detailed investigations for the female partner (Stephenson 1996). My study showed that seminal reactive oxygen species (ROS) in male partners of women with RPL were significantly increased in comparison with the age-matched control group. Seminal oxidative stress with high ROS levels could induce placental dysfunction through sperm DNA damage and subsequent implantation failure (Gupta et al. 2007). According to a study by Ko YE et al, excess ROS production could lead to sperm DNA fragmentation and high likelihood of arrested embryo development due to fertilization of the oocyte with fragmented sperm DNA (Ko, Sabanegh, and Agarwal 2014). Expectedly, sperm DNA fragmentation, which has been shown to correlate with the genetic health of the offspring (A. Agarwal et al. 2017) was also significantly higher in the RPL group compared to the control group. It is important to recognise that men in the RPL group had a significantly greater weight compared to the control group. This observation is in agreement with Agarwal et al. who also supported that ROS values depend upon individual male parameters (A. Agarwal et al. 2017) and can be high in the context of obesity (Agarwal et al. 2018). Although in my study the association of subject BMI with sperm DNA fragmentation was significant for men in the RPL group with BMI greater than 40kg/m^2, this association disappeared for BMI- matched subjects (Jayasena et al. 2019). A future study including

obese male partners of women with RPL and an obese control group would be required to investigate any possible association between ROS and BMI.

Recent review evidence suggest that obesity and the metabolic syndrome are associated with high level of seminal cytokines, sperm DNA fragmentation as well as systemic high sensitivity-CRP levels (Leisegang, Henkel, and Agarwal 2019). Over the last fifty years dietetic patterns changed dramatically to reflect 'westernisation' with higher intake of processed food and fats with less seafood, vegetables and whole grains. Consequently, western pattern diets are associated with linear decline of sperm concentration and normal sperm morphology (C. Y. Liu et al. 2015; Hayden, Flannigan, and Schlegel 2018). Since my RPL study did not explore the role of western pattern diets, an animal study was set up to investigate the effects of high fat diet induced obesity in the reproductive function of male mice. I noted slightly higher semen ROS generated in mice given high fat diet (HFD) compared to mice given standard diet (NCD). Also, slightly higher sperm DNA fragmentation was observed in the HFD group compared to NCD group. Unfortunately, obese mice were resistant to significant changes in their semen parameters, semen ROS and sperm DNA fragmentation as previously noted by Jasmine Aly et al. (Aly and Polotsky 2017). I also investigated the addition of an irreversible myeloperoxidase inhibitor, AZD5904 to prevent ROS production by myeloperoxidase in seminal neutrophils and unsurprisingly, only minor improvements were noted in the sperm function of obese male mice.

Semen ROS and sperm DNA fragmentation could be important additions to conventional semen analysis, especially on background of unexplained male factor infertility. For instance, male partners of women with idiopathic RPL had unremarkable semen parameters on conventional semen analysis but significantly higher seminal ROS and sperm DNA fragmentation. Therefore, modern semen analysis other than current WHO standards could be of value for couples with unexplained male factor infertility and correctly guide the use of antioxidants to reduce high semen oxidative stress as

measured by semen ROS. Similarly, weight loss via diet up to 12kg in obese men was shown to significantly increase their sperm concentration. Sperm motility and normal morphology did not change but this may be due to the high degree of variability or short study period. Weight loss of 12kg in obese infertile men could be an important milestone for these men to improve their reproductive potential and metabolic health via a better lifestyle.

In my RPL study, I cannot exclude that a proportion of men may have had asymptomatic genitourinary infections impossible to identify with standard semen analysis. Genitourinary infections although asymptomatic, could lead to high ROS (Agarwal et al. 2018) and would be important to examine semen microbial groups in future studies. Also, ROS measurement via chemiluminescence is time-consuming and novel technologies, such as Male Infertility Oxidative System (MiOXSYS) may be easier to implement in the assessment of seminal oxidation–reduction potential (Dutta, Majzoub, and Agarwal 2019). The possible resistance of mouse semen to oxidative stress due to high fat diet induced obesity could not be ruled out in my animal study, therefore future research with a more suitable animal model could identify novel pharmacotherapies for male infertility. Finally, my results from the weight loss study in obese men are encouraging however, they should be validated by longer studies including infertile obese men with controlled activity levels. Weight loss vial diet can be effective but possible difficulty in adherence to diet cannot be fully addressed. Behavioural changes and psychological support would be required to ensure successful weight loss via diet but cannot be guaranteed if these were to be implemented in complex healthcare systems.

It would be important to confirm my study findings with future work. Given that idiopathic RPL could be attributed to male factor infertility with high semen ROS, novel antioxidant therapies could be developed to target oxidative stress. Also, the role of seminal microbiome could be explored further to identify men with RPL and a specific microbial group preponderance, who would benefit from

suitable antibiotics. Equally it would be essential to conduct a randomised controlled weight loss study looking into the effects of exercise or 800kcal daily diet programme on semen parameters and seminal oxidative stress of obese infertile men. It is possible that exercise or diet alone affect different semen parameters. Therefore, examining these interventions separately over a longer time period to cover more than one wave of spermatogenesis could lead to a more effective study of sperm concentration, as well as sperm motility, normal sperm morphology and semen ROS.

