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Thesis submitted in the fulfilment of the requirements for the admission to  
the Degree of Doctor of Philosophy in Medicine

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**The Effect of Dietary Micronutrient Supplementation  
on the Reproductive Health of Obese Males**

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## Thesis Abstract

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According to the Australian Institute of Health and Welfare, 2 in 3 Australian men are overweight or obese. Sperm oxidative stress is a common factor in various forms of male infertility including obesity, and can lead to increased sperm intracellular reactive oxygen species (ROS) and oxidative DNA lesions (8-OHdG), impaired sperm function, reduced implantation and live birth rates and the transgenerational inheritance of disease for up to two generations. According to Cochrane Reviews, the use of dietary micronutrient and antioxidant supplementation has been shown efficient in improving live birth rates in infertile men despite recent reports suggesting the evidence is of low quality. There is limited research however, regarding the effectiveness of dietary micronutrient supplements in the growing population of obese males. Therefore, the aim of this thesis was to establish the impact of a combination of dietary micronutrient supplements on male reproductive health outcomes in obese men and diet-induced obese mice.

In the mouse model, male mice were fed a control diet (CD, 6% fat) or a high fat diet (HFD, 21% fat) for 10 weeks to induce obesity and then assigned to one of three dietary micronutrient supplement (selenium, vitamin C, lycopene, vitamin E, zinc, folic acid green tea extract) treatment durations: (i) 10 weeks; (ii) 5 weeks or (iii) 10 days in conjunction with their initially allocated control or HFD. In all three treatment durations, sperm morphology, intracellular ROS, 8-OHdG lesions, sperm-oocyte binding and fertilization rates were increased with micronutrient supplements in HFD-fed males compared to the un-supplemented HFD-fed mice. HFD-induced obese males treated with micronutrient supplementation for 10 days were mated with normal weight females and implantation rates were increased in addition to increased fetal weights. Also, immunofluorescence showed that 8-OHdG lesions were reduced in developing testicular



spermatogenic cells and the paternal pronucleus of the subsequent embryo in males fed a HFD supplemented with micronutrients.

In the retrospective and prospective studies, fertility treatment outcomes and sperm oxidative stress were assessed in non-smoking couples attending an infertility clinic whereby the male partner was aged <45 years old, of normal weight, overweight or obese BMI, and the female partner was non-obese and aged <38 years old. Couples were stratified by the male partner's BMI and whether they were consuming micronutrient supplements (Menevit or multivitamins) at the time of treatment. Retrospective data showed that fertilization rates and on-time embryo development were increased with micronutrient supplement intake in obese men compared to obese men not consuming dietary supplements. Also, implantation and live birth rates were improved with micronutrient supplementation in all men regardless of their BMI. The prospective data showed similar results in addition to reduced sperm intracellular ROS in obese men and reduced sperm 8-OHdG lesions in all men despite BMI with micronutrient supplement intake.

Overall, these findings demonstrate that micronutrient supplementation can increase implantation and live birth rates in obese males and lessen the inheritance of oxidative lesions in the male germline highlighting the importance of paternal preconception health. These studies warrant further research into potential molecular mechanisms in sperm, the testis and epididymis.

## **Declaration**

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I certify that this work contains no material which has been accepted for the award of any other degree or diploma in my name, in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. In addition, I certify that no part of this work will, in the future, be used in a submission in my name, for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint-award of this degree.

I give permission for the digital version of my thesis to be made available on the web, via the University's digital research repository, the Library Search and also through web search engines, unless permission has been granted by the University to restrict access for a period of time.

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## **Abstracts arising from this thesis**

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**2015** Australian Society of Medical Research (ASMR) SA Annual Scientific Meeting

*The impact of micronutrient supplements on sperm quality and function in obese males*

### **Best Oral Presentation in the Field of Reproductive Health**

**2015** South Australian Men's Health Research Symposium, Adelaide

*The impact of antioxidant and micronutrient supplementation on sperm quality and function in obese males*

**2015** Society for Reproductive Biology (SRB) Annual Scientific Meeting, Adelaide

*The impact of antioxidant and micronutrient supplementation on sperm quality and function in obese males*

**2015** Robinson Research Institute Symposium, Adelaide

*Dietary micronutrient supplementation of a high fat diet reduces sperm oxidative stress and improves fertilisation rates in the mouse*

### **Best Oral Presentation Prize**

**2016** Scientists in Reproductive Technology Annual Meeting, Adelaide

*The impact of antioxidant supplementation in sperm quality and sperm function in overweight and obese men*

**Early Career Research Award**

**2017** Fertility Society of Australia- National Conference, Adelaide

*The effectiveness of micronutrient supplementation on sperm quality, embryo development and pregnancy outcomes in overweight and obese men*

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Chapter 1

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**Literature Review**

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## 1.1 Introduction

Obesity is defined by the World Health Organization (WHO) as a body mass index (BMI) of 30kg/m<sup>2</sup> or greater (WHO 2015). The prevalence of obesity in men has more than doubled in the past 20 years across the globe (WHO 2015). Reports from the Australian Institute of Health and Welfare state that obesity has overtaken smoking as the leading cause of premature death emphasizing that this common and often preventable disease is of public health importance (AIHW 2016). According to the WHO, infertility is the third most serious health condition after cancer and cardiovascular disease. Infertility is a complex and multifactorial condition that can involve both the male and female partner and is defined as a failure to achieve a clinical pregnancy after 12 or more months of regular unprotected sexual intercourse (WHO 2016). Infertility affects approximately 15% of couples globally with up to 50% of cases attributed to the male partner (Kefer *et al.* 2009). Interestingly, the increase in the prevalence of obesity and the rise in infertility are simultaneous (Swan *et al.* 2000).

Based on current literature, there is an undeniable association between male obesity and compromised reproductive health characterized by reduced pregnancy and live birth rates in addition to elevated likelihood of miscarriage in their partners (Campbell *et al.* 2015). This compromise in male reproductive health is mainly measured by conventional sperm parameters including sperm motility, concentration and morphology. However, there is increasing evidence that conventional sperm parameters are only modestly affected by obesity (Campbell *et al.* 2015) and the larger impact of obesity is on an intracellular level in the form of sperm oxidative stress and oxidative DNA lesions (Bisht *et al.* 2017). Sperm cells are particularly susceptible to oxidative stress, which is characterized by the

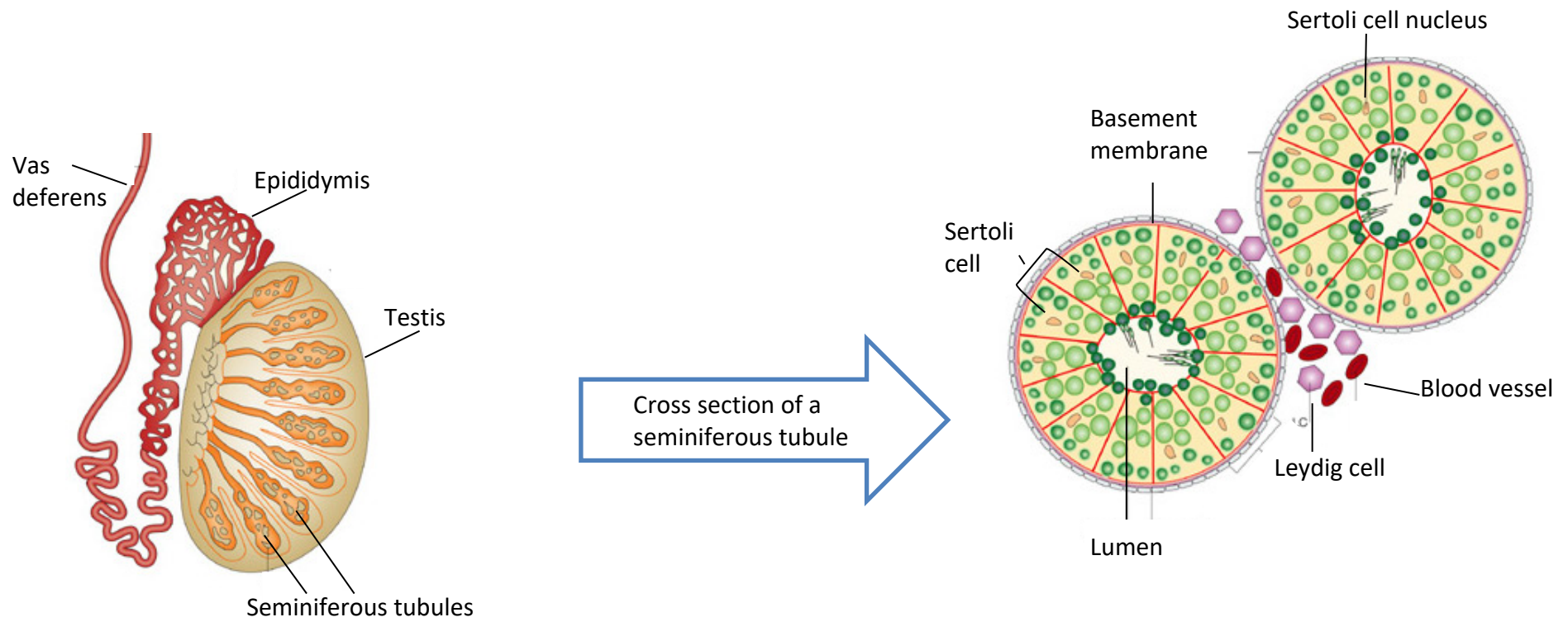
accumulation of reactive oxygen species (ROS) and/or the loss of antioxidant capacity. This susceptibility is due to shedding of the cytoplasm and thus subsequent loss of antioxidant enzymes as part of their maturation process during sperm cell generation and development (Gharagozloo and Aitken 2011).

Sperm oxidative stress is a common mediator for defective male reproductive potential (Aitken *et al.* 2014) with some major consequences including impaired DNA integrity and lipid peroxidation of the sperm plasma membrane. These factors collectively limit the fertilizing potential of sperm cells and if fertilization does occur, contribute to the transmission of impaired paternal DNA integrity to the embryo (Soubry *et al.* 2014) and increases the susceptibility of disease to potential offspring (Aitken *et al.* 2014; Lane *et al.* 2014). Micronutrient and antioxidant supplementation in an infertile population has been proposed as an effective treatment to ameliorate sperm oxidative stress and DNA damage, which has been demonstrated to increase pregnancy and live birth rates (Gharagozloo and Aitken 2011; Showell *et al.* 2014). However, the effectiveness of micronutrient and antioxidant supplementation treatment on the reproductive health of obese males is unknown (Barratt *et al.* 2017). Further, the optimal duration of supplement intake remains to be elucidated, as well as whether supplementation affects germ cell development during spermatogenesis and/or sperm maturation during epididymal transit. This chapter will review the implications of obesity and sperm oxidative stress on reproductive health and how micronutrient supplementation may protect against the detrimental consequences of obesity-induced sperm oxidative stress.

## **1.2 Obesity and Male Infertility**

### **1.2.1. Spermatogenesis**

Spermatogenesis is a complex process that occurs in the testis and epididymis by which diploid cells become haploid through meiosis that occurs concomitantly with major changes in both the structure and function of the sperm cell, ultimately generating mature haploid sperm (Guerriero *et al.* 2014). Spermatogenesis is comprised of a chronological series of events initiating in the seminiferous tubules of the testis and concluding with sperm maturation in the epididymis (Figure 1.1) and is regulated by several endocrine factors (Figure 1.2). The first phase of spermatogenesis occurs in the testis. The testis is composed of lobules that contain several seminiferous tubules. Seminiferous tubules consist of germ cells, Leydig cells, and Sertoli cells (Figure 1.1). Sertoli cells surround germ cells and their main functions include the nutrition of the developing spermatogenic germ cells, phagocytosis of spermatid remains, and the formation of the blood-testis barrier (BTB) (Griswold 1998). Leydig cells are located between the seminiferous tubules and their primary function is testosterone production. The epididymis, the site of the final phase of sperm maturation, extends from the upper to the caudal end of the testis (Figure 1.1) and is composed of the coiled epididymal duct lined by an epithelium with cilia and microvilli. The main functions of the epididymis include sperm maturation, where fertilization capacity is bestowed on the sperm, transport, protection, and storage.



**Figure 1.1:** Illustration depicting the location of the seminiferous tubules within the testis. The cross-section of the seminiferous tubules shows the structure of the tubule and the location of the developing sperm cells (green), Sertoli cells (yellow) and Leydig cells (pink).

Figure adapted from Cooke et al., *Nature Reviews Genetics* 2002; 3: 790-801.

### **1.2.2. Hormonal regulation of spermatogenesis disrupted with obesity**

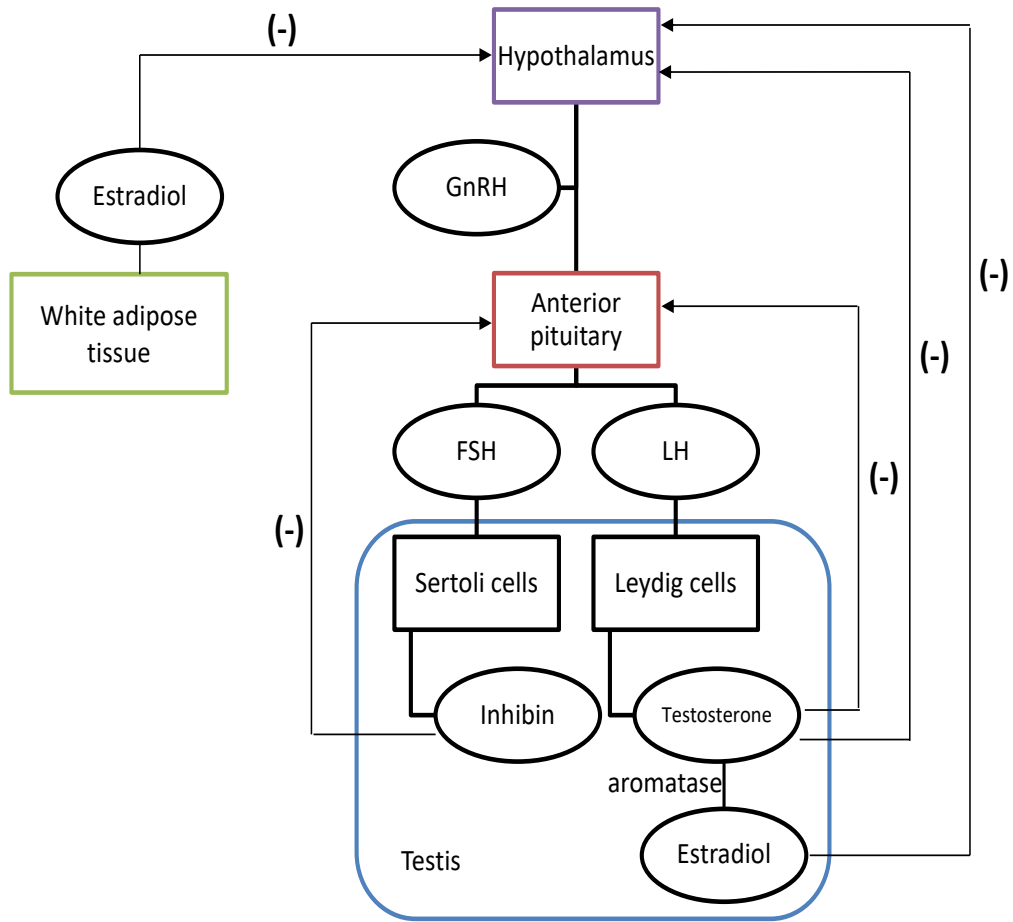
The production of mature sperm from primordial germ cells is regulated by hormones from the hypothalamus, pituitary gland, and locally from the testes. The hypothalamus secretes gonadotropin-releasing hormone (GnRH), which acts on the anterior pituitary gland to stimulate the release of follicle-stimulating hormone (FSH) and luteinizing hormone (LH), which act on Sertoli cells and Leydig cells, respectively (Figure 1.2). In response to FSH, Sertoli cells release the hormone inhibin, which acts on the anterior pituitary gland to inhibit the production of FSH generating a negative feedback loop (Figure 1.2) (Griswold 1998). Leydig cells located in the interstitial tissue of the testes are endocrine cells that produce testosterone and release it locally into the testis and also into blood (Mendis-Handagama 1997). Testosterone acts on Sertoli cells to regulate sperm differentiation. Testosterone concentrations in the body are relatively consistent due to a negative feedback loop that acts on the hypothalamus and pituitary gland inhibiting LH production and consequently, inhibiting the further release of testosterone (Figure 1.2) (Mendis-Handagama 1997). Serum testosterone is frequently measured to assess the fertility status of men as testosterone is essential for sperm production and maturation (Walker 2011), albeit that serum testosterone does not always reflect intratesticular testosterone levels limiting its clinical utility (Coviello *et al.* 2004).

Hormonal and endocrine changes with male obesity can dysregulate the hypothalamic-pituitary-gonadal (HPG) axis (Figure 1.2) and subsequently perturb the hormonal regulation of spermatogenesis. The most common and well-established hormonal changes with obesity are reduced concentrations of testosterone and sex hormone binding globulin (SHBG) and elevated concentrations of estradiol (Aggerholm *et al.* 2008; Chavarro *et al.* 2010; Jensen *et al.* 2004; Macdonald *et al.* 2013; Pauli *et al.* 2008; Tunc



*et al.* 2011). Although the specific mechanisms contributing to the impact of obesity on alterations to the concentrations of these hormones are still unclear, some explanations include hypogonadotropic hypogonadism, a reduction in SHBG production and elevated adipocyte aromatase activity resulting in an increase in the conversion of testosterone to estradiol leading to altered hypothalamic and pituitary feedback (Chambers and Richard 2015; Pasquali *et al.* 2007). Given that obesity is often a result of a long-term imbalance between caloric input and inadequate energy expenditure, each component of the HPG axis may be modified by altering the pulsatile secretion of GnRH and subsequently perturbing further levels of the HPG axis resulting in reduced serum SHBG and testosterone concentrations (Hill *et al.* 2008). Further, the increase in estradiol and reduction in testosterone and SHBG concentrations may be a result of the increased aromatization of testosterone (Mammi *et al.* 2012), and the increased conversion of androgens into estrogens due to increased adiposity and high bioavailability of these aromatase enzymes (Roth *et al.* 2008).

Overall, changes to the male reproductive hormone profile and endocrine disruptions that result from an increased BMI and increased adiposity may ultimately impact upon the process of spermatogenesis, acting via testosterone, thus compromising sperm quality and male fertility.



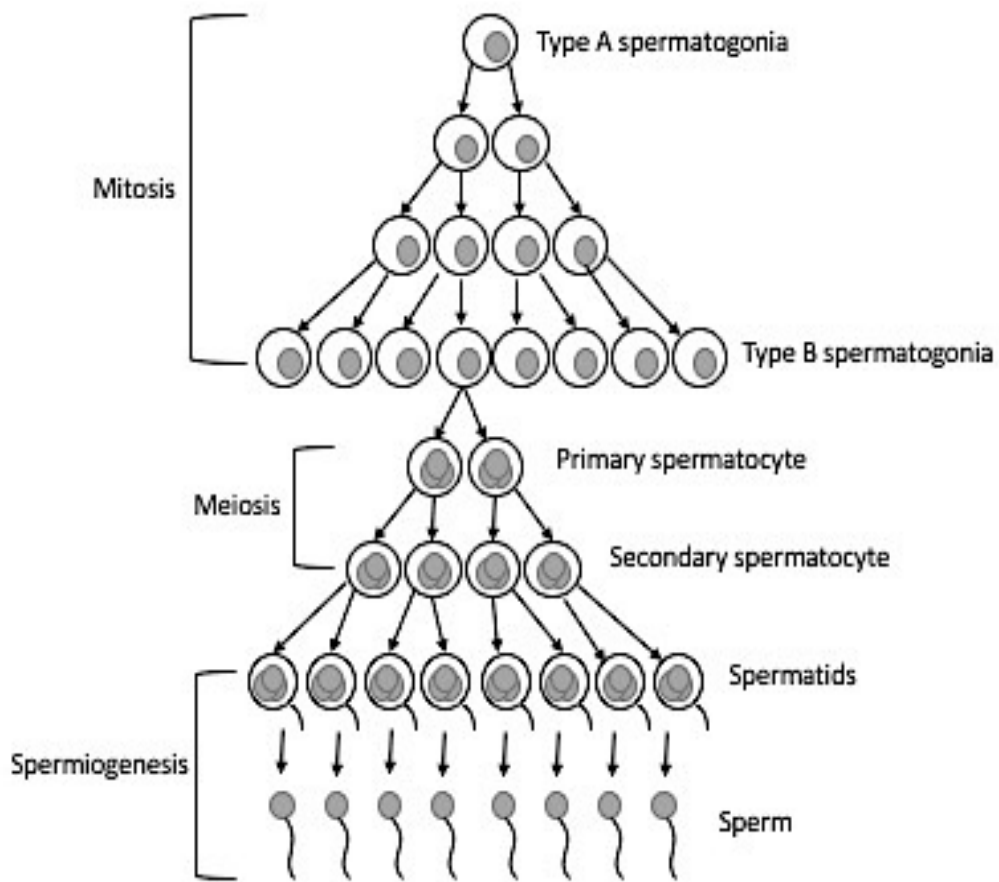
**Figure 1.2:** The Hypothalamic-Pituitary-Gonadal (HPG) Axis: hormonal regulation of spermatogenesis and the impact of obesity on male reproductive hormones. Spermatogenesis is regulated by gonadotropin-releasing hormone (GnRH), follicle-stimulating hormone (FSH), luteinizing hormone (LH) and by testosterone, estradiol and inhibin produced in the testis. In obesity, the increased concentrations of estradiol from white adipose tissue and from the aromatization of testosterone alter the normal regulatory mechanism of the HPG axis. The (-) icon represents negative feedback.

### **1.2.3. Stages of spermatogenesis perturbed with obesity**

In mice, one spermatogenic cycle occurs over approximately 34.5 days (Oakberg 1957) whereas in humans, one cycle occurs over approximately 74 days (Clermont 1972; Heller and Clermont 1963). Spermatogenesis is comprised of three main stages: mitosis, meiosis, and spermiogenesis. Firstly, the mitosis stage allows the proliferation of type A spermatogonia (renewing stem cells) into type B spermatogonia (differentiating progenitors) forming two daughter cells with the same DNA and chromosomal content as the original cell (Figure 1.3) (Clermont 1966). Secondly, meiosis occurs in type B spermatogonia, which progress into primary and secondary spermatocytes and then into early round spermatids, which generates four haploid gametes (Figure 1.3) (Sharpe 2010). Finally, spermatogenesis ends with spermiogenesis, a crucial stage involving vital changes to the structure and function of spermatids. During spermiogenesis, spermatids differentiate into sperm cells, whereby early round spermatids are converted to elongated spermatids with the shedding of excess cytoplasm and eventually form spermatozoa (Figure 1.3) (O'Donnell *et al.* 2011). Peritubular myoid cells, contractile cells that surround seminiferous tubules, expel sperm from the seminiferous tubule into the epididymis (O'Donnell *et al.* 2011).

Obesity, inflammation and elevated scrotal temperature can disturb the process of spermatogenesis. Sperm cells cannot mature appropriately at core body temperature and therefore optimal scrotal temperature is 1-3 degrees lower than internal body temperature (Paul *et al.* 2009; Shiraishi *et al.* 2010). Given that the processes of spermatogenesis in the testis and sperm maturation during epididymal transit are highly susceptible to structural and functional damage, it is vital that spermatogenic and mature sperm cells

are exposed to optimal temperatures (Aitken and Baker 2002; Aitken and Curry 2011b; Alvarez and Storey 1995).



**Figure 1.3:** Stages of spermatogenesis. The process of spermatogenesis is divided into three main stages; mitosis, meiosis and spermiogenesis. Briefly, type A spermatogonia proliferate into type B spermatogonia during the mitosis stage. Type B spermatogonia advance into primary and secondary spermatocytes and through meiotic divisions into early round spermatids generating four haploid gametes. In the final stage, spermiogenesis, early round spermatids are converted to elongated spermatids, which eventually form spermatozoa.

#### **1.2.4. Spermogenesis compromised by obesity**

Spermogenesis is the final stage of spermatogenesis and primarily occurs in the testes and this stage of development has been hypothesized to be one of the sites of perturbation of obesity. Spermogenesis is mainly comprised of four phases including nuclear condensation, acrosome formation, flagellum formation, and cytoplasmic reduction, and the impact of obesity for each stage is addressed below.

##### **1.2.4.1. Nuclear condensation**

Nuclear condensation is a key process of spermogenesis in which the sperm nucleus is condensed and compacted. The nuclear condensation process involves the gradual removal of the chromatin's original histone-based structure and substitution with smaller protamines (Braun 2001; Dadoune 1995; Miller *et al.* 2010; Rathke *et al.* 2013). The exact processes involved in chromatin re-organization and protamine substitution is not yet completely understood but it has been proposed that the compact protamine-based chromatin structure may be necessary for the protection of the paternal genome from chemical and physical damage (Braun 2001; Carrell *et al.* 2007). Although the majority of sperm chromatin is condensed and protaminated during spermogenesis, approximately 1% of the mouse genome and 10% of the human genome remain as histone-bound DNA (Miller *et al.* 2010). It is proposed that the retention of these histones is not random; these histone-bound regions of the paternal genome are associated with genes necessary for embryo development (Hammoud *et al.* 2011), are enriched in genes essential for DNA replication events post-fertilization and paternal genome transcription activation in the onset of embryo development (Shaman *et al.* 2007). Alterations in the numbers and placement of these histones in sperm has been associated with altered gene expression in the 2-cell embryo (Ihara 2014). Moreover, any alterations made to the

histone-bound regions of the paternal genome may be inherited by the embryo as these histone-bound regions are not replaced by the oocyte following fertilization and therefore would remain in the developing embryo (Ward 2010). In obese men, the nuclear condensation process during spermatogenesis is compromised and results in exposed and decondensed chromatin (Hammoud *et al.* 2011). It is therefore suggested that obesity compromises sperm DNA integrity from early germ cell development stages.

#### **1.2.4.2. Acrosome formation**

Acrosome formation is the formation of a cap (acrosome) containing enzymes that allow sperm penetration through the zona pellucida of the oocyte at fertilization. The acrosome is derived from proteins from the Golgi apparatus that create vesicles that then merge into a larger formation, which finally inverts itself, similar to a cap, over the largest part of the nucleus (Figure 1.4) (Wassarman 1999; Yasuno *et al.* 2013). After ejaculation, sperm undergo capacitation, an activation process, in the female tract to penetrate the zona pellucida of the oocyte and fuse with the oocyte plasma membrane to ultimately achieve successful fertilization. Although the mechanisms are unknown, sperm from high-fat diet (HFD)-induced obese male mice have shown to have reduced acrosome reaction and consequent reduced fertilizing potential (Bakos *et al.* 2010; Fan *et al.* 2015; Palmer *et al.* 2012b). These findings highlight the deleterious effects of obesity on sperm cell acrosomal function for successful acrosome reaction and subsequent fertilization.

#### **1.2.4.3. Flagellum formation**

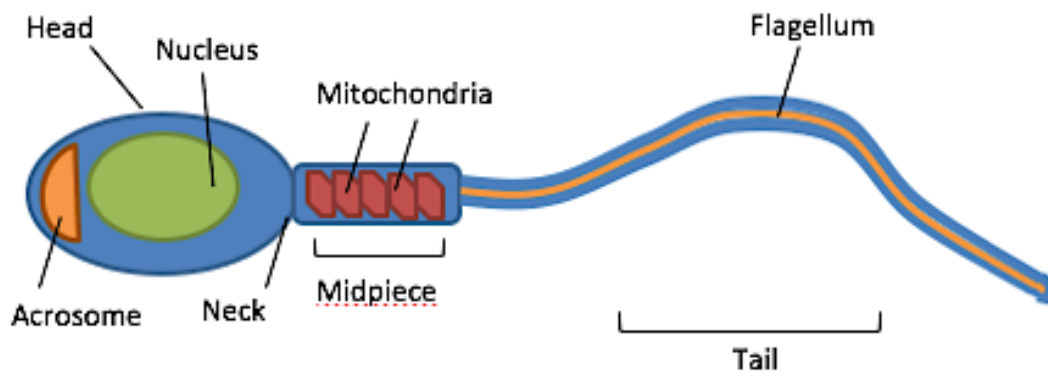
Flagellum formation is the generation of the sperm tail, whereby one centriole is assembled consisting of a bundle of nine peripheral double microtubules and two single microtubules in the centre (Carvalho-Santos *et al.* 2012). The rotation of the nucleus and

acrosomal vesicle controls flagellum development until the flagellum lies on the opposite side of the acrosome (Figure 1.4) (Wassarman 1999). Normal flagellum formation and morphology is required for progressive sperm motility, which is reduced with obesity in men and in diet-induced obese male rodent models (Hammoud *et al.* 2008; Palmer *et al.* 2012a). Also, obesity is associated with abnormal sperm tail morphology in mice (Ghosh and Mukherjee 2018) suggesting that obesity and its co-morbidities may affect the spermiogenesis stage, in particular morphogenesis of the sperm tail before it is released into the lumen of the seminiferous tubule for epididymal transit.

#### **1.2.4.4.        *Cytoplasmic reduction***

Cytoplasmic reduction is the removal of excess cytoplasm and organelles from the spermatids by Sertoli cell phagocytosis (Ho 2010). Cytoplasmic reduction is vital for the structural conversion of a spermatid into a functioning sperm cell. Interruptions to spermiogenesis and to cytoplasmic extrusion/ reduction via Sertoli cell phagocytosis can lead to excess residual cytoplasm in sperm cells and subsequently increased ROS production (Rengan *et al.* 2012; Sprando and Russell 1987). Obesity is often associated with increases in sperm intracellular ROS, which may be due to the limited availability of antioxidants and disruption of spermiogenesis by obesity-induced systemic metabolic changes and/or adiposity-induced elevated scrotal heat and temperature (McPherson and Lane 2015).





**Figure 1.4:** The structure of a mature sperm cell. The head contains a condensed protamine based nucleus and is capped anteriorly with the acrosome. Mitochondria are located in the sperm midpiece and the flagellum is composed of microtubules.

### **1.2.5. Epididymal maturation and transit altered with obesity**

Following the release of sperm into the lumen of the epididymis (to undergo the final stages of maturation), sperm are immobile and do not have fertilizing capacity until the completion of the maturation process that occurs during epididymal transit. During sperm epididymal transit, sperm acquire progressive motility (Bedford *et al.* 1973; Soler *et al.* 1994) and develop the capacity to undergo acrosome reaction, to ultimately bind and fuse with the zona pellucida of the oocyte (Lakoski *et al.* 1988). Sperm enter the epididymis via the testicular fluid and are transported throughout the epididymis by the muscular contractions lining the epididymis tubule. The minimal time required for sperm to mature and to transit through the epididymis is approximately 10 days regardless of the animal species (Robaire *et al.* 2006). Sperm are then stored in the epididymis until ejaculation and therefore; environmental factors such as elevated heat or inflammation, which can cause ROS, may impact sperm quality and function during their maturation period. Obese men commonly have fat deposits in the suprapubic and inner thigh areas which can cause both scrotal inflammation, including epididymitis, as well as increasing temperature ultimately altering the epididymal environment, epididymal function and sperm maturation likely contributing the observed elevated ROS (Katib 2015).

### **1.2.6. Blood-testis and blood-epididymal barrier atrophy with obesity**

Sertoli cells of the seminiferous tubule are bound to each other by tight junctions, gap junctions, adhesion junctions, and other junctional complex proteins to form the BTB (Jiang *et al.* 2014; Mruk and Cheng 2015). The BTB creates a specialized and immunologically privileged micro-environment that isolates developing germ cells from the cardiovascular and lymphatic systems (Mruk and Cheng 2015). The cellular junctions allow the entry of nutrients, hormones, and electrolytes into the seminiferous tubule but

block the entry of toxicants (Jiang *et al.* 2014). It is crucial that the cellular junctions of the BTB are fully functional and its integrity is not compromised as disturbances to the BTB caused by heat or toxins, result in reduced sperm count (Fan *et al.* 2015). Testicular structure and integrity have been shown to be compromised with obesity in rodent models. Normal testicular structure is illustrated by abundant seminiferous tubules with large diameters and intact basement membranes with normal spermatogenic cells. HFD-induced obese male mice had disorganized and atrophic seminiferous tubules with a disruption in cell adhesion between Sertoli cells and spermatogenic cells ultimately indicating impaired cell junctions and compromised BTB integrity (Fan *et al.* 2015). In HFD-fed rats, there was a reduced number of spermatogonia, Sertoli cells, and Leydig cells in testicular tissue in addition to small atrophic and distorted seminiferous tubules with a loss of testicular membrane integrity (Yan *et al.* 2015). The blood barrier extends past the testis and to the epididymis where tight junctions between epididymal epithelial cells form the blood-epididymis barrier (BEB). The BEB restricts the entry of certain ions, solutes, and macromolecules across the epididymal epithelium and into the lumen so that an appropriate environment surrounds sperm as they travel through the different segments of the epididymis (Turner 1991). The integrity of the BEB, has been shown to be compromised under stress conditions such as aging (Levy and Robaire 1999). Although the effects of obesity on the BEB integrity have not been investigated, the stressors associated with obesity may be similar to that found with aging. Inflammatory-associated pathologies such as interstitial inflammation of the epididymis have been shown to disrupt the BEB on both a structural and functional level (Gregory and Cyr 2014), which may be similar to obesity as obesity has been described as a constant state of inflammation (Monteiro and Azevedo 2010).

### **1.2.7. Obesity and conventional sperm parameters**

Clinical human studies have examined the effects of male BMI on conventional sperm parameters, including sperm concentration, motility and morphology, with conflicting findings (Eisenberg *et al.* 2014). The lack of consensus may be due to the age of the men, as advanced male age is also known to reduce sperm quality (Johnson *et al.* 2015), or due to uncontrolled confounding lifestyle factors such as diet, cigarette and marijuana smoking, regular alcohol consumption, and drug abuse, which also independently alter sperm quality (MacDonald *et al.* 2010). Additionally, the metabolic status of the male including leptin insensitivity, insulin resistance, glucose intolerance or diabetes may contribute to the conflicting data (Teerds *et al.* 2011). Moreover, the study design, sample size, patient recruitment, and methodology also differ between these studies and may contribute to the discrepancies (MacDonald *et al.* 2010). A systematic review of 5 studies, including a total of 6,800 men, reported no association between BMI and sperm concentration and sperm motility (MacDonald *et al.* 2010). However, an updated meta-analysis of 30 studies and a total of 115,158 men indicated a negative relationship between BMI and normal sperm morphology and motility despite the heterogeneity of the male participant population including general and subfertile populations (Campbell *et al.* 2015).

To avoid lifestyle factors that can confound human studies, controlled rodent models have been developed to investigate the effects of diet-induced obesity on conventional sperm parameters. In these studies, male obesity is associated with a reduced percentage of motile sperm, reduced sperm concentration, and increased abnormal sperm morphology (Bakos *et al.* 2010; Fullston *et al.* 2012; Ghanayem *et al.* 2010; Ibrahim *et al.* 2012; Palmer *et al.* 2012b; Palmer *et al.* 2011). However, these compromised

conventional sperm parameters with obesity vary to different degrees across studies unlike sperm oxidative stress, which is fairly consistent across the literature.

#### **1.2.8. Obesity and sperm oxidative stress**

Some recent studies suggest that underlying sperm measures beyond conventional sperm parameters including sperm oxidative stress markers: intracellular ROS and oxidative DNA lesions, and molecular changes to sperm DNA are changed with obesity which is significant as it may be passed to the embryo and affect resultant offspring (McPherson *et al.* 2016; Stuppia *et al.* 2015). Although the literature reports conflicting observations regarding the relationship between obesity and conventional sperm parameters, there is a consensus that obesity is associated with increased sperm oxidative stress. A positive relationship between BMI and sperm oxidative stress has been reported whereby sperm ROS production, measured using a photometric nitro blue tetrazolium (NBT) assay was increased in men with a BMI > 28 kg/m<sup>2</sup> (overweight and obese men) in addition to reduced clinical pregnancy rates (Qian *et al.* 1986; Yang *et al.* 2016). Obesity has been described as a chronic state of inflammation and systemic oxidative stress (Furukawa *et al.* 2004; Ozata *et al.* 2002) resulting from the production of pro-inflammatory cytokines from increased adipose tissue mass, which stimulate leukocyte generation of ROS ultimately inducing oxidative stress (Das *et al.* 2009), which is likely to extend to the testicular micro-environment (Tunc *et al.* 2011). Few studies have investigated the impact of metabolic syndrome, a common characteristic in obese men, on sperm parameters and seminal oxidative stress (Leisegang *et al.* 2014; Rosety *et al.* 2014) and only two studies have investigated the impact of obesity alone on seminal plasma (Tunc *et al.* 2011) and sperm oxidative stress (Yang *et al.* 2016). One study by Rosety *et al.* reports that men with metabolic syndrome have reduced sperm concentration, motility,

and normal morphology in addition to an increase in seminal malondialdehyde (MDA), a byproduct of lipid peroxidation compared with control healthy men (Rosety *et al.* 2014). Another study showed that obese and morbidly obese men with metabolic syndrome had reduced sperm concentration and motility compared to normal weight and overweight non-metabolic syndrome control group (Leisegang *et al.* 2014). However, smokers were included in this study, which may have led to confounding results given that there is a strong correlation between smoking and increased sperm oxidative stress (Fraga *et al.* 1996; Kovac *et al.* 2015). Only two human studies have investigated the effect of obesity on seminal oxidative stress and showed a positive relationship between an elevated BMI and seminal oxidative stress markers (Taha *et al.* 2016; Tunc *et al.* 2011). One study showed that this was primarily due to an increase in seminal macrophage activation which was reportedly mediated by an increase in local genital tract infections, however, this study did not conduct any bacteriology testing to confirm this link (Tunc *et al.* 2011). Also, this study did not report essential patient demographics including age and lifestyle factors such as smoking, in addition to no specifications of the BMI group sample size except a total participant number of 81 patients (Tunc *et al.* 2011). Another study that reported the number of non-smoking participants in each BMI group with no difference in mean age among them demonstrated increased seminal ROS in overweight and obese men compared to normal weight men, ultimately highlighting a positive association between BMI and sperm oxidative stress (Taha *et al.* 2016).