In summary, in this thesis I have identified a novel diagnostic marker for men with recurrent pregnancy loss. Abnormalities in sperm function are not always detected with standard semen examination. Using a direct chemiluminescence assay to measure the emission of light produced from oxidised luminol in the semen, I demonstrated markedly elevated oxidative stress in the semen of men, whose partners experienced more than 3 pregnancy losses before the 24th week of gestation. Considering that obesity is a main factor contributing to male infertility I identified for first time the effects of caloric restriction on sperm function in obese men in a randomised controlled setting. Daily caloric restriction to 800kcal led to significant improvements in the metabolic profile and sperm concentration in obese men. My data have important implications for the development of novel diagnostic tools and the management of obese men undergoing weight loss to improve their fertility as well as overall health.

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APPENDIX 1

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Endocrinology and Metabolism

Reduced Testicular Steroidogenesis and Increased Semen Oxidative Stress in Male Partners as Novel Markers of Recurrent Miscarriage

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BACKGROUND: Recurrent pregnancy loss, (RPL) affecting 1%–2% of couples, is defined as 3 consecutive pregnancy losses before 20-week' gestation. Women with RPL are routinely screened for etiological factors, but routine screening of male partners is not currently recommended. Recently it has been suggested that sperm quality is reduced in male partners of women with RPL, but the reasons underlying this lower quality are unclear. We hypothesized that these men may have underlying impairments of reproductive endocrine and metabolic function that cause reductions in sperm quality.

METHODS: After ethical approval, reproductive parameters were compared between healthy controls and male partners of women with RPL. Semen reactive oxygen species (ROS) were measured with a validated inhouse chemiluminescent assay. DNA fragmentation was measured with the validated Halosperm method.

RESULTS: Total sperm motility, progressive sperm motility, and normal morphology were all reduced in the RPL group vs controls. Mean SE morning serum testosterone (nmol/L) was 15% lower in RPL than in controls (controls, 19.01.0;RPL,16.00.8;*P*0.05).MeanSEserum estradiol (pmol/L) was 16% lower in RPL than in controls (controls,103.15.7;RPL,86.53.4;*P*0.01).Serum luteinizing hormone and follicle-stimulating hormone were similar between groups. Mean SE ROS (RLU/sec/10^6 sperm)were4-foldhigherinRPLthanincontrols(controls, 2.0 0.6; RPL, 9.1 4.1; *P* 0.01). Mean SE sperm DNA fragmentation (%) was 2-fold higher in RPL than in controls (controls, 7.3 1.0; RPL, 16.4 1.5; *P* 0.0001).

CONCLUSIONS: Our data suggest that male partners of women with RPL have impaired reproductive endocrine function, increased levels of semen ROS, and sperm DNA fragmentation. Routine reproductive assessment of the male partners may be beneficial in RPL.

Recurrent pregnancy loss (RPL[1]; recurrent miscarriage) may be defined as the loss of 3 or more consecutive pregnancies before 20 weeks' gestation, and affects 1%–2% of couples *(1, 2)*. Women with RPL are routinely screened for etiological factors such as antiphospholipid syndrome and thrombophilia *(3, 4)*. However, approximately 50% of RPL have been reported as idiopathic *(2, 5)*, which precludes development of targeted therapies. It is therefore imperative to identify novel markers associated with the pathogenesis of RPL to improve the management of affected couples.

Sperm DNA plays a critical role in placentation *(6)*, so it is biologically plausible that impairments in male reproductive function could increase the risk of RPL. Recent studies suggest that male partners affected by RPL have impaired sperm quality with reduced total motility and morphology *(7)* and increased sperm DNA damage *(8–12)*; however, the reasons underlying are not well understood. High levels of intratesticular synthesis of testosterone are required for spermatogenesis.

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[1] Nonstandard abbreviations: RPL, recurrent pregnancy loss; RLUs, relative light units; ROS, reactive oxygen species; DFI, DNA fragmentation index; LH, luteinizing hormone; FSH, follicle-stimulating hormone; AUC, area under the curve.

Therefore, impairment of the reproductive endocrine axis could feasibly impair sperm function in male partners of women with RPL. Reactive oxygen species (ROS) are unstable metabolic by-products containing unpaired outer shell electrons, causing oxidative cellular damage *(12)*. Spermatozoa and semen polymorphonuclear leukocytes are both sources of ROS generation. ROS, therefore, have the potential to impair sperm function and cause sperm DNA damage.

We hypothesized that male partners of women with RPL have significant abnormalities in reproductive endocrine and metabolic function that may impair sperm quality, when compared with the general male population. We therefore investigated serum levels of reproductive hormones, semen ROS, sperm DNA fragmentation, and sperm function in men affected and unaffected by RPL in a female partner.