Mouse models have shown a positive relationship between adiposity and sperm intracellular ROS levels demonstrated by increased mitochondrial ROS and total ROS production (Bakos *et al.* 2010; Fullston *et al.* 2015; Fullston *et al.* 2013; Palmer *et al.* 2012b). Further, sperm oxidative stress in HFD-induced obese mice is correlated with

compromised sperm quality, including increased DNA damage (Palmer *et al.* 2012b). In rats, HFD-induced obesity resulted in increased MDA and nitric oxide (NO) radical production in sperm (Chen *et al.* 2013). Also, this study showed reduced antioxidant enzyme activity of superoxide dismutase (SOD) and glutathione peroxidase (GPx) in sperm from rats fed a HFD, indicative of increased oxidative stress and sperm plasma membrane lipid peroxidation (Chen *et al.* 2013).

The link between increased sperm oxidative stress levels and obesity may be due to elevated systemic levels of lipids, including fatty acids and cholesterol, which affect the environment that sperm is exposed to during spermatogenesis and ultimately altering the content and function of sperm (Bakos *et al.* 2010; Palmer *et al.* 2012a). Also, increased serum concentrations of cytokines and ROS, common characteristics with obesity, may be causative factors for sperm oxidative stress as it has been suggested that serum cytokines and ROS may pass the BTB and/or the seminal fluid through seminal vesicles and into the testicular tissue where inflammatory cytokines and ROS may perturb sperm morphology, development, and DNA integrity (La Vignera *et al.* 2012).

#### **1.2.9. Obesity and sperm DNA damage**

The majority of studies that have assessed the effects of obesity on sperm DNA damage report an increase in DNA fragmentation and DNA lesions within sperm from obese men (Chavarro *et al.* 2010; Dupont *et al.* 2013; Fariello *et al.* 2012; Kort *et al.* 2006; La Vignera *et al.* 2012). DNA damage in sperm has been correlated with various pregnancy pathologies such as miscarriage (Kleinhaus *et al.* 2006), cancer in children (Ji *et al.* 1997; Lee *et al.* 2009) and neurological defects including autism, spontaneous schizophrenia, bipolar disease and epilepsy in children fathered by men with elevated DNA damage

(Frans *et al.* 2008; Reichenberg *et al.* 2006; Sipos *et al.* 2004). A recent meta-analysis of the effect of male obesity on sperm DNA fragmentation further supports the positive relationship between BMI and DNA fragmentation (Campbell *et al.* 2015). Therefore, suggesting that elevated BMI in men could be linked with poorer health outcomes for their offspring.

The link between male obesity and impaired sperm DNA integrity has also been shown in mouse models of diet induced obesity with all studies confirming a positive relationship between adiposity and/or body mass and sperm DNA fragmentation (Duale *et al.* 2014; Fullston *et al.* 2013; Mitchell *et al.* 2011b; Palmer *et al.* 2012b). In mice fed a HFD, sperm DNA fragmentation correlated positively with mitochondrial ROS and negatively with sperm motility, normal morphology and sperm-oocyte binding (Palmer *et al.* 2012b) with perturbations in germ cell gene expression (Duale *et al.* 2014).

Overall, these studies demonstrate that obesity leads not only to reduced sperm quality (conventional sperm parameters) but also to impaired DNA integrity, which may have lasting impacts on offspring health.

#### **1.2.10. Obesity, sperm function and fertilization**

In couples undergoing ART treatment, the relationship between male BMI and successful fertilization seems to depend on the fertilization method; standard insemination versus intracytoplasmic sperm injection (ICSI). The standard insemination method is conducted by placing cumulus-oocyte complexes (COCs) with sperm, whereas ICSI involves the injection of a single sperm directly into an oocyte, thereby by-passing sperm binding processes. Some clinical studies report no relationship between paternal BMI and successful fertilization however, these couples used the ICSI method to fertilize oocytes,



which may mask the sperm binding capacity of sperm (Bakos *et al.* 2011; Umul *et al.* 2015). Two studies have analyzed fertilization rates using the standard insemination method in 651 men and demonstrated that fertilization rates were lower among overweight and obese men compared to normal weight men (all men had a female partner with normal BMI) (Keltz *et al.* 2010; Yang *et al.* 2016). This difference in fertilization success based on the ART insemination method suggests a detrimental impact of obesity on the capacity of sperm to undergo capacitation, acrosome reaction, and sperm-oocyte binding as these events are by-passed using ICSI.

Animal models of obesity have investigated the fertilization process in more detail with the capacitation and acrosome reaction status of sperm examined. Capacitation is a process sperm must undergo for successful fertilization; and the proportion of capacitated sperm and acrosome-reacted sperm were reduced in HFD-fed male mice with reduced subsequent sperm-oocyte binding and fertilization rates (Bakos *et al.* 2010; Palmer *et al.* 2012b). These studies, both clinical and animal models, suggest that obesity impairs sperm capacitation, which in turn may contribute to the delay or interruption of the acrosome reaction and compromise subsequent sperm-oocyte fusion to ultimately perturb successful fertilization.

#### **1.2.11. Obesity and embryo development**

In couples undergoing ART treatment where the male partner is overweight or obese, most studies report no effect on cleavage stage embryo development assessed on day 3 post insemination (Bakos *et al.* 2011; Colaci *et al.* 2012; Schliep *et al.* 2015). However, there is one study that reports that high quality embryo morphology on day 3, defined as embryos with 6-8 cells with no or minimal fragmentation, was reduced with an increased

paternal BMI (Yang *et al.* 2016). The reduction in day 3 embryo quality with an increasing paternal BMI may be explained by the activation of the paternal genome which mainly occurs on day 3 (the 8-cell stage), which is generally accepted as there is limited transcription from the paternal genome at this stage and that embryo development to this stage is primarily influenced by maternal factors (Braude *et al.* 1988). However, after the 8-cell stage and embryonic genome activation is completed, embryo development is entirely dependent on embryonic transcripts. Analysis of embryo development after the 8-cell stage has shown a linear decrease in on-time and expanded blastocyst development on day 5 of embryo culture with an increasing paternal BMI (Bakos *et al.* 2011).

Mouse models of diet-induced paternal obesity are quite valuable as they enable a more detailed analysis of embryos which is limited in the human due to the observational nature and ethical restrictions of human studies. Delayed embryo development was reported from mice fed a HFD compared with mice fed a control diet (CD) at both the post-embryonic genome activation cleavage stages of development (Binder *et al.* 2012; McPherson *et al.* 2013; Mitchell *et al.* 2011b) and development to the morula and early blastocyst stages (McPherson *et al.* 2013; Mitchell *et al.* 2011b). Of the embryos that reached the blastocyst stage, inner cell mass (ICM) and trophoctoderm (TE) cell numbers were reduced in blastocysts produced from HFD-fed male mice (McPherson *et al.* 2013; Mitchell *et al.* 2011b). Further, the number of epiblast cells within the ICM (the cells which ultimately form the fetus) were reduced in blastocysts sired by HFD-induced obese mice (McPherson *et al.* 2013; McPherson *et al.* 2015). Interestingly, obese men also have an increase in early pregnancy loss and sac only implantations lacking a fetal pole, suggesting that a similar mechanism may also occur in humans (Bakos *et al.* 2011; Schliep *et al.* 2015; Umul *et al.* 2015).

### 1.2.12. Obesity and pregnancy outcome

Overweight and obese men with a female partner of normal BMI have an increased odds ratio for time to conceive compared to couples with normal weight men (Nguyen *et al.* 2007; Ramlau-Hansen *et al.* 2007). The majority of studies that have investigated the effect of male obesity on achieving a clinical pregnancy and live birth in couples undergoing infertility treatment report a reduced clinical pregnancy rate with an increasing male BMI (Bakos *et al.* 2011; Keltz *et al.* 2010; Umul *et al.* 2015) (Table 1.1) and reduced live birth rate for obese men compared with normal weight men (Bakos *et al.* 2011; Colaci *et al.* 2012; Petersen *et al.* 2013; Umul *et al.* 2015) (Table 1.1). This reduction in live birth rate was detected despite differences in the reporting of live birth including live birth per oocyte retrieval or live birth per embryo transfer.

In mouse models, it is possible to examine the fetuses generated from control and obese mice and by using blastocyst transfer into a pseudo-pregnant mother and control for any maternal gestational impacts on fetal growth. Blastocysts derived from *in vitro* fertilization with sperm from diet-induced obese mice had a reduced implantation rate following transfer (Binder *et al.* 2012). Also, fetal and placental weights were reduced from that sired by a HFD-fed male mice in addition to retarded limb morphology and decreased crown-rump length of the resultant fetuses (Binder *et al.* 2012). Embryos derived from *in vivo* fertilization by HFD-induced obese male mice also implanted at significantly lower rates compared with embryos produced by CD-fed males (McPherson *et al.* 2013; Mitchell *et al.* 2011b). Of the embryos that implanted successfully, significantly fewer went on to form a fetus with one study reporting reduced fetal weights, fetal lengths, and placental weights in those sired by HFD-induced obese mice

(McPherson *et al.* 2013) while another reported no difference (Mitchell *et al.* 2011b). The most likely explanation for this discrepancy between McPherson *et al.* and Mitchell *et al.* may be the differences in durations of diet exposure; 18 weeks versus 8 weeks, suggesting that duration of diet intake and subsequent increases in adiposity and metabolic syndrome may also affect pregnancy outcomes.

**Table 1.1:** A summary of literature investigating the effects of an elevated male BMI on pregnancy and live birth rates.

Study	Population of Participants	Total Number of Participants	Mean Age of Participants	Number of Participants within each BMI (kg/m <sup>2</sup> )	Pregnancy Outcomes with increasing BMI	Potential Confounders or Limitations
Keltz et al., 2010	Infertility patients	290	38	<24.9 n=62 ≥25 n=228	↓ clinical pregnancy rates	BMI was self-reported
Bakos et al., 2011	Infertility patients	305	36	<24.9; n=63 25-29.9; n=148 ≥30; n=94	↓ clinical pregnancy and live birth rates	Lifestyle factors were not reported e.g. smoking
Colaci et al., 2012	Infertility patients	114	37	<.9; n=38 25 -29.9 n=53; ≥30; n=23	↓ live birth rates	Smokers included
Petersen et al., 2013	Infertility patients	1,906	35	< 20; n=9 20-24.9; n=905 25-29.9 n=742; ≥30; n=250	↓ live birth rates (with IVF not ICSI)	Smokers included
Schliep et al., 2015	Infertility patients	735	34	<24.9; n=224 25 -29.9 n=334; ≥30; n=177	No effect	Lifestyle factors were not reported e.g. smoking
Umul et al., 2015	Infertility patients	155	34	<24.9; n=52 25 -29.9 n=75; ≥30; n=28	↓ clinical pregnancy and live birth rates	Lifestyle factors were not reported e.g. smoking

### **1.2.13. Paternal obesity and offspring health**

In recent years there has been an increasing number of reports linking paternal health, at conception and the programming of their offspring's health. Examples of paternal conditions that have been demonstrated to impact on offspring health include occupation exposures to solvents; pesticides; toxic fumes, advanced age, smoking, diet, as well as obesity (El-Helaly *et al.* 2011; Frans *et al.* 2008; Gianicolo *et al.* 2010; Johnson *et al.* 2015; Li *et al.* 2009).

In epidemiological studies, paternal obesity at conception is associated with alterations in fetal growth and disease in offspring in a sex specific manner including lower birth weight in babies (McCowan *et al.* 2011), increased body fat in pre-pubertal daughters (Figuroa-Colon *et al.* 2000) and early mortality from chronic diseases in sons (Pembrey 2010; Pembrey *et al.* 2006). Also, ultrasound measurements in late gestation including head circumference, abdominal diameter, abdominal circumference and pectoral diameter, and birth weight in male newborns are correlated positively with an increased paternal BMI (Chen *et al.* 2012). Infants from obese fathers had elevated growth curves from birth to 3.5 years old compared with children from normal weight fathers (Linabery *et al.* 2013). Paternal metabolic syndrome together with obesity has been shown to be associated with the development of polycystic ovary syndrome in their daughters (Leibel *et al.* 2006), identifying another example of paternal non-genetic transmission of disease to the next generation. Recently, the National Institutes of Health (NIH) found that children of obese fathers were 75% more likely to fail measures of personal-social functioning in a study that involved approximately 5000 children (Yeung *et al.* 2017). However, it should be noted that these measures were tested using a questionnaire completed by parents at home after performing a series of activities with their children

and therefore may be potentially biased (Yeung *et al.* 2017). Taken together, evidence is accumulating that an obese father transmits perturbed health cues to their children.

Animal models have enabled the rapid assessment of the impacts of male obesity on multiple generations including the assessment of invasive measures, which are not able to be performed in an investigative clinical setting. Female rat offspring sired by diet-induced obese and diabetic fathers developed early onset impaired insulin secretion, glucose intolerance, and altered expression of multiple genes related to normal pancreatic function (de Castro Barbosa *et al.* 2016; Ng *et al.* 2010). In a mouse model of paternal diet induced obesity in the absence of diabetes, perturbed metabolic health including glucose intolerance and insulin resistance were observed in two generations of both female and male offspring (Fullston *et al.* 2013). Moreover, the same model of paternal obesity at conception resulted in female offspring who produced oocytes with reduced meiotic competence and reduced mitochondrial membrane potential (Fullston *et al.* 2012). Further, male offspring produced sperm with reduced motility, increased sperm ROS, and increased DNA damage with many changes evident in the second generation (Fullston *et al.* 2012).

Overall, these findings suggest a link between paternal obesity at conception and delayed embryo development and impaired subsequent offspring health (de Castro Barbosa *et al.* 2016). Given that the prevalence of male obesity continues to rise with evidence that paternal health and BMI before conception influence the development and health of the fetus, infant and child, strategies to address and intervene are warranted to maintain optimal childhood health.

### **1.3 Sperm Oxidative Stress – A Proposed Mechanism**

ROS and reactive nitrogen species (RNS) are molecules that have one or more unpaired electrons and are able to modify molecules such as membrane lipids, proteins, and nucleic acids by transferring these unpaired electrons to other molecules causing oxidation. Examples of ROS and RNS include superoxide, hydrogen peroxide, hydroxyl, peroxy, nitric oxide and peroxynitrite. Oxidative stress is defined as an imbalance between ROS and/or RNS and antioxidant levels, and occurs when the concentration of ROS and/or RNS exceeds the concentration of antioxidants and overwhelms the antioxidant free radical scavenging protective systems (Agarwal *et al.* 2006), leading to loss of function and in some cases apoptosis (Agarwal *et al.* 2006).

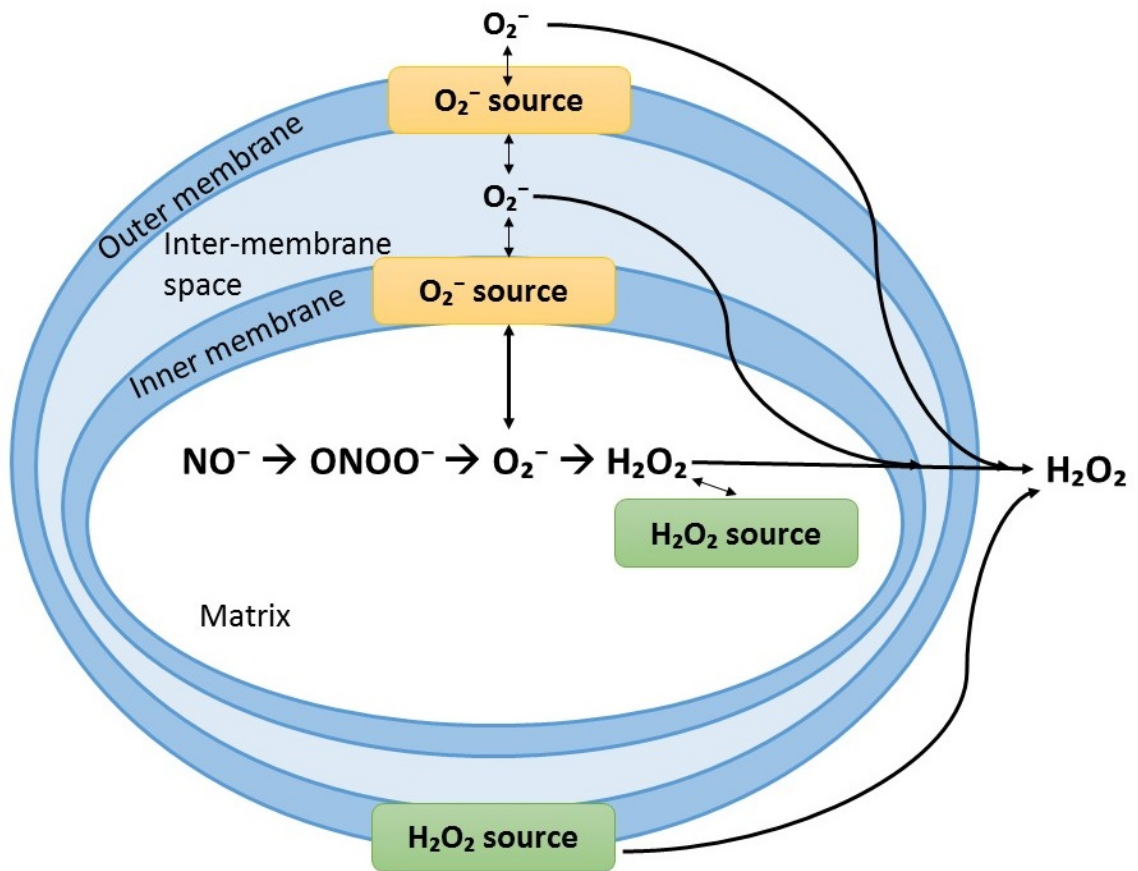
#### **1.3.1 Main sources of ROS**

##### ***1.3.1.1. Mitochondria***

ROS are a normal by-product of mitochondrial metabolism and are required for normal physiological sperm cell functions, such as sperm capacitation and acrosome reaction (Leclerc *et al.* 1997). In normal cell metabolism, ROS is predominately produced from mitochondrial Complex I whereas in sperm cells, Koppers *et al.* found that stimulation of Complex I and Complex III of the inner mitochondrial matrix increased superoxide formation (Koppers *et al.* 2008) (Figure 1.5). Superoxide is then rapidly dismutated to hydrogen peroxide via SOD antioxidant enzymes, and subsequently released into the cytoplasm of sperm which can result in peroxidative damage to the mid-piece of the sperm leading to a loss of motility as a consequence of membrane lipid peroxidation (Amaral *et al.* 2013; Koppers *et al.* 2008) (Figure 1.5). Further, aldehydes generated from



sperm membrane lipid peroxidation have been shown to disrupt the mitochondrial electron transport chain ultimately inducing more ROS generation in a self-perpetuating cycle (Aitken *et al.* 2012).



**Figure 1.5:** Mitochondria are a main source of ROS in sperm. Several complexes within the inner mitochondrial matrix results in superoxide formation, which is then dismutated to hydrogen peroxide and released into the cytoplasm of sperm.

### **1.3.1.2.        *Leukocytes and seminal oxidative stress***

Leukocytes, particularly neutrophils and macrophages, are considered a major source of the production of ROS found in semen. Infection and/or inflammation of the male organs contribute to oxidative stress as leukocytes are generated in response to infection and inflammation (Tremellen and Tunc 2010). Although the exact mechanism for how seminal leukocyte ROS production impacts sperm is unknown, it is postulated that hydrogen peroxide, produced by leukocytes, can penetrate the sperm plasma membrane and cause intracellular damage whereas other ROS such as superoxide and hydroxyl radicals cannot permeate the sperm plasma membrane or their short half-lives can be difficult to detect (Henkel *et al.* 2005). Hydrogen peroxide exposure can induce further intracellular ROS production via transition metal reactions such as Fenton type reaction or reaction with other ROS and can thus damage DNA integrity. This mechanism has been shown to be active *in vitro* as adding hydrogen peroxide to a sperm suspension resulted in increased DNA fragmentation (Ramos and Wetzels 2001). However, non-permeable leukocyte-derived ROS such as superoxide or hydroxyl radicals can damage sperm structure and function by oxidizing the lipids of the sperm plasma membrane resulting in lipid peroxidation (Henkel *et al.* 2005), thereby reducing sperm-oocyte fusion and fertilization capacity (Lackner *et al.* 2010; Sharma *et al.* 2001).

### **1.3.1.3.        *Excess residual cytoplasm***

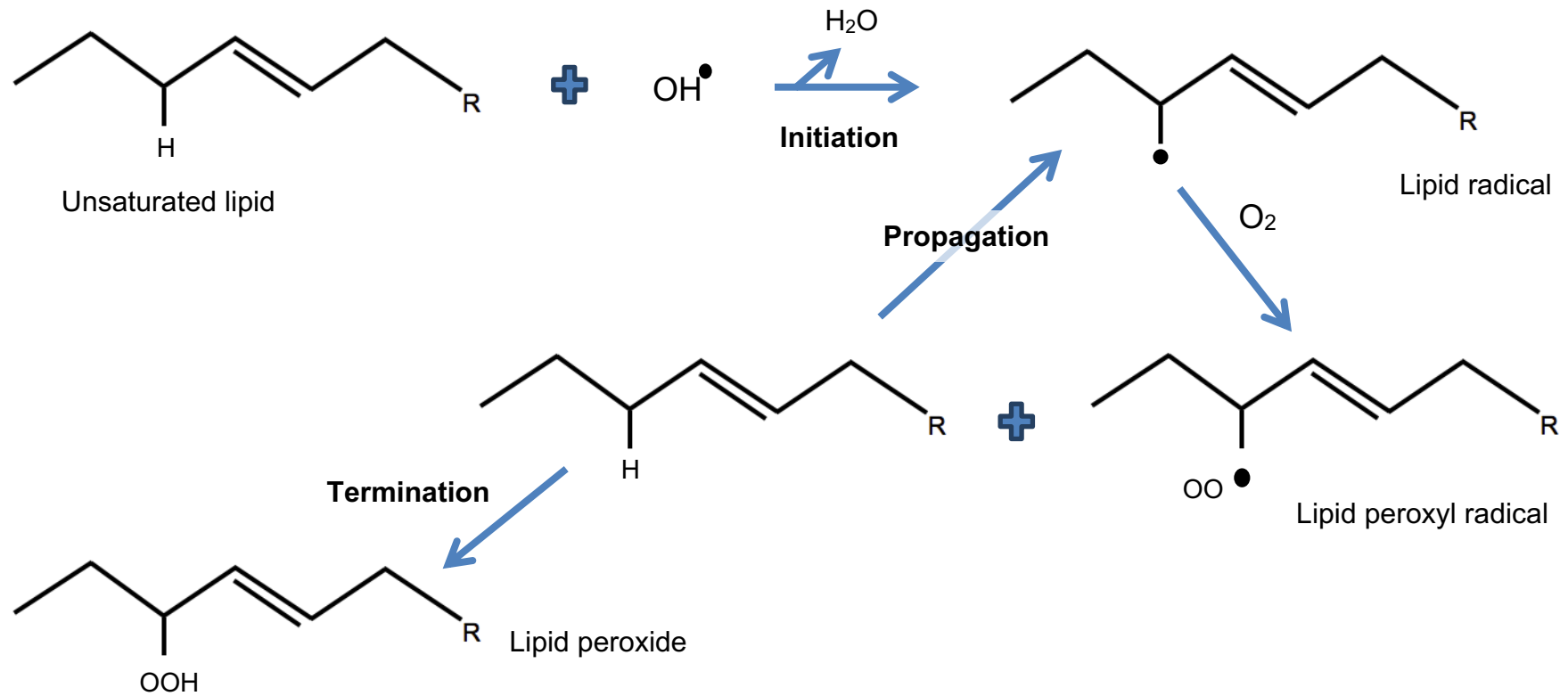
During sperm maturation, excess residual cytoplasm may remain in the morphologically abnormal sperm heads and contribute to oxidative stress (Rengan *et al.* 2012; Sprando and Russell 1987). Immature sperm, particularly sperm with excess cytoplasmic retention and abnormal head morphology, contain high ROS concentrations (Gomez *et al.* 1996). This emphasizes the notion that oxidative stress is considered a paradox that involves

both favorable and unfavorable consequences. The crux of this paradox is that the cytoplasm is shed as part of their maturation process, reducing susceptibility to ROS generation but in doing so, their antioxidant defense mechanisms are also reduced, as antioxidant enzymes are mainly located in the cytoplasm (Aitken and Curry 2011a). In stress conditions such as obesity, the physiological levels of antioxidants in sperm with excess residual cytoplasm cannot counteract the surplus production of ROS ultimately limiting the sperm cell's capacity to eliminate the excess ROS (Rengan *et al.* 2012).

#### ***1.3.1.4. Lipid peroxidation***

Lipid peroxidation is considered to be both a source of ROS production and consequence of increased ROS and oxidative stress (Storey 1997). The sperm plasma membrane contains an abundance of polyunsaturated fatty acids, which are vulnerable to lipid peroxidation, generating a cascade of lipid peroxides and aldehydes resulting in the rapid immobilization of sperm (Jones *et al.* 1979). Lipid peroxidation of the sperm plasma membrane is a cascade of self-propagating oxidative reactions composed of three main steps: initiation, propagation, and termination initiated by hydroxyl (OH) radicals (Henkel 2011). Lipid peroxidation is initiated by hydrogen atom abstraction from the lipid molecules of the polyunsaturated fatty acids in the sperm plasma membrane (Figure 1.6). The abstraction of hydrogen atoms from the unconjugated carbon double bonds results in free radical formation. These free radicals react with fatty acid chains and form lipid radicals, which then react with oxygen to form peroxy radicals (Figure 1.6). The peroxy radicals react with hydrogen to form lipid peroxides in the propagation stage resulting in the formation of cytotoxic aldehydes and the destabilization of the sperm plasma membrane (Henkel 2011), ultimately impairing the structure and function of the sperm plasma membrane and its membrane proteins (ATP-dependent ion pumps and

voltage-regulated ion channels) that are critical for sperm motility (Lundbaek and Andersen 1994). During the propagation stage, the peroxy and alkyl radical byproducts are regenerated until they react with each other to form stable end products and lipoproteins (Figure 1.6). When stable lipoproteins are formed, lipid peroxidation is at the termination stage, ultimately resulting in the generation of electrophilic lipid aldehydes including 4-hydroxynonenal (4HNE), acrolein, and MDA (Moazamian *et al.* 2015).



**Figure 1.6:** Lipid peroxidation of the sperm plasma membrane is a cascade of self-propagating oxidative reactions composed of three main steps: initiation, propagation and termination. Initiation is triggered by free radical hydrogen atom abstraction from the unsaturated lipid molecules resulting in the formation of lipid radicals. Lipid radicals react with oxygen to form lipid peroxy radicals, which then react with more unsaturated lipids from the sperm plasma membrane during the propagation stage. Lipid peroxidation is terminated when peroxy radicals react with hydrogen to generate the stable lipoprotein end product, lipid peroxide.

### **1.3.2. Oxidative stress and sperm function**

The cellular generation of ROS in human sperm was first reported in 1943 whereby hydrogen peroxide generation by sperm was concomitant with a reduction in sperm motility (MacLeod 1943). There are various ROS that at physiological concentrations are responsible for and modulate sperm capacitation including hydrogen peroxide (Aitken *et al.* 1995; Rivlin *et al.* 2004), superoxide anion (de Lamirande and Gagnon 1993) and the peroxy nitrite radical (Herrero and Gagnon 2001). However, at pathological levels, inducing oxidative stress results in a loss of sperm motility likely due to the by-products of lipid peroxidation as described in more detail above, which form adducts with the flagellar proteins resulting in a loss of flagellar function and ultimately loss of progressive motility (Baker *et al.* 2015; Moazamian *et al.* 2015). Also, the aldehyde, 4HNE, has the capacity to bind to mitochondrial proteins in sperm, which triggers mitochondrial electron leakage and leads to additional ROS generation (Koppers *et al.* 2010). An overabundance of ROS dysregulate protein tyrosine phosphatase activity, leading to the dephosphorylation and deactivation of phospholipase A<sub>2</sub>, which is necessary for the fluidity of the sperm plasma membrane and ultimately sperm-oocyte fusion (Calamera *et al.* 2003). Many studies have established that impaired sperm function is induced by oxidative stress, affecting capacitation, acrosome reaction, and sperm-oocyte fusion (Aitken *et al.* 1991; Kao *et al.* 2008; Morielli and O'Flaherty 2015; Sanocka *et al.* 1996; Sharma and Agarwal 1996).

### **1.3.3. Oxidative stress and sperm DNA damage**

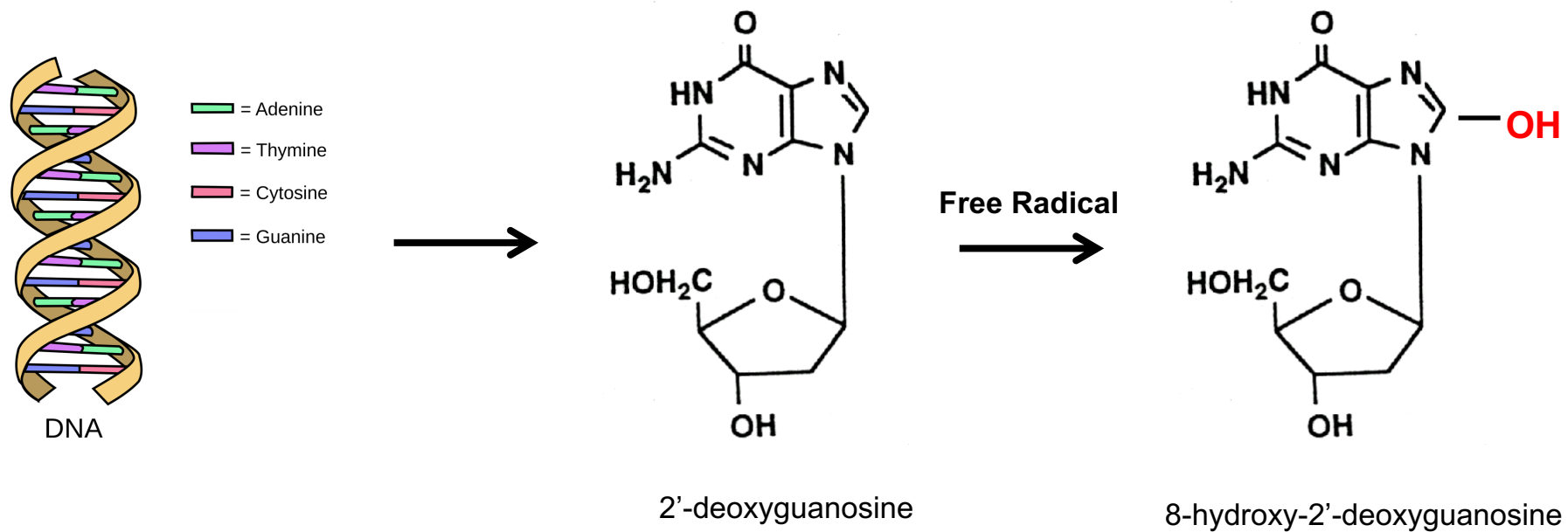
In addition to impaired sperm function, sperm oxidative stress can also damage the intracellular components of sperm, in particular DNA. Sperm chromatin integrity is compromised by high concentrations of sperm intracellular ROS (Aitken *et al.* 2003; De

Iuliis *et al.* 2009). Sperm DNA damage can be initiated by oxidative damage (De Iuliis *et al.* 2009) that forms oxidative base adducts such as 8-hydroxy-2'-deoxyguanosine (8-OHdG) (De Iuliis *et al.* 2009; Noblanc *et al.* 2013) (Figure 1.7). 8-OHdG is an oxidized guanine residue within sperm DNA, resulting from the oxidant attack of ROS by such molecules as hydroxyl radicals and hydrogen peroxide (Noblanc *et al.* 2013) (Figure 1.7). This then leads to a loss of the affected guanine base with an abasic site, which has a strong destabilizing effect on the DNA backbone and the DNA strand eventually breaks (Mitchell *et al.* 2011a), which may explain the increase of single and double stranded breaks observed in obese men (Campbell *et al.* 2015).

Sperm cells do not have the capacity to repair their DNA damage and base modifications, as sperm possess only one enzyme from the base excision repair (BER) pathway: 8-oxoguanine DNA glycosylase (OGG1) (Bruner *et al.* 2000; Smith *et al.* 2013). The enzyme OGG1 actively excises the 8-OHdG base adduct and releases it into the extracellular space however, sperm do not have the remaining components of the BER pathway: apurinic endonuclease 1 (APE1) and X-ray repair cross-complementing protein 1 (XRCC1) (Smith *et al.* 2013). As a result of this reduced DNA repair capacity, abasic sites are formed at locations affected by 8-OHdG generation leading to not only fragmentation of the DNA but also potential mutagenic base lesions (Bruner *et al.* 2000; Smith *et al.* 2013). 8-OHdG generation can also lead to the transmission of abasic sites into the oocyte for continuation of the repair pathway immediately after fertilization (Gawecka *et al.* 2013; Lord and Aitken 2015; Shimura *et al.* 2002). Moreover, 8-OHdG lesions are co-localized to chromatin regions of sperm that have less compacted and condensed chromatin, which are associated with loci that have retained histones (Noblanc *et al.* 2013). The relatively more exposed histone-bound regions in the paternal genome



are thus more susceptible to oxidative damage (Noblanc *et al.* 2013), but are also necessary for paternal genome transcription activation and paternal DNA replication (Shaman *et al.* 2007). Alterations or damage to the DNA bound to these histones in the paternal genome may be transmitted to the embryo without repair, resulting in delayed embryo development and blastocyst cell differentiation, and ultimately impaired health of potential offspring (Ward 2010).



**Figure 1.7:** Formation of the oxidative base adduct: 8-hydroxy-2'-deoxyguanosine (8-OHdG). 8-OHdG is a common oxidized base modification resulting from oxidant attack by reactive oxygen species (ROS) including hydroxyl radicals, hydrogen peroxide, singlet oxygen and superoxide.

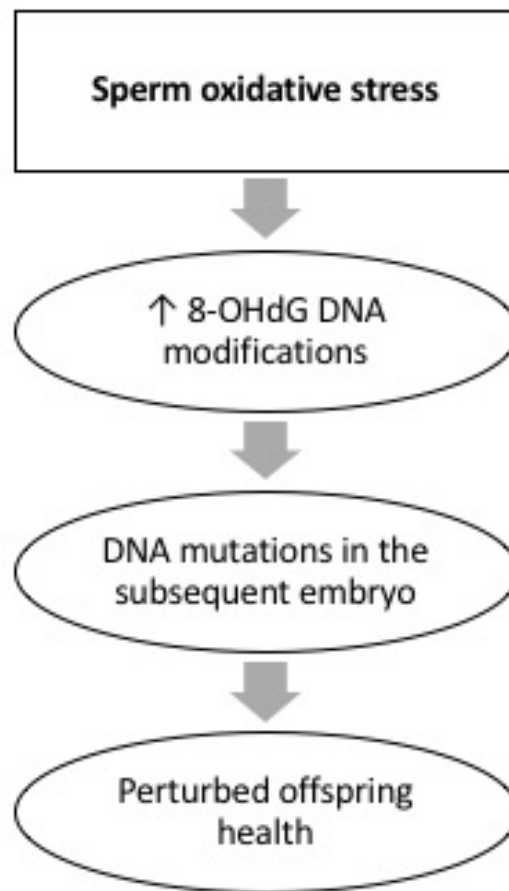
#### **1.3.4. Transmission of paternal DNA damage**

Current literature suggests that sperm intracellular ROS leads to oxidative damage to not only DNA but RNA, proteins, and microRNA. As a result, this damage may trigger paternal programming by alterations to the genome and/or epigenome of sperm (Aitken and Baker 2002; Aitken and Curry 2011a). Epidemiological studies demonstrate that there is a range of environmental and lifestyle factors that can impair DNA integrity in the male germ line due to increased sperm oxidative stress, which may induce subsequent DNA mutations in the embryo and ultimately perturb offspring health (Figure 1.8). High levels of ROS in seminal plasma and elevated levels of oxidative DNA modifications (e.g. 8-OHdG) in sperm DNA were observed in sperm from smokers compared to the sperm of non-smokers (Fraga *et al.* 1996). The oxidative DNA modifications and DNA fragmentation in sperm cells have been associated with elevated levels of childhood cancer in their offspring (Ji *et al.* 1997). Oxidative damage to sperm DNA such as deletions, abasic sites, and oxidative base change, have been proposed to contribute to an increased mutational load in the embryo (Trasler 2009). These mutational changes would be created in the fertilized oocyte as a result of abnormal DNA repair during DNA replication in the first cleavage division, highlighting a potential pathway for genetic damage to be transmitted through the male germ line, which may then impact offspring health.