Methods

PARTICIPANT RECRUITMENT AND SAMPLE COLLECTION

Ethical approval was granted by the West London and GTAC Local Research Ethics Committee (Ref 14/LO/ 1038), and the study was performed in accordance with the Declaration of Helsinki. The study protocol is summarized in Fig. 1 in the online Data Supplement that accompanies the online version of this article at http:// www.clinchem.org/content/vol65/issue1. Cases were recruited from the recurrent miscarriage clinic at St. Mary's Hospital, between September 2016 and May 2017. RPL was defined by the Royal College of Obstetrics and Gynaecologists criteria *(5)*. Exclusion criteria were history of anemia, current symptoms of genitourinary tract infection, alcohol excess, active treatment for severe systemic disease, antioxidant nutritional supplement use within the previous 6 months, recent febrile illness, and female cause of RPL. Healthy male controls were recruited through local advertisements and completed a questionnaire to screen for conditions impairing their fertility, including the following: testicular surgery; orchidopexy; varicocele; history of systemic illness or sexually transmitted infection; medications; smoking; recreational drug use. Following informed consent, participants attended a single study visit to complete a questionnaire, undergo height and weight measurement, and provide semen and blood samples. Five study participants were excluded after the first study visit owing to excess alcohol intake 21 U/week. Two participants were excluded following study recruitment owing to azoospermia and active hepatitis B virus infection. To enable age-matched comparisons, subanalyses were performed between the Recurrent Miscarriage Clinic (RMC) group (n 50) and all control participants more than 30 years of age (n 33; mean age 36.4 0.9, *P* 0.43 vs RMC group).

SEMEN ANALYSIS

All samples were analyzed within the Department of Andrology, Hammersmith Hospital, UK, according to WHO 2010 guidelines and UK NEQAS accreditation

(13). All samples were produced on site following 2–7 days of sexual abstinence and incubated at 36 1 °C for liquefaction, up to 60 min before analysis. Sperm morphology was analyzed on Papanicolaou prestained slides with Kruger strict criteria. Reference intervals were as follows: volume 1.5 mL; sperm concentration 15 million/mL; total motility 40%; progressive motility 32%; normal morphology 4%; total motile count 20 million.

MEASUREMENT OF SEMINAL ROS LEVELS

ROS were measured according to a previously described method *(14)*. In brief, 400 L of undiluted (native) semen was mixed with 100 L of stock solution containing 5-mmol/L luminol (5-amino-2,3-dihydro-1,4-phthalazinedione), which is oxidized, resulting in chemiluminescence. Each sample was gently mixed immediately before taking luminometer readings (GloMax; Promega Corporation). Chemiluminescence was measured as relative light units per second (RLU/sec), as measured over 10 min at 1-min intervals, reported as a mean. Negative control was 400 L of PBS with 100 L of luminol working solution. Positive control contained 395 L of PBS, 5 L of 30% H_2O_2, and 100 L of luminol working solution. Methods for the initial assay validation are described in Vessey et al. *(15)*.Inhousevalidationwasperformeddailytoensureconsistent positive and negative calibration. Before commencingthestudy,theassayhadbeenrundailyforoverayear.All analysis runs contained negative and positive control samples.ThereferenceintervalforsemenROSwas 3.8RLU/ sec/million sperm.

DNA FRAGMENTATION ANALYSIS

DNA fragmentation was measured with the Halosperm G2 kit (Halotech DNA SL) according to the method described by Ferna´ndez et al. in 2005 *(16, 17)*. In brief, semen samples were mixed with heated inert agarose and cooled on pretreated glass slides. A denaturant agent and lysis solution were added, followed by staining with eosin and thiazine. Slides were subsequently viewed under bright-field light microscopy to assess sperm chromatin dispersion. With this method, a large halo is seen around sperm without substantial DNA breakage, due to spreading DNA loops emerging from a central core. However, no halo or a minimal halo is seen around sperm containing fragmented DNA. The Halosperm test kit was internally validated in the Andrology department at Hammersmith Hospital. Data were accumulated from QC tested sample using lot numbers G21701026 and G21701026 and analyzed between December 1, 2016, and February 23, 2017. Negative internal QC imprecision based on 28 analyses resulted in a CV of 2.6%. Positive IQC imprecision based on 64 analyses resulted in a CV of 2.7%. Samples with a DNA fragmentation index (DFI) 15% were considered normal, as directed by the kit *(18)*.

ENDOCRINE BIOCHEMISTRY

Morning blood samples were analyzed for serum luteinizing hormone (LH), follicle-stimulating hormone (FSH), estradiol, testosterone, and sex hormone– binding globulin in the clinical biochemistry department of Charing Cross Hospital, by use of the automated immunoassay platforms under UK Accreditation System standards of quality control and reporting. Reference intervals were as follows: LH, 2–12 IU/L; FSH, 1.7–8 IU/L; estradiol, 190pmol/L; sex-hormone–binding globulin, 15–55nmol/L; testosterone, 10–30nmol/L; free androgen index, 30–150.