In mice, treatment of sperm with hydrogen peroxide to induce elevated intracellular ROS did not affect fertilization but delayed on-time embryo development at the 8-cell stage, reduced the ratio of blastocyst ICM to TE cells, reduced embryo implantation rates, and reduced fetal: placental weights (Lane *et al.* 2014). Furthermore, sex-specific impacts on offspring were demonstrated, whereby female offspring were smaller in size, glucose

intolerant, and accumulated increased levels of adipose tissue when born from sperm treated with hydrogen peroxide-treated sperm compared to female offspring sired by sperm not treated with hydrogen peroxide. Male offspring however, did not display the phenotypes found in female offspring. Interestingly, sex-specific changes in the phenotype of offspring are similar to those reported in other models of paternal obesity and offspring programming (Fullston *et al.* 2013; Fullston *et al.* 2012; Ng *et al.* 2010) whereby many of the measured outcomes in female offspring were similar to that reported for obesity.

Overall, amongst the proposed mechanisms contributing to the transmission of paternal DNA damage, sperm oxidative stress is a prime central candidate mechanism that enacts programming of impaired offspring health via obesity-induced sperm oxidative stress.



**Figure 1.8:** A summary of the impact of sperm oxidative stress on offspring health in mice. Sperm oxidative stress and elevated intracellular ROS levels increases oxidative DNA modifications (8-OHdG), which in turn increases the incidence of DNA mutations in the subsequent embryo. Further, if a pregnancy and live birth is achieved, perturbed offspring health is observed with paternal sperm oxidative stress.

## 1.4 Dietary Micronutrient Supplementation

Oxidative stress and the production of damaging ROS within cells can be alleviated with antioxidants and antioxidant enzymes such as SOD, catalase and GPx. However, there is increasing interest in dietary impacts of antioxidants including micronutrients such as vitamins and minerals. Current literature is mixed with some supporting dietary micronutrient supplementation to treat sperm oxidative stress and improve sperm function in subfertile men (Showell *et al.* 2014), whilst others claim the evidence is of low quality (Barratt *et al.* 2017). The data in support of dietary micronutrient supplementation is considered as poor quality due to the inconsistencies in study design, patient phenotype, investigative outcomes, duration of intake and the intake of a single micronutrient versus a combination of micronutrients. Further, some micronutrients are not antioxidants but have anti-oxidative properties that combat oxidative stress indirectly (Table 1.2). Although the general functional capacity of these micronutrients during oxidative stress is understood (Table 1.2), the exact mechanism(s) involved in dietary supplements and improved sperm function is not known albeit proposed mechanisms via the passing of the BTB and BEB during spermatogenesis and sperm maturation, respectively.

**Table 1.2:** Commonly studied micronutrients (including antioxidants and elements) as dietary supplements for treatment of male subfertility and their anti-oxidative functions.

<b>Compound</b>	<b>Micronutrient type</b>	<b>Proposed Anti-oxidative function/ mechanism</b>
Vitamin E	Antioxidant (lipophilic)	Inhibits lipid peroxidation primarily of the plasma membrane
Vitamin C	Antioxidant (hydrophilic)	Electron donor and neutralizes free radicals
Zinc	Element	Inhibits ROS production through redox-active transition metals
Selenium	Element	Major constituent of antioxidant enzyme glutathione peroxidase (GPx)
Lycopene	Carotenoid (lipophilic)	Scavenges ROS primarily singlet oxygen
Folic acid	Antioxidant (B vitamin)	Free radical scavenger
Co-enzyme Q10	Antioxidant (lipophilic)	Inhibits lipid peroxidation and superoxide formation during cellular metabolism
N-acetyl cysteine	Amino acid	Indirectly as a precursor of glutathione peroxidase (GPx) and directly as a free radical scavenger
Carnitines	Antioxidant (lipophilic)	Indirectly as it inhibits fatty acid oxidation in mitochondria and directly as a free radical scavenger

### **1.4.1. Dietary micronutrient supplementation in normal weight males or subfertile men with unspecified BMIs**

#### ***1.4.1.1 Sperm oxidative stress and DNA integrity***

Gharagozloo and Aitken evaluated 20 trials that investigated the effects of various oral micronutrient supplements on sperm oxidative stress and DNA damage in men undergoing ART treatment. They found that 19 of the 20 trials reported reduced sperm oxidative stress or DNA fragmentation with micronutrient intake (Gharagozloo and Aitken 2011). Sperm oxidative stress has been shown to be reduced with a range of dietary micronutrient supplementation despite the variance in the types of micronutrients used, the duration of intake and other variables and potential confounders. This may be due to the indirect and direct anti-oxidative pathways and properties of micronutrients, which include antioxidants and trace elements (Table 1.2).

Zinc is a trace element that indirectly functions as an antioxidant by reducing hydroxyl radical formation from hydrogen peroxide through the antagonism of redox-active transition metals (Powell 2000). In asthenozoospermic men, the intake of various zinc-based supplements were compared including zinc only, zinc with vitamin E and zinc in combination with vitamins E and C supplementation for 3 months (Omu *et al.* 2008). Antioxidant enzyme concentrations of SOD and GPx (antioxidant enzymes) in both serum and semen in men taking all variations of these zinc supplements were increased compared to no treatment control with no differences in outcome measures when comparing the three different types of zinc-variant supplements, suggesting that the zinc is the primary agent for elucidating this effect (Omu *et al.* 2008).



Vitamin E is a readily diffused lipophilic antioxidant primarily localized in the cell's plasma membrane. The major and direct anti-oxidative function of vitamin E is the inhibition of lipid peroxidation to maintain membrane fluidity and integrity (Traber and Atkinson 2007). In men undergoing ART treatment, vitamin E supplementation for 6 months was associated with reduced lipid peroxidation observed by reduced sperm MDA concentrations (byproduct of lipid peroxidation) compared to placebo-treated patients (Suleiman *et al.* 1996). Vitamin C (ascorbic acid) is a hydrophilic antioxidant, which neutralizes free radicals by electron donation. Vitamin C in cells protects against protein, lipid and DNA damage by directly reducing radical species to maintain intracellular and DNA integrity (Padayatty *et al.* 2003). The protective role of dietary intake of vitamin C on sperm oxidative stress and sperm DNA integrity was demonstrated when the dose of vitamin C supplementation was reduced from 250mg to 5mg/day, sperm 8-OHdG lesions increased after 28 days (Fraga *et al.* 1991). In couples undergoing ART treatment, men with elevated sperm DNA fragmentation were selected to consume both vitamin C and vitamin E supplementation or a placebo and following 2 months, men treated with vitamin C and vitamin E supplementation had reduced sperm DNA fragmentation compared to placebo treated patients (Greco *et al.* 2005). Furthermore, protection against sperm DNA fragmentation has been also demonstrated with *in vitro* lycopene supplementation. Lycopene is a lipophilic carotenoid with high singlet oxygen-quenching potency (Rao *et al.* 2006). Sperm DNA fragmentation from fertile men was reduced in hydrogen peroxide-treated sperm by treatment with lycopene for 2 hours *in vitro* highlighting the protective role of lycopene in maintaining DNA integrity (Zini *et al.* 2010).

The protection of micronutrient supplement intake has also been shown in animal models whereby oral green tea extract supplementation, a catechin and natural antioxidant reduced sperm oxidative stress by increasing catalase and SOD antioxidant enzyme activity in sperm from rats compared to sperm from the no treatment control (Awoniyi *et al.* 2012). Given that sperm are transcriptionally and translationally silent, this alteration in gene expression and protein synthesis must have occurred during the early stages of spermatogenesis. In mice fed a restricted diet to mimic under-nutrition, the intake of a combination of micronutrient supplements (selenium, lycopene, vitamin E, vitamin C, green tea extract, zinc and folic acid) reduced levels of sperm 8-OHdG lesions compared to sperm from under-nourished controls (McPherson *et al.* 2016). In mice, an epididymis specific *GPx5*-knockout model was generated to have the hydrogen peroxide-scavenging activity of GPx5 in the epididymis nullified to expose maturing sperm to hydrogen peroxide-mediated oxidative stress (Chabory *et al.* 2009). In this model, sperm 8-OHdG lesions were reduced with a 8 week intake of a combination of micronutrient supplementation consisting of a carnitine blend, folic acid, lycopene, selenium, vitamin C, vitamin E, and zinc (Gharagozloo *et al.* 2016). Overall, these studies suggest an important role of micronutrient supplementation in combating oxidative stress in the male germ line and reducing sperm oxidative stress byproducts including oxidative DNA damage and DNA fragmentation. Interestingly, these mouse models suggest that micronutrient supplementation may have a vital role in both the testes and epididymis in protecting against sperm oxidative stress.

#### ***1.4.1.2 Conventional sperm parameters***

Current literature suggests that the consumption of micronutrient supplements in subfertile men is not only associated with improved sperm oxidative stress and DNA

integrity but also improved conventional sperm parameters. The largest meta-analysis of 48 randomized-controlled trials is a Cochrane systematic review by Showell *et al.* that included 4,179 couples and compared any type or dose of micronutrient supplement with a placebo or no treatment (Showell *et al.* 2014). These micronutrients included antioxidants and trace elements (Table 1.2). The overall findings from the systematic review reported that data were inconsistent to draw conclusive findings regarding conventional sperm parameters due to the extremely high heterogeneity between studies. However, when analyzing studies with low heterogeneity, the systematic review reported a combination of micronutrients and antioxidants for 3 months increased sperm motility compared to placebo or no treatment (Showell *et al.* 2014).

The impact of a variety of dietary micronutrient and antioxidant supplementation on sperm motility for different durations has been previously investigated. An increase in sperm motility was found after three months of N-acetyl cysteine, vitamin E and selenium, zinc or selenium supplement intake in infertile and asthenozoospermic men (Ciftci *et al.* 2009; Keskes-Ammar *et al.* 2003; Nozha *et al.* 2001; Omu *et al.* 1998; Scott *et al.* 1998), six months of carnitine, co-enzyme Q10 or vitamin E supplementation in infertile and oligoasthenoteratozoospermic men (Balercia *et al.* 2005; Cavallini *et al.* 2004; Lenzi *et al.* 2003; Lenzi *et al.* 2004; Safarinejad 2009; Safarinejad and Safarinejad 2009; Suleiman *et al.* 1996), and nine months of co-enzyme Q10 in infertile men (Balercia *et al.* 2009). N-acetyl cysteine and selenium are vital for the synthesis of the antioxidant enzyme GPx, whereby n-acetyl cysteine is a precursor and selenium is a major constituent through selenoproteins, which are required for flagellum formation and maintain progressive sperm motility (Aruoma *et al.* 1989; Boitani and Puglisi 2008). Whereas carnitines have direct and indirect anti-oxidative properties by neutralizing free

radicals and inhibition of fatty acid oxidation endogenously (Agarwal and Said 2004). Similar to vitamin E, endogenous co-enzyme Q10 is an antioxidant primarily located in the plasma membrane and inhibits lipid peroxidation and ROS formation, primarily superoxide (Crane 2001). Collectively, these micronutrients are examples of compounds that have indirect and direct anti-oxidative functions in free radical scavenging, which maintain sperm cell mitochondrial metabolism and support progressive sperm motility which is regulated by the mitochondria. Further, one study showed an improvement in sperm motility after vitamin C supplementation of three weeks (Dawson *et al.* 1990). Given that a full round of spermatogenesis is approximately 3 months in men, improvement in sperm motility over a much shorter time would suggest that micronutrient supplementation can modify sperm during sperm maturation during epididymal transit, which is of a much shorter duration (10 days).

The concentration of lycopene in the testes is relatively high compared to other parts of the body (Zini *et al.* 2010), which suggests that lycopene may have a vital role in sperm morphogenesis during spermatogenesis. An increase in sperm concentration and normal morphology was also reported after six months of carnitine or co-enzyme Q10 in infertile men (Cavallini *et al.* 2004; Lenzi *et al.* 2003; Safarinejad 2009; Safarinejad and Safarinejad 2009) and lycopene intake was positively associated with normal sperm morphology in young healthy men (Zareba *et al.* 2013). A double-blinded, randomized, controlled trial found that sperm motility, concentration and normal morphology were increased with folic acid combined with zinc for 6 months in both fertile and subfertile men to similar extents (Wong *et al.* 2002). Although the underlying mechanisms are unknown, it is likely that the anti-oxidative properties of folic acid and zinc in scavenging free radicals and inhibition of ROS production respectively, may contribute to normal

spermatogenesis as micronutrient concentrations were elevated in blood and seminal plasma of men taking dietary folic acid and zinc supplementation (Wong *et al.* 2002).

The impact of micronutrient supplement intake on conventional sperm parameters have also been studied in various animal models. A study in cyclophosphamide treated rats, a chemotherapy drug, investigated the protective role of lycopene on sperm morphology and found that dietary lycopene consumption significantly improved normal sperm morphology compared to rats that did not consume lycopene (Ceribasi *et al.* 2010). Some studies report that certain forms of garlic (*allitridum*) when added to epididymal sperm *in vitro* have dose dependant deleterious effects on sperm motility from various species including ram, hamster and rat (Chakrabarti *et al.* 2003; Qian *et al.* 1986). In contrast, beneficial effects of garlic oil intake have been reported whereby an increase in sperm count was found when adding garlic to drinking water in mice (al-Bekairi *et al.* 1990), which may be due to the antioxidants, allicin, alliin, allyl disulfide and allyl cysteine, found in garlic compounds (Chung 2006). The discrepancies between these studies may be due to the differences in use of garlic *in vitro* (adding it to collected epididymal sperm) and *in vivo* (adding it to drinking water) in addition to the differences in doses and concentrations of garlic used. Green tea extract has been shown to improve sperm motility and normal morphology in nicotine-induced oxidative stress in rats (Mosbah *et al.* 2015). Also in rats, selenium deficiency was associated with reduced sperm motility and concentration, and abnormal sperm morphology (Olson *et al.* 2004). Overall, these animal models highlight the effectiveness of dietary micronutrient supplement intake in improving conventional sperm parameters including sperm motility, concentration and morphology particularly in stress-induced models.

#### ***1.4.1.3 Sperm function and pregnancy outcome***

The impact of dietary micronutrient supplementation on sperm function including sperm-oocyte binding and fertilization rates in men has also been assessed. In a blinded, randomized controlled trial, otherwise healthy men but who presented with high levels of seminal ROS received either vitamin E supplements or a placebo for three months. After three months, blood serum vitamin E concentrations were increased in men taking the vitamin E supplementation with a concomitant increase in sperm-oocyte binding (Kessopoulou *et al.* 1995). Also, vitamin E supplementation for one month was associated with increased fertilization rates from 15 normospermic men that had low fertilization rates in their previous IVF cycles (Geva *et al.* 1996). An similar increase in fertilization rates has also been reported with zinc supplementation in asthenozoospermic men after one month of intake (Omu *et al.* 1998).

The association between dietary micronutrient supplementation and pregnancy rates has been reviewed and the majority of studies report a positive relationship between micronutrient supplementation in men and pregnancy rates after undergoing ART treatment (Gharagozloo and Aitken 2011; Showell *et al.* 2011; Showell *et al.* 2014). The micronutrients used in these studies included vitamin E in asthenozoospermic men (Suleiman *et al.* 1996), L-carnitine/ acetyl L-carnitine in oligoasthenozoospermic men (Cavallini *et al.* 2004; Li *et al.* 2005) and a combination of micronutrients including selenium, zinc, folic acid, vitamin E, vitamin C, lycopene, and garlic oil in men with severe male factor infertility (Tremellen *et al.* 2007).

The increase in pregnancy rates with micronutrient supplementation persisted into increased live birth rates whereby a Cochrane systematic review found that 3 of 34

randomized controlled trials investigated the correlation between supplement intake and live birth rates and found that all 3 studies showed an increase in live birth rate compared to the control group (Showell *et al.* 2011). Dietary vitamin E supplementation (Suleiman *et al.* 1996) and zinc supplementation in asthenozoospermic men (Omu *et al.* 1998), and a combination of micronutrients including selenium, zinc, folic acid, vitamin E, vitamin C, lycopene, and garlic oil in men with severe male factor infertility (Tremellen *et al.* 2007) increased live birth rates after IVF compared to placebo or no treatment.

Overall, the efficacy of dietary micronutrient supplementation has been established in normal weight or sub-fertile men whereby improved conventional sperm parameters and reduced sperm oxidative stress, oxidative DNA damage, and DNA fragmentation were observed.

#### **1.4.2. Dietary micronutrient supplementation in obese males**

Despite being a disease of over-nutrition, obesity is commonly associated with a micronutrient-poor diet (Gillis and Gillis 2005). Increased adiposity and BMI are associated with reduced serum concentrations of essential vitamins and minerals with anti-oxidative properties including vitamin C, vitamin E, selenium, folate and zinc (Aasheim *et al.* 2008; Ernst *et al.* 2009; Garcia *et al.* 2012; Gobato *et al.* 2014). In men with unspecified BMIs, micronutrient deficiencies have been associated with subfertility, such as selenium deficiency, which is associated with increased abnormal sperm morphology (Krol *et al.* 2012), and reduced sperm motility and concentration, (Hardy and Hardy 2004; Olson *et al.* 2004; Sanchez-Gutierrez *et al.* 2008) and increased sperm oxidative damage (Sanchez-Gutierrez *et al.* 2008). Also, men with vitamin C deficiency

have demonstrated increased 8-OHdG in their sperm, which is reduced with vitamin C supplementation (Fraga *et al.* 1991).

To date, there have been no studies that have investigated the impact of dietary micronutrient supplement intake on male fertility in overweight and obese men. However, in animal models, particularly rodent models, the benefits of micronutrient supplementation on sperm quality and function in HFD-induced obese males have been demonstrated to a certain extent. In 2012, Ibrahim *et al.* used a mouse model of obesity and hyperlipidemia induced by a HFD (15% fat) and showed that a HFD supplemented with selenium for approximately 11 weeks reduced the number and degree of irregular and collapsed seminiferous tubules, increased the number of spermatocytes, increased testosterone levels, sperm motility and count, and improved sperm morphology (Ibrahim *et al.* 2012). In rats, a HFD consisting of approximately 60% fat supplemented with vitamin E, vitamin C, and astaxanthin (carotenoid) for 12 weeks increased sperm count, motility, and normal morphology in addition to increases in the number of spermatogonia and Sertoli cells in testicular tissue compared to rats that consumed an un-supplemented HFD (Mortazavi *et al.* 2014). Further, the rats fed the un-supplemented HFD had increased serum triglyceride and cholesterol concentrations compared to that treated with dietary vitamin E, vitamin C, and astaxanthin supplementation suggesting that systemic metabolic issues may also contribute to impaired reproductive health in these rats (Mortazavi *et al.* 2014). Thymoquinone, an anti-inflammatory and antioxidant compound found in plants was injected daily and intraperitoneally into rats fed a 40% fat diet and after 6 weeks, the treated rats showed no differences in sperm concentration or motility but displayed increased levels of normal sperm morphology in addition to a reduction in testicular and seminiferous tubule structural atrophy, and an increase in the



number of spermatogenic and Leydig cells compared to the non-treatment control (Tufek *et al.* 2015).