STATISTICAL ANALYSIS

Data analysis was performed with GraphPad Prism v.7. Quantitative data were assessed for normality with the D'Agostino–Pearson normality test, followed by appropriate parametric (unpaired *t* test) or nonparametric (Wilcoxon rank-sum test) analysis. Group comparisons with respect to categorical variables were performed with the Fisher exact test or chi-squar test. All hypothesis testing was 2 tailed; P 0.05 was considered statistically significant. Data are presented as either mean

(SE) of mean (SE) or median and interquartile range, as applicable.

Results

CLINICAL CHARACTERISTICS OF MALE PARTNERS OF WOMEN WITH RPL

Controls and the RPL group had similar clinical characteristics with regard to ethnicity, smoking, and alcohol intake (Table 1). Furthermore, the RPL group had no apparent increase in exposure to comorbidities known to be associated with seminal ROS generation, including genitourinary diseases such as sexually transmitted infection, orchidopexy, or varicocele (see Table 1 in the online Data Supplement). Mean age and body mass index were higher in the RPL group than in the controls. However, neither age nor body mass index were associated with seminal ROS levels, sperm DNA fragmentation, serum testosterone, or serum estradiol in the control or RPL groups (see Fig. 2 and Table 2 in the online Data Supplement). Nine of the control group and 18 of the RPL group had fathered children previously (Table 1).

REPRODUCTIVE HORMONE PROFILING OF MEN WITH RPL Serum reproductive hormone levels in both groups are shown in Table 2. Levels of serum morning testosterone were approximately 15% lower in the RPL group than in the control group (mean SE serum testosterone in nmol/L: control, 19.0 1.0; RPL, 16.0 0.8; *P* 0.05). Furthermore, levels of serum estradiol (which is predominantly synthesized in the testes with testosterone) were 16% lower in the RPL group than in the control group (mean SE serum estradiol in pmol/L: 103.1 5.7, control; 86.5 3.4, RPL, *P* 0.01). Serum levels of LH were lower in the RPL group than in the controls, but this difference was nonsignificant (mean SE serum LH in iU/L: control, 3.9 0.7; RPL, 2.7 0.2; *P* 0.10). Serum FSH levels were similar in both groups (mean SE serum FSH in iU/L: control, 3.3 0.2; RPL, 3.6 0.2; *P* 0.30). Sex hormone–binding globulin levels were similar between men with RPL and healthy controls.

Table 1. Clinical characteristics of participants.[a]

Parameter	Controls (*n* = 63)	Partners of women with RPL (N = 50)[b]
Age, years	30.8 ± 1.0	37.3 ± 0.7[c]
BMI, kg/m^2	24.8 ± 0.4	27.6 ± 0.6[c]
Ethnicity		
White	43	34
Asian, Indian	5	5
Asian, Other	6	3
Afro-Caribbean	4	3
Other	5	5
Smoker, %	14	12
Alcohol, %	73	66
Alcohol intake (units/week)[d]	11.4	13.6
Previous children	9	18

[a] Data for age and body mass index presented as mean ± SE. [b] RPL, recurrent pregnancy loss; BMI, body mass index.
[c] $P < 0.05$, vs with healthy controls, with unpaired Student *t* test or Wilcoxon ranksum test. [d] Alcohol intake presented as mean.

Table 2. Endocrine parameters of participants.[a]

Parameter	Controls (*n* = 63)	Partners of women with RPL (*n* = 50)[b]
LH, IU/L	3.9 ± 0.7	2.7 ± 0.2
FSH, IU/L	3.3 ± 0.2	3.6 ± 0.2
Estradiol, pmol/L	103.1 ± 5.7	86.5 ± 3.4[c]
SHBG, nmol/L	32.7 ± 1.6	29.4 ± 1.3
Testosterone, nmol/L	19.0 ± 1.0	16.0 ± 0.8[d]
Free androgen index	60.8 ± 2.8	56.7 ± 2.6

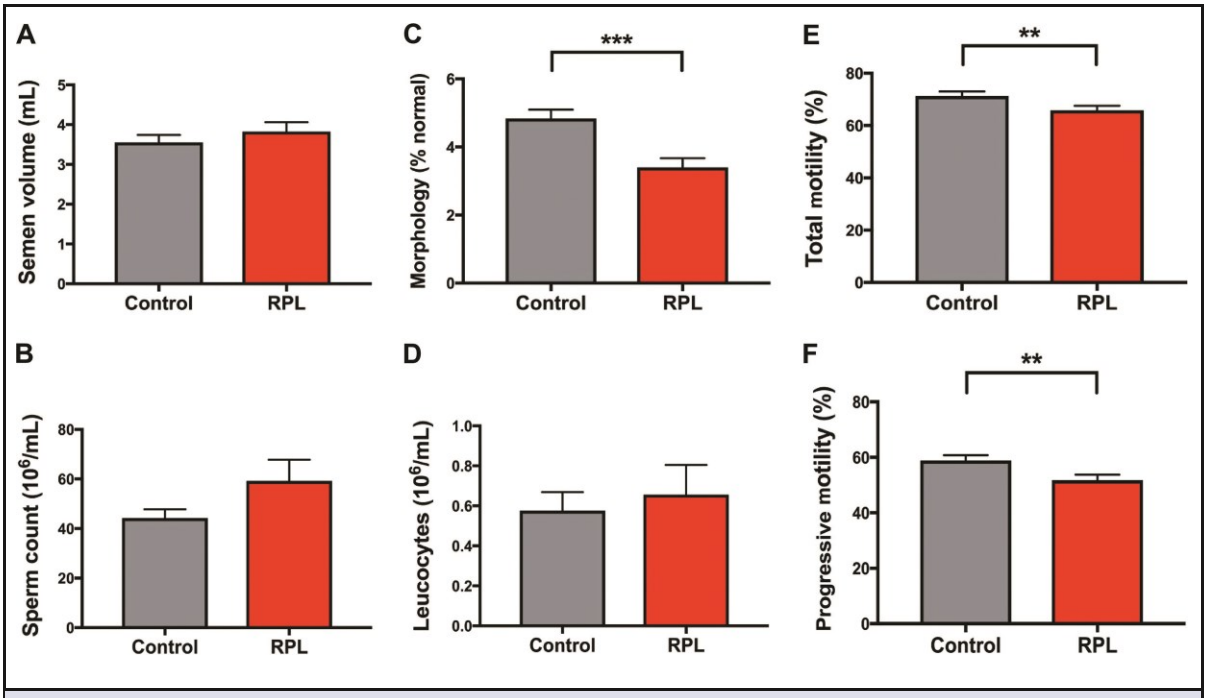

Fig. 1. Sperm characteristics of male partners of women with recurrent pregnancy loss. Bargraphscomparesemenvolume(A),sperm count (B), normal sperm morphology (C), leukocyte count (D), total sperm motility (E), and progressive sperm motility (F) in recurrent pregnancy loss (RPL) group *versus* control group. Data are mean ±SE. $^{**}P < 0.01$; $^{***}P < 0.001$.

[a] Free androgen index calculated as (serum testosterone × 100)/SHBG. Data presented as mean ± SE.

[b] RPL, recurrent pregnancy loss; LH, luteinizing hormone; FSH, follicle stimulating hormone; SHBG, sex hormone binding globulin.

[c] $P < 0.01$. [d] $P < 0.05$.

SPERM FUNCTION IN MEN WITH RPL

Semen analysis parameters are summarized in Fig. 1 and see Table 1 in the online Data Supplement. Reduced levels of sperm motility, progressive motility, sperm morphology, ejaculate volume, and sperm count are established markers of the failure to conceive (i.e., infertility) in affected couples *(13)*. We were therefore interested in investigating whether these factors also were reduced in male partners of women with RPL. Ejaculate volume and sperm count were not significantly different between the study groups. Men in the RPL group had significantly fewer motile sperm than controls (mean SE total percentage of total sperm motility: control, 65.8 1.7; RPL, 71.3 1.7; *P* 0.01) and fewer progressively motile sperm than controls (mean SE percentage of progressively motile sperm: control, 58.9 1.8; RPL, 51.8 2.0; *P* 0.01). The RPL group had a significantly lower proportion of morphologically normal sperm according to WHO criteria (reference range is 4% or above) than controls (% sperm SE with normal morphology: control, 5.0 0.3; RPL, 3.0 0.3; *P* 0.001). Latent genitourinary infection may cause sperm damage through semen ROS generation; it is therefore important to note that levels of semen leukocytes (which are an important source of semen ROS) were similar between

control and RPL groups (Fig. 1D; see Fig. 3D in the online Data Supplement).

MOLECULAR SPERM CHARACTERISTICS IN MEN WITH RPL Having observed that male partners of women with RPL