Overall, these studies suggest that that micronutrients may improve male fertility compromised by obesity. However, the optimal duration for administration and whether embryo development and pregnancy is improved is unclear. It is also not clear whether the effect is related to obesity or related metabolic disorders such as hyperinsulinemia or hyperlipidemia. Importantly, no studies to date have examined the impact of micronutrient supplement intake on sperm oxidative stress and pregnancy outcomes in men with an increased BMI. Therefore, investigations regarding the impact of commonly used dietary micronutrient supplements on pregnancy and fertility treatment outcomes in addition to the potential underlying oxidative stress markers in sperm from obese males are warranted.

## 1.5 Conclusion

The prevalence of male obesity and its detrimental effects on both male reproductive health and the health of their offspring is becoming more evident. The improvement of methods for the diagnosis, management, and prevention of obesity and male mediated reproductive failure is crucial. This literature review emphasizes the significant and central role that sperm oxidative stress plays in the induction of defective sperm quality and function, loss of DNA integrity, and transmission of disease to offspring. Methods for the treatment and prevention, with appropriate micronutrients, to reduce the severity of oxidative damage to sperm need to be investigated to try and improve sperm function, pregnancy outcomes and ultimately the health of offspring.

Dietary micronutrient supplementation can improve sperm function and even improve pregnancy rates after ART in sub-fertile men. However, whether micronutrient supplementation can reverse or minimize the detrimental downstream effects of obesity on sperm remains unknown, or how this influences the embryo and pregnancy. This area of research requires studies to establish an understanding of the correlation between dietary micronutrient supplementation and obese male reproductive health, and the mechanisms for improvements of not only sperm quality and function and oxidative markers, but also subsequent pregnancy outcomes.

Therefore, my hypothesis is: dietary micronutrient supplementation in obese males improves fertilization rates, embryo development, implantation, and pregnancy rates by reducing sperm oxidative stress and oxidative DNA damage.

## 1.6 References

- Aasheim, E.T., Hofso, D., Hjelmessaeth, J., Birkeland, K.I., and Bohmer, T. (2008) Vitamin status in morbidly obese patients: a cross-sectional study. *Am J Clin Nutr* **87**, 362-369.
- Agarwal, A., Prabakaran, S., and Allamaneni, S. (2006) What an andrologist/urologist should know about free radicals and why. *Urology* **67**, 2-8.
- Agarwal, A., and Said, T.M. (2004) Carnitines and male infertility. *Reprod Biomed Online* **8**, 376-384.
- Aggerholm, A.S., Thulstrup, A.M., Toft, G., Ramlau-Hansen, C.H., and Bonde, J.P. (2008) Is overweight a risk factor for reduced semen quality and altered serum sex hormone profile? *Fertil Steril* **90**, 619-626.
- AIHW (2016) Australia's Health series no.15, Cat. no. AUS 199, Australia's Health 2016. Australian Institute of Health and Welfare, Canberra: AIHW.
- Aitken, R.J., and Baker, M.A. (2002) Reactive oxygen species generation by human spermatozoa: a continuing enigma. *Int J Androl* **25**, 191-194.
- Aitken, R.J., Baker, M.A., and Sawyer, D. (2003) Oxidative stress in the male germ line and its role in the aetiology of male infertility and genetic disease. *Reprod Biomed Online* **7**, 65-70.
- Aitken, R.J., Buckingham, D.W., Brindle, J., Gomez, E., Baker, H.W.G., and Irvine, D.S. (1995) Analysis of Sperm Movement in Relation to the Oxidative Stress Created by Leukocytes in Washed Sperm Preparations and Seminal Plasma. *Human Reproduction* **10**, 2061-2071. [In English]
- Aitken, R.J., and Curry, B.J. (2011a) Redox Regulation of Human Sperm Function: From the Physiological Control of Sperm Capacitation to the Etiology of Infertility and DNA Damage in the Germ Line. *Antioxidants & Redox Signaling* **14**, 367-381. [In English]
- Aitken, R.J., and Curry, B.J. (2011b) Redox regulation of human sperm function: from the physiological control of sperm capacitation to the etiology of infertility and DNA damage in the germ line. *Antioxid Redox Signal* **14**, 367-381.
- Aitken, R.J., Irvine, D.S., and Wu, F.C. (1991) Prospective analysis of sperm-oocyte fusion and reactive oxygen species generation as criteria for the diagnosis of infertility. *Am J Obstet Gynecol* **164**, 542-551.
- Aitken, R.J., Jones, K.T., and Robertson, S.A. (2012) Reactive oxygen species and sperm function--in sickness and in health. *J Androl* **33**, 1096-1106.
- Aitken, R.J., Smith, T.B., Jobling, M.S., Baker, M.A., and De Iuliis, G.N. (2014) Oxidative stress and male reproductive health. *Asian J Androl* **16**, 31-38.

- al-Bekairi, A.M., Shah, A.H., and Qureshi, S. (1990) Effect of *Allium sativum* on epididymal spermatozoa, estradiol-treated mice and general toxicity. *J Ethnopharmacol* **29**, 117-125.
- Alvarez, J.G., and Storey, B.T. (1995) Differential incorporation of fatty acids into and peroxidative loss of fatty acids from phospholipids of human spermatozoa. *Mol Reprod Dev* **42**, 334-346.
- Amaral, A., Lourenco, B., Marques, M., and Ramalho-Santos, J. (2013) Mitochondria functionality and sperm quality. *Reproduction* **146**, R163-174.
- Aruoma, O.I., Halliwell, B., Hoey, B.M., and Butler, J. (1989) The antioxidant action of N-acetylcysteine: its reaction with hydrogen peroxide, hydroxyl radical, superoxide, and hypochlorous acid. *Free Radic Biol Med* **6**, 593-597.
- Awoniyi, D.O., Aboua, Y.G., Marnewick, J., and Brooks, N. (2012) The effects of rooibos (*Aspalathus linearis*), green tea (*Camellia sinensis*) and commercial rooibos and green tea supplements on epididymal sperm in oxidative stress-induced rats. *Phytother Res* **26**, 1231-1239.
- Baker, M.A., Weinberg, A., Hetherington, L., Villaverde, A.I., Velkov, T., Baell, J., and Gordon, C.P. (2015) Defining the mechanisms by which the reactive oxygen species by-product, 4-hydroxynonenal, affects human sperm cell function. *Biol Reprod* **92**, 108.
- Bakos, H.W., Henshaw, R.C., Mitchell, M., and Lane, M. (2011) Paternal body mass index is associated with decreased blastocyst development and reduced live birth rates following assisted reproductive technology. *Fertil Steril* **95**, 1700-1704.
- Bakos, H.W., Mitchell, M., Setchell, B.P., and Lane, M. (2010) The effect of paternal diet-induced obesity on sperm function and fertilization in a mouse model. *Int J Androl* **34**, 402-410.
- Balercia, G., Buldreghini, E., Vignini, A., Tiano, L., Paggi, F., Amoroso, S., Ricciardo-Lamonica, G., Boscaro, M., Lenzi, A., and Littarru, G. (2009) Coenzyme Q10 treatment in infertile men with idiopathic asthenozoospermia: a placebo-controlled, double-blind randomized trial. *Fertil Steril* **91**, 1785-1792.
- Balercia, G., Regoli, F., Armeni, T., Koverech, A., Mantero, F., and Boscaro, M. (2005) Placebo-controlled double-blind randomized trial on the use of L-carnitine, L-acetylcarnitine, or combined L-carnitine and L-acetylcarnitine in men with idiopathic asthenozoospermia. *Fertil Steril* **84**, 662-671.
- Barratt, C.L.R., Bjorndahl, L., De Jonge, C.J., Lamb, D.J., Osorio Martini, F., McLachlan, R., Oates, R.D., van der Poel, S., St John, B., Sigman, M., *et al.* (2017) The diagnosis of male infertility: an analysis of the evidence to support the development of global WHO guidance-challenges and future research opportunities. *Hum Reprod Update* **23**, 660-680.

- Bedford, J.M., Calvin, H., and Cooper, G.W. (1973) The maturation of spermatozoa in the human epididymis. *J Reprod Fertil Suppl* **18**, 199-213.
- Binder, N.K., Hannan, N.J., and Gardner, D.K. (2012) Paternal diet-induced obesity retards early mouse embryo development, mitochondrial activity and pregnancy health. *PLoS One* **7**, e52304.
- Bisht, S., Faiq, M., Tolahunase, M., and Dada, R. (2017) Oxidative stress and male infertility. *Nat Rev Urol*.
- Boitani, C., and Puglisi, R. (2008) Selenium, a key element in spermatogenesis and male fertility. *Adv Exp Med Biol* **636**, 65-73.
- Braude, P., Bolton, V., and Moore, S. (1988) Human gene expression first occurs between the four- and eight-cell stages of preimplantation development. *Nature* **332**, 459-461.
- Braun, R.E. (2001) Packaging paternal chromosomes with protamine. *Nat Genet* **28**, 10-12.
- Bruner, S.D., Norman, D.P., and Verdine, G.L. (2000) Structural basis for recognition and repair of the endogenous mutagen 8-oxoguanine in DNA. *Nature* **403**, 859-866.
- Calamera, J., Buffone, M., Ollero, M., Alvarez, J., and Doncel, G.F. (2003) Superoxide dismutase content and fatty acid composition in subsets of human spermatozoa from normozoospermic, asthenozoospermic, and polyzoospermic semen samples. *Mol Reprod Dev* **66**, 422-430.
- Campbell, J.M., Lane, M., Owens, J.A., and Bakos, H.W. (2015) Paternal obesity negatively affects male fertility and assisted reproduction outcomes: a systematic review and meta-analysis. *Reproductive BioMedicine Online* **31**, 593-604.
- Carrell, D.T., Emery, B.R., and Hammoud, S. (2007) Altered protamine expression and diminished spermatogenesis: what is the link? *Hum Reprod Update* **13**, 313-327.
- Carvalho-Santos, Z., Machado, P., Alvarez-Martins, I., Gouveia, S.M., Jana, S.C., Duarte, P., Amado, T., Branco, P., Freitas, M.C., Silva, S.T., *et al.* (2012) BLD10/CEP135 is a microtubule-associated protein that controls the formation of the flagellum central microtubule pair. *Dev Cell* **23**, 412-424.
- Cavallini, G., Ferraretti, A.P., Gianaroli, L., Biagiotti, G., and Vitali, G. (2004) Cinnocicam and L-carnitine/acetyl-L-carnitine treatment for idiopathic and varicocele-associated oligoasthenospermia. *J Androl* **25**, 761-770; discussion 771-762.

- Ceribasi, A.O., Turk, G., Sonmez, M., Sakin, F., and Atessahin, A. (2010) Toxic effect of cyclophosphamide on sperm morphology, testicular histology and blood oxidant-antioxidant balance, and protective roles of lycopene and ellagic acid. *Basic Clin Pharmacol Toxicol* **107**, 730-736.
- Chabory, E., Damon, C., Lenoir, A., Kauselmann, G., Kern, H., Zevnik, B., Garrel, C., Saez, F., Cadet, R., Henry-Berger, J., *et al.* (2009) Epididymis seleno-independent glutathione peroxidase 5 maintains sperm DNA integrity in mice. *J Clin Invest* **119**, 2074-2085.
- Chakrabarti, K., Pal, S., and Bhattacharyya, A.K. (2003) Sperm immobilization activity of *Allium sativum* L. and other plant extracts. *Asian J Androl* **5**, 131-135.
- Chambers, T.J., and Richard, R.A. (2015) The impact of obesity on male fertility. *Hormones (Athens)* **14**, 563-568.
- Chavarro, J.E., Toth, T.L., Wright, D.L., Meeker, J.D., and Hauser, R. (2010) Body mass index in relation to semen quality, sperm DNA integrity, and serum reproductive hormone levels among men attending an infertility clinic. *Fertil Steril* **93**, 2222-2231.
- Chen, X.L., Gong, L.Z., and Xu, J.X. (2013) Antioxidative activity and protective effect of probiotics against high-fat diet-induced sperm damage in rats. *Animal* **7**, 287-292.
- Chen, Y.P., Xiao, X.M., Li, J., Reichetzeder, C., Wang, Z.N., and Hocher, B. (2012) Paternal body mass index (BMI) is associated with offspring intrauterine growth in a gender dependent manner. *PLoS One* **7**, e36329.
- Chung, L.Y. (2006) The antioxidant properties of garlic compounds: allyl cysteine, alliin, allicin, and allyl disulfide. *J Med Food* **9**, 205-213.
- Ciftci, H., Verit, A., Savas, M., Yeni, E., and Erel, O. (2009) Effects of N-acetylcysteine on semen parameters and oxidative/antioxidant status. *Urology* **74**, 73-76.
- Clermont, Y. (1966) Renewal of spermatogonia in man. *Am J Anat* **118**, 509-524.
- Clermont, Y. (1972) Kinetics of spermatogenesis in mammals: seminiferous epithelium cycle and spermatogonial renewal. *Physiol Rev* **52**, 198-236.
- Colaci, D.S., Afeiche, M., Gaskins, A.J., Wright, D.L., Toth, T.L., Tanrikut, C., Hauser, R., and Chavarro, J.E. (2012) Men's body mass index in relation to embryo quality and clinical outcomes in couples undergoing in vitro fertilization. *Fertil Steril* **98**, 1193-1199 e1191.
- Coviello, A.D., Bremner, W.J., Matsumoto, A.M., Herbst, K.L., Amory, J.K., Anawalt, B.D., Yan, X., Brown, T.R., Wright, W.W., Zirkin, B.R., *et al.* (2004) Intratesticular testosterone concentrations comparable with serum levels are not sufficient to

maintain normal sperm production in men receiving a hormonal contraceptive regimen. *J Androl* **25**, 931-938.

Crane, F.L. (2001) Biochemical functions of coenzyme Q10. *J Am Coll Nutr* **20**, 591-598.

Dadoune, J.P. (1995) The nuclear status of human sperm cells. *Micron* **26**, 323-345.

Das, S.K., Varadhan, S., Gupta, G., Mukherjee, S., Dhanya, L., Rao, D.N., and Vasudevan, D.M. (2009) Time-dependent effects of ethanol on blood oxidative stress parameters and cytokines. *Indian J Biochem Biophys* **46**, 116-121.

Dawson, E.B., Harris, W.A., and Powell, L.C. (1990) Relationship between ascorbic acid and male fertility. *World Rev Nutr Diet* **62**, 1-26.

de Castro Barbosa, T., Ingerslev, L.R., Alm, P.S., Versteyhe, S., Massart, J., Rasmussen, M., Donkin, I., Sjogren, R., Mudry, J.M., Vetterli, L., *et al.* (2016) High-fat diet reprograms the epigenome of rat spermatozoa and transgenerationally affects metabolism of the offspring. *Mol Metab* **5**, 184-197.

De Iuliis, G.N., Thomson, L.K., Mitchell, L.A., Finnie, J.M., Koppers, A.J., Hedges, A., Nixon, B., and Aitken, R.J. (2009) DNA Damage in Human Spermatozoa Is Highly Correlated with the Efficiency of Chromatin Remodeling and the Formation of 8-Hydroxy-2'-Deoxyguanosine, a Marker of Oxidative Stress. *Biology of Reproduction* **81**, 517-524. [In English]

de Lamirande, E., and Gagnon, C. (1993) A positive role for the superoxide anion in triggering hyperactivation and capacitation of human spermatozoa. *Int J Androl* **16**, 21-25.

Duale, N., Steffensen, I.L., Andersen, J., Brevik, A., Brunborg, G., and Lindeman, B. (2014) Impaired sperm chromatin integrity in obese mice. *Andrology* **2**, 234-243.

Dupont, C., Faure, C., Sermondade, N., Boubaya, M., Eustache, F., Clement, P., Briot, P., Berthaut, I., Levy, V., Cedrin-Durnerin, I., *et al.* (2013) Obesity leads to higher risk of sperm DNA damage in infertile patients. *Asian J Androl* **15**, 622-625.

Eisenberg, M.L., Kim, S., Chen, Z., Sundaram, R., Schisterman, E.F., and Louis, G.M.B. (2014) The relationship between male BMI and waist circumference on semen quality: data from the LIFE study. *Human Reproduction* **29**, 193-200. [In English]

El-Helaly, M., Abdel-Elah, K., Haussein, A., and Shalaby, H. (2011) Paternal occupational exposures and the risk of congenital malformations--a case-control study. *Int J Occup Med Environ Health* **24**, 218-227.

Ernst, B., Thurnheer, M., Schmid, S.M., and Schultes, B. (2009) Evidence for the necessity to systematically assess micronutrient status prior to bariatric surgery. *Obes Surg* **19**, 66-73.

- Fan, Y., Liu, Y., Xue, K., Gu, G., Fan, W., Xu, Y., and Ding, Z. (2015) Diet-induced obesity in male C57BL/6 mice decreases fertility as a consequence of disrupted blood-testis barrier. *PLoS One* **10**, e0120775.
- Fariello, R.M., Pariz, J.R., Spaine, D.M., Cedenho, A.P., Bertolla, R.P., and Fraietta, R. (2012) Association between obesity and alteration of sperm DNA integrity and mitochondrial activity. *BJU Int* **110**, 863-867.
- Figuroa-Colon, R., Arani, R.B., Goran, M.I., and Weinsier, R.L. (2000) Paternal body fat is a longitudinal predictor of changes in body fat in premenarcheal girls. *Am J Clin Nutr* **71**, 829-834. [In eng]
- Fraga, C.G., Motchnik, P.A., Shigenaga, M.K., Helbock, H.J., Jacob, R.A., and Ames, B.N. (1991) Ascorbic acid protects against endogenous oxidative DNA damage in human sperm. *Proc Natl Acad Sci U S A* **88**, 11003-11006.
- Fraga, C.G., Motchnik, P.A., Wyrobek, A.J., Rempel, D.M., and Ames, B.N. (1996) Smoking and low antioxidant levels increase oxidative damage to sperm DNA. *Mutat Res* **351**, 199-203.
- Frans, E.M., Sandin, S., Reichenberg, A., Lichtenstein, P., Langstrom, N., and Hultman, C.M. (2008) Advancing paternal age and bipolar disorder. *Arch Gen Psychiatry* **65**, 1034-1040.
- Fullston, T., McPherson, N.O., Owens, J.A., Kang, W.X., Sandeman, L.Y., and Lane, M. (2015) Paternal obesity induces metabolic and sperm disturbances in male offspring that are exacerbated by their exposure to an "obesogenic" diet. *Physiol Rep* **3**.
- Fullston, T., Ohlsson Teague, E.M., Palmer, N.O., DeBlasio, M.J., Mitchell, M., Corbett, M., Print, C.G., Owens, J.A., and Lane, M. (2013) Paternal obesity initiates metabolic disturbances in two generations of mice with incomplete penetrance to the F2 generation and alters the transcriptional profile of testis and sperm microRNA content. *FASEB J* **27**, 4226-4243.
- Fullston, T., Palmer, N.O., Owens, J.A., Mitchell, M., Bakos, H.W., and Lane, M. (2012) Diet-induced paternal obesity in the absence of diabetes diminishes the reproductive health of two subsequent generations of mice. *Hum Reprod* **27**, 1391-1400.
- Furukawa, S., Fujita, T., Shimabukuro, M., Iwaki, M., Yamada, Y., Nakajima, Y., Nakayama, O., Makishima, M., Matsuda, M., and Shimomura, I. (2004) Increased oxidative stress in obesity and its impact on metabolic syndrome. *J Clin Invest* **114**, 1752-1761.
- Garcia, O.P., Ronquillo, D., Caamano Mdel, C., Camacho, M., Long, K.Z., and Rosado, J.L. (2012) Zinc, vitamin A, and vitamin C status are associated with leptin concentrations and obesity in Mexican women: results from a cross-sectional study. *Nutr Metab (Lond)* **9**, 59.