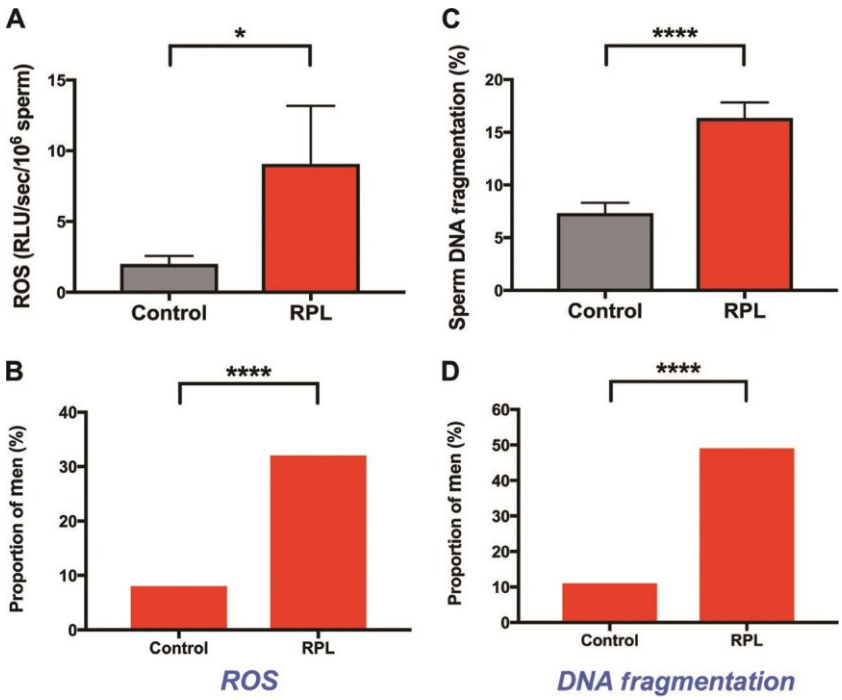

Fig. 2. Sperm DNA damage and oxidative stress in male partners of women with recurrent pregnancy loss.

Bar graphs compare semen reactive oxygen species (ROS; A) and sperm DNA fragmentation (B) in recurrent pregnancy loss (RPL) versus controls.Dataaremean+SE.HistogramscomparetheproportionofparticipantswithincreasedsemenROS(C)andspermDNAfragmentation (D). *$P < 0.01$; ****$P < 0.0001$.

had significant impairments in sperm function, we investigated whether these men also had abnormally increased levels of semen oxidative stress and sperm DNA damage, which are known to impair sperm function. Mean semen ROS levels were more than 4-fold higher in the RPL group than either controls (mean semen ROS in RLU/sec/10^6: control, 2.0 0.6; RPL, 9.1 4.1; *P* 0.01; Fig. 2A). Male partners of women with RPL were 4-fold more likely to have abnormally increased levels of semen ROS than either controls [proportion (%) of men with semen ROS above reference interval: control, 5/63 (7.9); RPL, 16/50 (32.0); *P* 0.0001; Fig. 2B].

Mean levels of sperm DNA fragmentation were more than 2-fold higher in the RPL group than in either controls (mean DFI: control, 7.3 1.0; RPL, 16.4 1.5; *P* 0.0001; Fig. 2C). Furthermore, male partners of women with RPL were 4-fold more likely to have abnormally increased levels of sperm DNA fragmentation than controls [proportion (%) of men with DFI above refer-

ence range: control, 7/63 (11.1); RPL, 22/50 (44.0); *P* 0.0001; Fig. 2D].

COMPARING REPRODUCTIVE PARAMETERS BETWEEN CONTROLS AND MEN WITH RPL

Of the investigated markers, sperm morphology, semen ROS, and sperm DNA fragmentation had the greatest mean or median difference (30%) between controls and RPL cases. We therefore investigated the potential of these factors to distinguish men with ROS from controls in the study, using ROC analyses (Fig. 3). ROC analysis suggested that

sperm morphology, semen ROS, and sperm DNA fragmentation each discriminated significantly between controls and men with ROS. The greatest discriminator between control and ROS groups was sperm DNA fragmentation, which had an ROC curve area under the curve (AUC) value of 81% (*P* 0.0001 vs line of nondiscrimination).

SUBANALYSIS WITH CONTROL PARTICIPANTS OLDER THAN 30 YEARS

Similar patterns in hormone analysis were observed when analysis was restricted to the 30 control participants older than 30 years of age; mean serum testosterone and estradiol were higher than in the RPL group, although neither comparison reached statistical significance (see Table 3 in the online Data Supplement). No significant differences in semen volume, sperm concentration, total motility, or progressive motility were observed (see Fig. 3 in the online Data Supplement). However, the RPL group had a significantly lower proportion of morphologically normal sperm than age-matched controls (% sperm SE with normal morphology: controls 30 years, 5.0 0.4; RPL, 3.0 0.3; *P* 0.001). Mean semen ROS levels were more than 4-fold higher in the RPL group than in age-matched controls (mean SE semen ROS in RLU/ sec/10^6: controls 30 years, 2.0 0.8; RPL, 9.1 4.1, *P* 0.05; see Fig. 4A in the online Data Supplement). Mean SE levels of sperm DNA fragmentation were more than 2-fold higher in the RPL group than in agematched controls (mean DFI: controls 30 years, 7.7 7.0; RPL, 16.4 1.5, *P* 0.0001; see Fig. 4C in the online Data Supplement). Furthermore, the RPL group were 3-fold more likely to have abnormally increased ROS or sperm DNA fragmentation than age-matched controls (see Fig. 4, B and D, in the online Data Supplement). ROC curve analyses suggested that total motility, morphology, ROS, and DNA fragmentation were all dis-

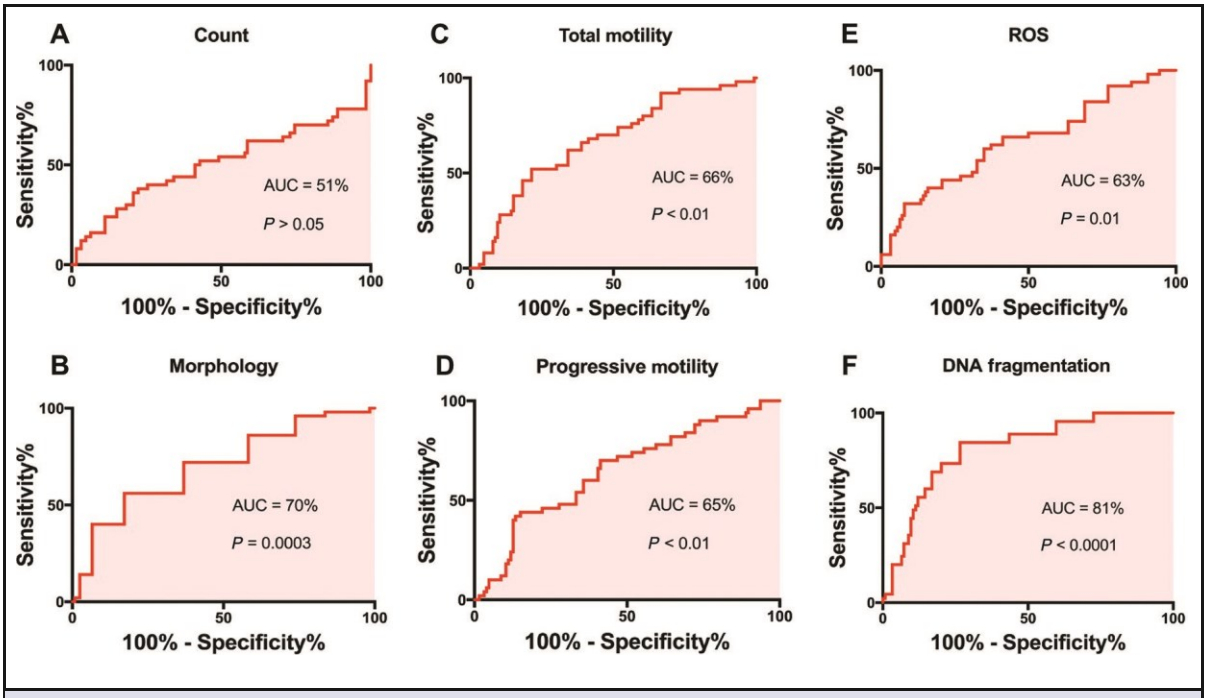

Fig. 3. Receiver operation characteristics of reproductive parameters in male partners of women with recurrent pregnancy loss.

ROC analyses for sperm count (A), normal sperm morphology (B), total sperm motility (C), progressive sperm motility (D), semen reactive oxygen species (E), and sperm DNA fragmentation (F). Area under curve (AUC) values are presented for each parameter.

criminatory between controls older than 30 years and the RPL group (see Fig. 5 in the online Data Supplement); the greatest discriminator was sperm DNA fragmentation, which had an ROC curve AUC value of 79% (*P*

0.0001 vs line of nondiscrimination).

Discussion

No underlying cause can be found in half of all couples with RPL *(19)*, and current guidelines do not recommend the routine diagnostic investigation of male partners. We have performed the first study evaluating reproductive endocrine and metabolic sperm function in male partners of women with RPL. We report that male partners of women with RPL have reduced concentrations of serum testosterone and estrogen when compared with controls, which warrant further investigation. We also report markedly increased levels of semen ROS and sperm DNA fragmentation and reduced functional sperm parameters when compared with control participants. Our data suggest that male partners may benefit from diagnostic assessment in the routine management of couples with RPL.

No previous study has investigated endocrine function in male partners of women with RPL. Intratesticular production of testosterone is critical for the final stages of spermatogenesis, and testosterone deficiency is associated with male infertility *(20)*. Testosterone and estrogen synthesis from testicular Leydig cells is driven by the pulsatile secretion of LH from the pituitary gland *(20)*. We observed that testosterone and estradiol were reduced by 15% and 16%, respectively, in the RPL group when compared with controls, although these differences became nonsignificant when excluding controls older

than 30 years. Primary hypogonadism (i.e., low testicular production of testosterone) tends to increase serum LH levels owing to reduced feedback. However, levels of serum LH were not increased in the RPL when compared with the control group, which might be consistent with a partial secondary hypogonadism due to hypothalamopituitary impairment. It would be important to confirm these data with more detailed endocrine and metabolic phenotyping in a large age-matched cohort.