Gawecka, J.E., Marh, J., Ortega, M., Yamauchi, Y., Ward, M.A., and Ward, W.S. (2013) Mouse zygotes respond to severe sperm DNA damage by delaying paternal DNA replication and embryonic development. *PLoS One* **8**, e56385.

Geva, E., Bartoov, B., Zabludovsky, N., Lessing, J.B., Lerner-Geva, L., and Amit, A. (1996) The effect of antioxidant treatment on human spermatozoa and fertilization rate in an in vitro fertilization program. *Fertil Steril* **66**, 430-434.

Ghanayem, B.I., Bai, R., Kissling, G.E., Travlos, G., and Hoffler, U. (2010) Diet-induced obesity in male mice is associated with reduced fertility and potentiation of acrylamide-induced reproductive toxicity. *Biol Reprod* **82**, 96-104.

Gharagozloo, P., and Aitken, R.J. (2011) The role of sperm oxidative stress in male infertility and the significance of oral antioxidant therapy. *Hum Reprod* **26**, 1628-1640.

Gharagozloo, P., Gutierrez-Adan, A., Champroux, A., Noblanc, A., Kocer, A., Calle, A., Perez-Cerezales, S., Pericuesta, E., Polhemus, A., Moazamian, A., *et al.* (2016) A novel antioxidant formulation designed to treat male infertility associated with oxidative stress: promising preclinical evidence from animal models. *Hum Reprod* **31**, 252-262.

Ghosh, S., and Mukherjee, S. (2018) Testicular germ cell apoptosis and sperm defects in mice upon long-term high fat diet feeding. *J Cell Physiol*.

Gianicolo, E.A., Cresci, M., Ait-Ali, L., Foffa, I., and Andreassi, M.G. (2010) Smoking and congenital heart disease: the epidemiological and biological link. *Curr Pharm Des* **16**, 2572-2577.

Gillis, L., and Gillis, A. (2005) Nutrient inadequacy in obese and non-obese youth. *Can J Diet Pract Res* **66**, 237-242.

Gobato, R.C., Seixas Chaves, D.F., and Chaim, E.A. (2014) Micronutrient and physiologic parameters before and 6 months after RYGB. *Surg Obes Relat Dis* **10**, 944-951.

Gomez, E., Buckingham, D.W., Brindle, J., Lanzafame, F., Irvine, D.S., and Aitken, R.J. (1996) Development of an image analysis system to monitor the retention of residual cytoplasm by human spermatozoa: Correlation with biochemical markers of the cytoplasmic space, oxidative stress, and sperm function. *Journal of Andrology* **17**, 276-287. [In English]

Greco, E., Iacobelli, M., Rienzi, L., Ubaldi, F., Ferrero, S., and Tesarik, J. (2005) Reduction of the incidence of sperm DNA fragmentation by oral antioxidant treatment. *J Androl* **26**, 349-353.

Gregory, M., and Cyr, D.G. (2014) The blood-epididymis barrier and inflammation. *Spermatogenesis* **4**, e979619.

- Griswold, M.D. (1998) The central role of Sertoli cells in spermatogenesis. *Semin Cell Dev Biol* **9**, 411-416.
- Guerriero, G., Trocchia, S., Abdel-Gawad, F.K., and Ciarcia, G. (2014) Roles of Reactive Oxygen Species in the Spermatogenesis Regulation. *Front Endocrinol (Lausanne)* **5**, 56.
- Hammoud, A.O., Gibson, M., Peterson, C.M., Meikle, A.W., and Carrell, D.T. (2008) Impact of male obesity on infertility: a critical review of the current literature. *Fertil Steril* **90**, 897-904. [In eng]
- Hammoud, S.S., Nix, D.A., Hammoud, A.O., Gibson, M., Cairns, B.R., and Carrell, D.T. (2011) Genome-wide analysis identifies changes in histone retention and epigenetic modifications at developmental and imprinted gene loci in the sperm of infertile men. *Hum Reprod* **26**, 2558-2569.
- Hardy, G., and Hardy, I. (2004) Selenium: the Se-XY nutraceutical. *Nutrition* **20**, 590-593.
- Heller, C.G., and Clermont, Y. (1963) Spermatogenesis in man: an estimate of its duration. *Science* **140**, 184-186.
- Henkel, R., Kierspel, E., Stalf, T., Mehnert, C., Menkveld, R., Tinneberg, H.R., Schill, W.B., and Kruger, T.F. (2005) Effect of reactive oxygen species produced by spermatozoa and leukocytes on sperm functions in non-leukocytospermic patients. *Fertil Steril* **83**, 635-642.
- Henkel, R.R. (2011) Leukocytes and oxidative stress: dilemma for sperm function and male fertility. *Asian J Androl* **13**, 43-52.
- Herrero, M.B., and Gagnon, C. (2001) Nitric oxide: a novel mediator of sperm function. *J Androl* **22**, 349-356.
- Hill, J.W., Elmquist, J.K., and Elias, C.F. (2008) Hypothalamic pathways linking energy balance and reproduction. *Am J Physiol Endocrinol Metab* **294**, E827-832.
- Ho, H.C. (2010) Redistribution of nuclear pores during formation of the redundant nuclear envelope in mouse spermatids. *J Anat* **216**, 525-532.
- Ibrahim, H.A., Zhu, Y., Wu, C., Lu, C., Ezekwe, M.O., Liao, S.F., and Huang, K. (2012) Selenium-enriched probiotics improves murine male fertility compromised by high fat diet. *Biol Trace Elem Res* **147**, 251-260.
- Jensen, T.K., Andersson, A.M., Jorgensen, N., Andersen, A.G., Carlsen, E., Petersen, J.H., and Skakkebaek, N.E. (2004) Body mass index in relation to semen quality and reproductive hormones among 1,558 Danish men. *Fertil Steril* **82**, 863-870.

- Ji, B.T., Shu, X.O., Linet, M.S., Zheng, W., Wacholder, S., Gao, Y.T., Ying, D.M., and Jin, F. (1997) Paternal cigarette smoking and the risk of childhood cancer among offspring of nonsmoking mothers. *J Natl Cancer Inst* **89**, 238-244.
- Jiang, X.H., Bukhari, I., Zheng, W., Yin, S., Wang, Z., Cooke, H.J., and Shi, Q.H. (2014) Blood-testis barrier and spermatogenesis: lessons from genetically-modified mice. *Asian J Androl* **16**, 572-580.
- Johnson, S.L., Dunleavy, J., Gemmell, N.J., and Nakagawa, S. (2015) Consistent age-dependent declines in human semen quality: a systematic review and meta-analysis. *Ageing Res Rev* **19**, 22-33.
- Jones, R., Mann, T., and Sherins, R. (1979) Peroxidative Breakdown of Phospholipids in Human-Spermatozoa, Spermicidal Properties of Fatty-Acid Peroxides, and Protective Action of Seminal Plasma. *Fertility and Sterility* **31**, 531-537. [In English]
- Kao, S.H., Chao, H.T., Chen, H.W., Hwang, T.I., Liao, T.L., and Wei, Y.H. (2008) Increase of oxidative stress in human sperm with lower motility. *Fertil Steril* **89**, 1183-1190.
- Katib, A. (2015) Mechanisms linking obesity to male infertility. *Cent European J Urol* **68**, 79-85.
- Kefer, J.C., Agarwal, A., and Sabanegh, E. (2009) Role of antioxidants in the treatment of male infertility. *Int J Urol* **16**, 449-457.
- Keltz, J., Zapantis, A., Jindal, S.K., Lieman, H.J., Santoro, N., and Polotsky, A.J. (2010) Overweight men: clinical pregnancy after ART is decreased in IVF but not in ICSI cycles. *J Assist Reprod Genet* **27**, 539-544. [In eng]
- Keskes-Ammar, L., Feki-Chakroun, N., Rebai, T., Sahnoun, Z., Ghozzi, H., Hammami, S., Zghal, K., Fki, H., Damak, J., and Bahloul, A. (2003) Sperm oxidative stress and the effect of an oral vitamin E and selenium supplement on semen quality in infertile men. *Arch Androl* **49**, 83-94.
- Kessopoulou, E., Powers, H.J., Sharma, K.K., Pearson, M.J., Russell, J.M., Cooke, I.D., and Barratt, C.L. (1995) A double-blind randomized placebo cross-over controlled trial using the antioxidant vitamin E to treat reactive oxygen species associated male infertility. *Fertil Steril* **64**, 825-831.
- Kleinhaus, K., Perrin, M., Friedlander, Y., Paltiel, O., Malaspina, D., and Harlap, S. (2006) Paternal age and spontaneous abortion. *Obstet Gynecol* **108**, 369-377.
- Koppers, A.J., De Iuliis, G.N., Finnie, J.M., McLaughlin, E.A., and Aitken, R.J. (2008) Significance of mitochondrial reactive oxygen species in the generation of oxidative stress in spermatozoa. *Journal of Clinical Endocrinology & Metabolism* **93**, 3199-3207.

- Koppers, A.J., Garg, M.L., and Aitken, R.J. (2010) Stimulation of mitochondrial reactive oxygen species production by unesterified, unsaturated fatty acids in defective human spermatozoa. *Free Radic Biol Med* **48**, 112-119.
- Kort, H.I., Massey, J.B., Elsner, C.W., Mitchell-Leef, D., Shapiro, D.B., Witt, M.A., and Roudebush, W.E. (2006) Impact of body mass index values on sperm quantity and quality. *J Androl* **27**, 450-452. [In eng]
- Kovac, J.R., Khanna, A., and Lipshultz, L.I. (2015) The effects of cigarette smoking on male fertility. *Postgrad Med* **127**, 338-341.
- Krol, M.B., Gromadzinska, J., and Wasowicz, W. (2012) SeP, ApoER2 and megalin as necessary factors to maintain Se homeostasis in mammals. *J Trace Elem Med Biol* **26**, 262-266.
- La Vignera, S., Condorelli, R.A., Vicari, E., and Calogero, A.E. (2012) Negative effect of increased body weight on sperm conventional and nonconventional flow cytometric sperm parameters. *J Androl* **33**, 53-58.
- Lackner, J.E., Agarwal, A., Mahfouz, R., du Plessis, S.S., and Schatzl, G. (2010) The association between leukocytes and sperm quality is concentration dependent. *Reprod Biol Endocrinol* **8**, 12.
- Lakoski, K.A., Carron, C.P., Cabot, C.L., and Saling, P.M. (1988) Epididymal maturation and the acrosome reaction in mouse sperm: response to zona pellucida develops coincident with modification of M42 antigen. *Biol Reprod* **38**, 221-233.
- Lane, M., McPherson, N.O., Fullston, T., Spillane, M., Sandeman, L., Kang, W.X., and Zander-Fox, D.L. (2014) Oxidative stress in mouse sperm impairs embryo development, fetal growth and alters adiposity and glucose regulation in female offspring. *PLoS One* **9**, e100832.
- Leclerc, P., de Lamirande, E., and Gagnon, C. (1997) Regulation of protein-tyrosine phosphorylation and human sperm capacitation by reactive oxygen derivatives. *Free Radic Biol Med* **22**, 643-656.
- Lee, K.M., Ward, M.H., Han, S., Ahn, H.S., Kang, H.J., Choi, H.S., Shin, H.Y., Koo, H.H., Seo, J.J., Choi, J.E., *et al.* (2009) Paternal smoking, genetic polymorphisms in CYP1A1 and childhood leukemia risk. *Leuk Res* **33**, 250-258.
- Leibel, N.I., Baumann, E.E., Kocherginsky, M., and Rosenfield, R.L. (2006) Relationship of adolescent polycystic ovary syndrome to parental metabolic syndrome. *J Clin Endocrinol Metab* **91**, 1275-1283. [In eng]
- Leisegang, K., Bouic, P.J., Menkveld, R., and Henkel, R.R. (2014) Obesity is associated with increased seminal insulin and leptin alongside reduced fertility parameters in a controlled male cohort. *Reprod Biol Endocrinol* **12**, 34.

- Lenzi, A., Lombardo, F., Sgro, P., Salacone, P., Caponecchia, L., Dondero, F., and Gandini, L. (2003) Use of carnitine therapy in selected cases of male factor infertility: a double-blind crossover trial. *Fertil Steril* **79**, 292-300.
- Lenzi, A., Sgro, P., Salacone, P., Paoli, D., Gilio, B., Lombardo, F., Santulli, M., Agarwal, A., and Gandini, L. (2004) A placebo-controlled double-blind randomized trial of the use of combined l-carnitine and l-acetyl-carnitine treatment in men with asthenozoospermia. *Fertil Steril* **81**, 1578-1584.
- Levy, S., and Robaire, B. (1999) Segment-specific changes with age in the expression of junctional proteins and the permeability of the blood-epididymis barrier in rats. *Biol Reprod* **60**, 1392-1401.
- Li, L., Law, C., Lo Conte, R., and Power, C. (2009) Intergenerational influences on childhood body mass index: the effect of parental body mass index trajectories. *Am J Clin Nutr* **89**, 551-557. [In eng]
- Li, Z., Chen, G.W., Shang, X.J., Bai, W.J., Han, Y.F., Chen, B., Teng, X.M., Meng, F.H., Zhang, B., Chen, D.N., *et al.* (2005) [A controlled randomized trial of the use of combined L-carnitine and acetyl-L-carnitine treatment in men with oligoasthenozoospermia]. *Zhonghua Nan Ke Xue* **11**, 761-764.
- Linabery, A.M., Nahhas, R.W., Johnson, W., Choh, A.C., Towne, B., Odegaard, A.O., Czerwinski, S.A., and Demerath, E.W. (2013) Stronger influence of maternal than paternal obesity on infant and early childhood body mass index: the Fels Longitudinal Study. *Pediatr Obes* **8**, 159-169.
- Lord, T., and Aitken, R.J. (2015) Fertilization stimulates 8-hydroxy-2'-deoxyguanosine repair and antioxidant activity to prevent mutagenesis in the embryo. *Dev Biol* **406**, 1-13.
- Lundbaek, J.A., and Andersen, O.S. (1994) Lysophospholipids modulate channel function by altering the mechanical properties of lipid bilayers. *J Gen Physiol* **104**, 645-673.
- MacDonald, A.A., Herbison, G.P., Showell, M., and Farquhar, C.M. (2010) The impact of body mass index on semen parameters and reproductive hormones in human males: a systematic review with meta-analysis. *Hum Reprod Update* **16**, 293-311. [In eng]
- Macdonald, A.A., Stewart, A.W., and Farquhar, C.M. (2013) Body mass index in relation to semen quality and reproductive hormones in New Zealand men: a cross-sectional study in fertility clinics. *Hum Reprod* **28**, 3178-3187.
- MacLeod, J. (1943) The role of oxygen in the metabolism and motility of human spermatozoa. *American Journal of Physiology* **138**, 0512-0518. [In English]

- Mammi, C., Calanchini, M., Antelmi, A., Cinti, F., Rosano, G.M., Lenzi, A., Caprio, M., and Fabbri, A. (2012) Androgens and adipose tissue in males: a complex and reciprocal interplay. *Int J Endocrinol* **2012**, 789653.
- McCowan, L.M., North, R.A., Kho, E.M., Black, M.A., Chan, E.H., Dekker, G.A., Poston, L., Taylor, R.S., and Roberts, C.T. (2011) Paternal contribution to small for gestational age babies: a multicenter prospective study. *Obesity (Silver Spring)* **19**, 1035-1039.
- McPherson, N.O., Bakos, H.W., Owens, J.A., Setchell, B.P., and Lane, M. (2013) Improving metabolic health in obese male mice via diet and exercise restores embryo development and fetal growth. *PLoS One* **8**, e71459.
- McPherson, N.O., Bell, V.G., Zander-Fox, D.L., Fullston, T., Wu, L.L., Robker, R.L., and Lane, M. (2015) When two obese parents are worse than one! Impacts on embryo and fetal development. *Am J Physiol Endocrinol Metab* **309**, E568-581.
- McPherson, N.O., Fullston, T., Kang, W.X., Sandeman, L.Y., Corbett, M.A., Owens, J.A., and Lane, M. (2016) Paternal under-nutrition programs metabolic syndrome in offspring which can be reversed by antioxidant/vitamin food fortification in fathers. *Sci Rep* **6**, 27010.
- McPherson, N.O., and Lane, M. (2015) Male obesity and subfertility, is it really about increased adiposity? *Asian J Androl* **17**, 450-458.
- Mendis-Handagama, S.M. (1997) Luteinizing hormone on Leydig cell structure and function. *Histol Histopathol* **12**, 869-882.
- Miller, D., Brinkworth, M., and Iles, D. (2010) Paternal DNA packaging in spermatozoa: more than the sum of its parts? DNA, histones, protamines and epigenetics. *Reproduction* **139**, 287-301.
- Mitchell, L.A., De Iuliis, G.N., and Aitken, R.J. (2011a) The TUNEL assay consistently underestimates DNA damage in human spermatozoa and is influenced by DNA compaction and cell vitality: development of an improved methodology. *Int J Androl* **34**, 2-13.
- Mitchell, M., Bakos, H.W., and Lane, M. (2011b) Paternal diet-induced obesity impairs embryo development and implantation in the mouse. *Fertil Steril* **95**, 1349-1353.
- Moazamian, R., Polhemus, A., Connaughton, H., Fraser, B., Whiting, S., Gharagozloo, P., and Aitken, R.J. (2015) Oxidative stress and human spermatozoa: diagnostic and functional significance of aldehydes generated as a result of lipid peroxidation. *Mol Hum Reprod* **21**, 502-515.
- Monteiro, R., and Azevedo, I. (2010) Chronic inflammation in obesity and the metabolic syndrome. *Mediators Inflamm* **2010**.