Paternally imprinted genes play an important role in the regulation of placentation, which is critical to embryo viability *(21)*. This characteristic is illustrated by observing that mouse embryos from 2 paternal genomes (androgenotes) have deficient embryo formation but relatively preserved placental formation; conversely, mouse embryos from 2 maternal genomes (parthenogenotes) have deficient placental formation with relative sparing of embryo formation *(6)*. It is therefore clinically important to investigate whether novel diagnostic markers of sperm function may cause miscarriage. Furthermore, these studies may enable an improved understanding of how the paternal genome regulates placentation and embryo development. Multiple studies have reported that sperm DNA fragmentation is increased in male partners affected by RPL when compared with unaffected men *(7–12, 22, 23)*, although failure to demonstrate this relationship has also been reported *(24)*. Our results are in agreement with these previous studies by observing that men with RPL had a much higher risk of increased sperm DNA fragmentation than controls. The mechanisms underlying increased sperm DNA fragmentation and reduced sperm function in couples with RPL have been poorly understood. Previous studies have implicated oxidative stress as a major cause of sperm DNA fragmentation *(12, 25, 26)*. We used a previously described and validated chemiluminescent assay using luminol, which detects both intracellular- and extracellular-produced ROS, including superoxide, hydrogen peroxide, hydroxyl, and hypochlorite *(15)*. We observed that mean levels of ROS were 4-fold higher in men with RPL than in controls. Furthermore, one-third of men with RPL had increased ROS, whereas only 10% of controls had increased ROS. Mean levels of seminal leukocytes were similar in men with RPL and controls. However, some of the included participants may have had asymptomatic infection, which may occur in the absence of leukocytospermia. It would be interesting to investigate if the relatively high levels of semen ROS observed in men with RPL are sperm or leukocyte derived. Varicocele and genitourinary infection are 2 major known causes of semen ROS elevation; we did not observe any increased risk of varicocele or genitourinary infection in men with RPL when compared with controls, although our sample size was small. Interestingly, a randomized controlled trial by Ghanaie et al. observed that varicocele repair significantly improved pregnancy rates and reduced miscarriage risk when compared with nontreatment of varicocele in couples with recurrent miscarriage *(27)*. Furthermore, a retrospective analysis by Negri et al. has suggested that miscarriage rates were similar to the general population following varicocele repair for the male partners of couples with infertility *(28)*. In addition, Kanakas et al. performed a case–control study in couples undergoing IVF after the male partner had been tested for seminal *Ureaplasma urealyticum* infection; abortion rates following IVF were significantly higher in the infected group than in the noninfected group *(29)*. Future studies should investigate whether male partners affected by RPL are more likely to have varicocele and genitourinary infection than other men. It would also be interesting to

further investigate whether treatment of varicocele and genitourinary infection in male partners of women with RPL reduces the risk of future miscarriage. Several ongoing studies are investigating whether the administration of dietary or pharmaceutical antioxidants in men who had a complete clinical investigation, excluding infectious or surgical causes of increased ROS and DNA damage, could be used to improve clinical outcomes in couples with infertility *(30, 31)*. It is therefore possible that seminal ROS measurement has diagnostic and therapeutic potential for couples with RPL, which warrants further investigation.

We finally investigated the performance of the 3 most promising potential diagnostic factors to distinguish men with RPL from controls by using ROC analysis. All factors had significant diagnostic performance (i.e., ROC AUC significantly different from the line of nondiscrimination), although sperm DNA was the bestperforming test to distinguish men with RPL from controls. Large prospective cohort studies are required to further investigate if sperm indices influence the risk of miscarriage in couples.

It is important to consider limitations of the study. Since commencement of the current study, new guidelines have been released by the European Society of Human Reproduction and Embryology *(32)*, defining RPL as 2 rather than 3 consecutive miscarriages. It is therefore important to consider that accurate comparison with future studies may be limited by heterogeneity in the definition of RPL. Several methods of sperm DNA fragmentation measurement are available. We used the Halosperm method, which is as an index of abnormal chromatin packaging rather than a direct assessment of DNA damage itself *(33)*. It would be interesting to compare results from the current study using Halosperm with other methods such as SCSA, TUNEL, and COMET, which more directly measure sperm DNA damage and have higher reported sensitivities for detecting sperm DNA fragmentation *(22, 34)*. It is important to consider that mean concentrations of serum testosterone and estradiol in the RPL group were within the reference interval for men. Furthermore, levels of sperm DNA fragmentation and semen ROS associated with male infertility are usually much higher than the mean levels reported in the RPL group of the current study *(35, 36)*. Further studies are required to determine if the observed abnormalities of endocrine and sperm function in RPL translate to pathogenic changes leading to pregnancy loss. Finally, we chose not to stipulate fatherhood as an inclusion criterion within our control group; it is therefore plausible that a small minority of our controls might later experience reproductive disorders. Selecting fathers as controls may have increased the power of the current study. However, one could argue that our choice of control group with unproven fertility increases the robustness of our conclusions, by revealing genuine abnormalities in the reproductive physiology of men affected by RPL when compared with the general male population, rather than just fathers *(37)*.

In summary, DNA fragmentation and ROS are recently identified markers of male reproductive dysfunction *(38, 27)*. We report that male partners of women with RPL have multiple abnormalities in reproductive function including testicular steroidogenesis, sperm function, sperm DNA damage, and semen oxidative stress. Our data have important implications for the management of couples with RPL. Endocrine and molecular sperm profiling may offer a potential novel approach to stratifying future miscarriage risk. Further studies will investigate whether endocrine and molecular sperm abnormalities may be

ameliorated by lifestyle, dietary interventions, and hormonal interventions, to optimize chances of successful conception in couples with RPL.

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