- Morielli, T., and O'Flaherty, C. (2015) Oxidative stress impairs function and increases redox protein modifications in human spermatozoa. *Reproduction* **149**, 113-123.
- Mortazavi, M., Salehi, I., Alizadeh, Z., Vahabian, M., and Roushandeh, A.M. (2014) Protective Effects of Antioxidants on Sperm Parameters and Seminiferous Tubules Epithelium in High Fat-fed Rats. *J Reprod Infertil* **15**, 22-28.
- Mosbah, R., Yousef, M.I., and Mantovani, A. (2015) Nicotine-induced reproductive toxicity, oxidative damage, histological changes and haematotoxicity in male rats: the protective effects of green tea extract. *Exp Toxicol Pathol* **67**, 253-259.
- Mruk, D.D., and Cheng, C.Y. (2015) The Mammalian Blood-Testis Barrier: Its Biology and Regulation. *Endocr Rev* **36**, 564-591.
- Ng, S.-F., Lin, R.C.Y., Laybutt, D.R., Barres, R., Owens, J.A., and Morris, M.J. (2010) Chronic high-fat diet in fathers programs [bgr]-cell dysfunction in female rat offspring. *Nature* **467**, 963-966.
- Nguyen, R.H., Wilcox, A.J., Skjaerven, R., and Baird, D.D. (2007) Men's body mass index and infertility. *Hum Reprod* **22**, 2488-2493.
- Nobranc, A., Damon-Soubeyrand, C., Karrich, B., Henry-Berger, J., Cadet, R., Saez, F., Guiton, R., Janny, L., Pons-Rejraji, H., Alvarez, J.G., *et al.* (2013) DNA oxidative damage in mammalian spermatozoa: where and why is the male nucleus affected? *Free Radic Biol Med* **65**, 719-723.
- Nozha, C.F., Leila, A.K., Zouhir, S., Hanen, G., Khled, Z., and Tarek, R. (2001) Oxidative stress and male infertility: comparative study of combined vitamin E/selenium treatment versus vitamin B. *Human Reproduction* **16**, 111-112. [In English]
- O'Donnell, L., Nicholls, P.K., O'Bryan, M.K., McLachlan, R.I., and Stanton, P.G. (2011) Spermiation: The process of sperm release. *Spermatogenesis* **1**, 14-35.
- Oakberg, E.F. (1957) Duration of spermatogenesis in the mouse. *Nature* **180**, 1137-1138.
- Olson, G.E., Winfrey, V.P., Hill, K.E., and Burk, R.F. (2004) Sequential development of flagellar defects in spermatids and epididymal spermatozoa of selenium-deficient rats. *Reproduction* **127**, 335-342.
- Omu, A.E., Al-Azemi, M.K., Kehinde, E.O., Anim, J.T., Oriowo, M.A., and Mathew, T.C. (2008) Indications of the mechanisms involved in improved sperm parameters by zinc therapy. *Med Princ Pract* **17**, 108-116.
- Omu, A.E., Dashti, H., and Al-Othman, S. (1998) Treatment of asthenozoospermia with zinc sulphate: andrological, immunological and obstetric outcome. *Eur J Obstet Gynecol Reprod Biol* **79**, 179-184.

- Ozata, M., Mergen, M., Oktenli, C., Aydin, A., Sanisoglu, S.Y., Bolu, E., Yilmaz, M.I., Sayal, A., Isimer, A., and Ozdemir, I.C. (2002) Increased oxidative stress and hypozincemia in male obesity. *Clin Biochem* **35**, 627-631.
- Padayatty, S.J., Katz, A., Wang, Y., Eck, P., Kwon, O., Lee, J.H., Chen, S., Corpe, C., Dutta, A., Dutta, S.K., *et al.* (2003) Vitamin C as an antioxidant: evaluation of its role in disease prevention. *J Am Coll Nutr* **22**, 18-35.
- Palmer, N.O., Bakos, H.W., Fullston, T., and Lane, M. (2012a) Impact of obesity on male fertility, sperm function and molecular composition. *Spermatogenesis* **2**, 253-263.
- Palmer, N.O., Bakos, H.W., Owens, J.A., Setchell, B.P., and Lane, M. (2012b) Diet and exercise in an obese mouse fed a high-fat diet improve metabolic health and reverse perturbed sperm function. *Am J Physiol Endocrinol Metab* **302**, E768-780.
- Palmer, N.O., Fullston, T., Mitchell, M., Setchell, B.P., and Lane, M. (2011) SIRT6 in mouse spermatogenesis is modulated by diet-induced obesity. *Reprod Fertil Dev* **23**, 929-939.
- Pasquali, R., Patton, L., and Gambineri, A. (2007) Obesity and infertility. *Curr Opin Endocrinol Diabetes Obes* **14**, 482-487.
- Paul, C., Teng, S., and Saunders, P.T. (2009) A single, mild, transient scrotal heat stress causes hypoxia and oxidative stress in mouse testes, which induces germ cell death. *Biol Reprod* **80**, 913-919.
- Pauli, E.M., Legro, R.S., Demers, L.M., Kunselman, A.R., Dodson, W.C., and Lee, P.A. (2008) Diminished paternity and gonadal function with increasing obesity in men. *Fertil Steril* **90**, 346-351. [In eng]
- Pembrey, M.E. (2010) Male-line transgenerational responses in humans. *Hum Fertil (Camb)* **13**, 268-271.
- Pembrey, M.E., Bygren, L.O., Kaati, G., Edvinsson, S., Northstone, K., Sjöström, M., Golding, J., and Team, A.S. (2006) Sex-specific, male-line transgenerational responses in humans. *Eur J Hum Genet* **14**, 159-166.
- Petersen, G.L., Schmidt, L., Pinborg, A., and Kamper-Jørgensen, M. (2013) The influence of female and male body mass index on live births after assisted reproductive technology treatment: a nationwide register-based cohort study. *Fertil Steril* **99**, 1654-1662.
- Powell, S.R. (2000) The antioxidant properties of zinc. *J Nutr* **130**, 1447S-1454S.
- Qian, Y.X., Shen, P.J., Xu, R.Y., Liu, G.M., Yang, H.Q., Lu, Y.S., Sun, P., Zhang, R.W., Qi, L.M., and Lu, Q.H. (1986) Spermicidal effect in vitro by the active principle of garlic. *Contraception* **34**, 295-302.



Ramlau-Hansen, C.H., Thulstrup, A.M., Nohr, E.A., Bonde, J.P., Sorensen, T.I., and Olsen, J. (2007) Subfecundity in overweight and obese couples. *Hum Reprod* **22**, 1634-1637.

Ramos, L., and Wetzels, A.M. (2001) Low rates of DNA fragmentation in selected motile human spermatozoa assessed by the TUNEL assay. *Hum Reprod* **16**, 1703-1707.

Rao, A.V., Ray, M.R., and Rao, L.G. (2006) Lycopene. *Adv Food Nutr Res* **51**, 99-164.

Rathke, C., Baarends, W.M., Awe, S., and Renkawitz-Pohl, R. (2013) Chromatin dynamics during spermiogenesis. *Biochim Biophys Acta*.

Reichenberg, A., Gross, R., Weiser, M., Bresnahan, M., Silverman, J., Harlap, S., Rabinowitz, J., Shulman, C., Malaspina, D., Lubin, G., *et al.* (2006) Advancing paternal age and autism. *Arch Gen Psychiatry* **63**, 1026-1032.

Rengan, A.K., Agarwal, A., van der Linde, M., and du Plessis, S.S. (2012) An investigation of excess residual cytoplasm in human spermatozoa and its distinction from the cytoplasmic droplet. *Reprod Biol Endocrinol* **10**, 92.

Rivlin, J., Mendel, J., Rubinstein, S., Etkovitz, N., and Breitbart, H. (2004) Role of hydrogen peroxide in sperm capacitation and acrosome reaction. *Biol Reprod* **70**, 518-522.

Robaire, B., Hinton, B.T., and Orgebin-Crist, M. (2006) The Epididymis. In 'Knobil and Neill's Physiology of Reproduction.' (Ed. JD Neill). (Elsevier)

Rosety, I., Elosegui, S., Pery, M.T., Fornieles, G., Rosety, J.M., Diaz, A.J., Rodriguez-Pareja, A., Brenes, F., Rosety-Rodriguez, M., Ordonez, F.J., *et al.* (2014) [Association between abdominal obesity and seminal oxidative damage in adults with metabolic syndrome]. *Rev Med Chil* **142**, 732-737.

Roth, M.Y., Amory, J.K., and Page, S.T. (2008) Treatment of male infertility secondary to morbid obesity. *Nat Clin Pract Endocrinol Metab* **4**, 415-419.

Safarinejad, M.R. (2009) Efficacy of coenzyme Q10 on semen parameters, sperm function and reproductive hormones in infertile men. *J Urol* **182**, 237-248.

Safarinejad, M.R., and Safarinejad, S. (2009) Efficacy of selenium and/or N-acetylcysteine for improving semen parameters in infertile men: a double-blind, placebo controlled, randomized study. *J Urol* **181**, 741-751.

Sanchez-Gutierrez, M., Garcia-Montalvo, E.A., Izquierdo-Vega, J.A., and Del Razo, L.M. (2008) Effect of dietary selenium deficiency on the in vitro fertilizing ability of mice spermatozoa. *Cell Biol Toxicol* **24**, 321-329.

Sanocka, D., Miesel, R., Jedrzejczak, P., and Kurpisz, M.K. (1996) Oxidative stress and male infertility. *J Androl* **17**, 449-454.

Schliep, K.C., Mumford, S.L., Ahrens, K.A., Hotaling, J.M., Carrell, D.T., Link, M., Hinkle, S.N., Kissell, K., Porucznik, C.A., and Hammoud, A.O. (2015) Effect of male and female body mass index on pregnancy and live birth success after in vitro fertilization. *Fertil Steril* **103**, 388-395.

Scott, R., MacPherson, A., Yates, R.W., Hussain, B., and Dixon, J. (1998) The effect of oral selenium supplementation on human sperm motility. *Br J Urol* **82**, 76-80.

Shaman, J.A., Yamauchi, Y., and Ward, W.S. (2007) The sperm nuclear matrix is required for paternal DNA replication. *J Cell Biochem* **102**, 680-688.

Sharma, R.K., and Agarwal, A. (1996) Role of reactive oxygen species in male infertility. *Urology* **48**, 835-850.

Sharma, R.K., Pasqualotto, A.E., Nelson, D.R., Thomas, A.J., Jr., and Agarwal, A. (2001) Relationship between seminal white blood cell counts and oxidative stress in men treated at an infertility clinic. *J Androl* **22**, 575-583.

Sharpe, R.M. (2010) Environmental/lifestyle effects on spermatogenesis. *Philos Trans R Soc Lond B Biol Sci* **365**, 1697-1712.

Shimura, T., Inoue, M., Taga, M., Shiraishi, K., Uematsu, N., Takei, N., Yuan, Z.M., Shinohara, T., and Niwa, O. (2002) p53-dependent S-phase damage checkpoint and pronuclear cross talk in mouse zygotes with X-irradiated sperm. *Mol Cell Biol* **22**, 2220-2228.

Shiraishi, K., Takihara, H., and Matsuyama, H. (2010) Elevated scrotal temperature, but not varicocele grade, reflects testicular oxidative stress-mediated apoptosis. *World J Urol* **28**, 359-364.

Showell, M.G., Brown, J., Yazdani, A., Stankiewicz, M.T., and Hart, R.J. (2011) Antioxidants for male subfertility. *Cochrane Database Syst Rev*, CD007411. [In eng]

Showell, M.G., Mackenzie-Proctor, R., Brown, J., Yazdani, A., Stankiewicz, M.T., and Hart, R.J. (2014) Antioxidants for male subfertility. *Cochrane Database Syst Rev*, CD007411.

Sipos, A., Rasmussen, F., Harrison, G., Tynelius, P., Lewis, G., Leon, D.A., and Gunnell, D. (2004) Paternal age and schizophrenia: a population based cohort study. *BMJ* **329**, 1070.

Smith, T.B., Dun, M.D., Smith, N.D., Curry, B.J., Connaughton, H.S., and Aitken, R.J. (2013) The presence of a truncated base excision repair pathway in human spermatozoa that is mediated by OGG1. *J Cell Sci* **126**, 1488-1497.

- Soler, C., Yeung, C.H., and Cooper, T.G. (1994) Development of sperm motility patterns in the murine epididymis. *Int J Androl* **17**, 271-278.
- Soubry, A., Hoyo, C., Jirtle, R.L., and Murphy, S.K. (2014) A paternal environmental legacy: evidence for epigenetic inheritance through the male germ line. *Bioessays* **36**, 359-371.
- Sprando, R.L., and Russell, L.D. (1987) Comparative study of cytoplasmic elimination in spermatids of selected mammalian species. *Am J Anat* **178**, 72-80.
- Storey, B.T. (1997) Biochemistry of the induction and prevention of lipoperoxidative damage in human spermatozoa. *Molecular Human Reproduction* **3**, 203-213. [In English]
- Stuppia, L., Franzago, M., Ballerini, P., Gatta, V., and Antonucci, I. (2015) Epigenetics and male reproduction: the consequences of paternal lifestyle on fertility, embryo development, and children lifetime health. *Clin Epigenetics* **7**, 120.
- Suleiman, S.A., Ali, M.E., Zaki, Z.M., el-Malik, E.M., and Nasr, M.A. (1996) Lipid peroxidation and human sperm motility: protective role of vitamin E. *J Androl* **17**, 530-537.
- Swan, S.H., Elkin, E.P., and Fenster, L. (2000) The question of declining sperm density revisited: an analysis of 101 studies published 1934-1996. *Environ Health Perspect* **108**, 961-966.
- Taha, E.A., Sayed, S.K., Gaber, H.D., Abdel Hafez, H.K., Ghandour, N., Zahran, A., and Mostafa, T. (2016) Does being overweight affect seminal variables in fertile men? *Reprod Biomed Online* **33**, 703-708.
- Teerds, K.J., de Rooij, D.G., and Keijer, J. (2011) Functional relationship between obesity and male reproduction: from humans to animal models. *Hum Reprod Update* **17**, 667-683. [In eng]
- Traber, M.G., and Atkinson, J. (2007) Vitamin E, antioxidant and nothing more. *Free Radic Biol Med* **43**, 4-15.
- Trasler, J.M. (2009) Epigenetics in spermatogenesis. *Mol Cell Endocrinol* **306**, 33-36.
- Tremellen, K., Miari, G., Froiland, D., and Thompson, J. (2007) A randomised control trial examining the effect of an antioxidant (Menevit) on pregnancy outcome during IVF-ICSI treatment. *Aust N Z J Obstet Gynaecol* **47**, 216-221.
- Tremellen, K., and Tunc, O. (2010) Macrophage activity in semen is significantly correlated with sperm quality in infertile men. *Int J Androl* **33**, 823-831.

- Tufek, N.H., Altunkaynak, M.E., Altunkaynak, B.Z., and Kaplan, S. (2015) Effects of thymoquinone on testicular structure and sperm production in male obese rats. *Syst Biol Reprod Med* **61**, 194-204.
- Tunc, O., Bakos, H.W., and Tremellen, K. (2011) Impact of body mass index on seminal oxidative stress. *Andrologia* **43**, 121-128.
- Turner, T.T. (1991) Spermatozoa are exposed to a complex microenvironment as they traverse the epididymis. *Ann N Y Acad Sci* **637**, 364-383.
- Umul, M., Kose, S.A., Bilen, E., Altuncu, A.G., Oksay, T., and Guney, M. (2015) Effect of increasing paternal body mass index on pregnancy and live birth rates in couples undergoing intracytoplasmic sperm injection. *Andrologia* **47**, 360-364.
- Walker, W.H. (2011) Testosterone signaling and the regulation of spermatogenesis. *Spermatogenesis* **1**, 116-120.
- Ward, W.S. (2010) Function of sperm chromatin structural elements in fertilization and development. *Mol Hum Reprod* **16**, 30-36.
- Wassarman, P.M. (1999) Mammalian fertilization: molecular aspects of gamete adhesion, exocytosis, and fusion. *Cell* **96**, 175-183.
- WHO (2015) Obesity and Overweight, Fact Sheet no. 311, Body Mass Index Classifications. World Health Organization.
- WHO (2016) Revised Glossary on Assisted Reproductive Terminology, Clinical definitions; International Committee for Monitoring Assisted Reproductive Technology.
- Wong, W.Y., Merkus, H.M., Thomas, C.M., Menkveld, R., Zielhuis, G.A., and Steegers-Theunissen, R.P. (2002) Effects of folic acid and zinc sulfate on male factor subfertility: a double-blind, randomized, placebo-controlled trial. *Fertil Steril* **77**, 491-498.
- Yan, W.J., Mu, Y., Yu, N., Yi, T.L., Zhang, Y., Pang, X.L., Cheng, D., and Yang, J. (2015) Protective effects of metformin on reproductive function in obese male rats induced by high-fat diet. *J Assist Reprod Genet* **32**, 1097-1104.
- Yang, Q., Zhao, F., Hu, L., Bai, R., Zhang, N., Yao, G., and Sun, Y. (2016) Effect of paternal overweight or obesity on IVF treatment outcomes and the possible mechanisms involved. *Sci Rep* **6**, 29787.
- Yasuno, Y., Kawano, J., Inoue, Y.H., and Yamamoto, M.T. (2013) Distribution and morphological changes of the Golgi apparatus during *Drosophila* spermatogenesis. *Dev Growth Differ* **55**, 635-647.
- Yeung, E.H., Sundaram, R., Ghassabian, A., Xie, Y., and Buck Louis, G. (2017) Parental Obesity and Early Childhood Development. *Pediatrics* **139**.

Zareba, P., Colaci, D.S., Afeiche, M., Gaskins, A.J., Jorgensen, N., Mendiola, J., Swan, S.H., and Chavarro, J.E. (2013) Semen quality in relation to antioxidant intake in a healthy male population. *Fertil Steril* **100**, 1572-1579.

Zini, A., San Gabriel, M., and Libman, J. (2010) Lycopene supplementation in vitro can protect human sperm deoxyribonucleic acid from oxidative damage. *Fertil Steril* **94**, 1033-1036.

## Chapter 2

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### **Materials and Methods**

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## **| 2.1 MOUSE EXPERIMENTAL METHODS**

### **2.1.1 Animal ethics approval**

The use and care of all mice were approved by The University of Adelaide's Animal Ethics Committee (project number M-2013-165B) and were handled in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (NHMRC, 2013).

### **2.1.2 Animals and the initial dietary regimen**

Five-week old male C57BL6 mice (n=144) were randomly assigned to the first phase of the dietary regimen; a control diet (CD) or high fat diet (HFD) for a duration of 10 weeks (Specialty Feeds, Perth, Australia, Figure 2.1). Mice were housed individually and were maintained in a 14:10 hour light:dark cycle with *ad libitum* access to food and water. Individual body weights were recorded weekly.

### **2.1.3 Animals and the intervention dietary regimen**

After 10 weeks of the initial dietary regimen, male mice were assigned to the second dietary phase; one of four diets whereby mice initially fed a CD either (i) continued a CD, or were fed a (ii) CD supplemented with micronutrients (CD+S; Specialty Feeds, Table 2.1), and mice initially fed a HFD either (iii) continued a HFD or were fed a (iv) HFD supplemented with micronutrients (HFD+S; Specialty Feeds, Table 2.1).

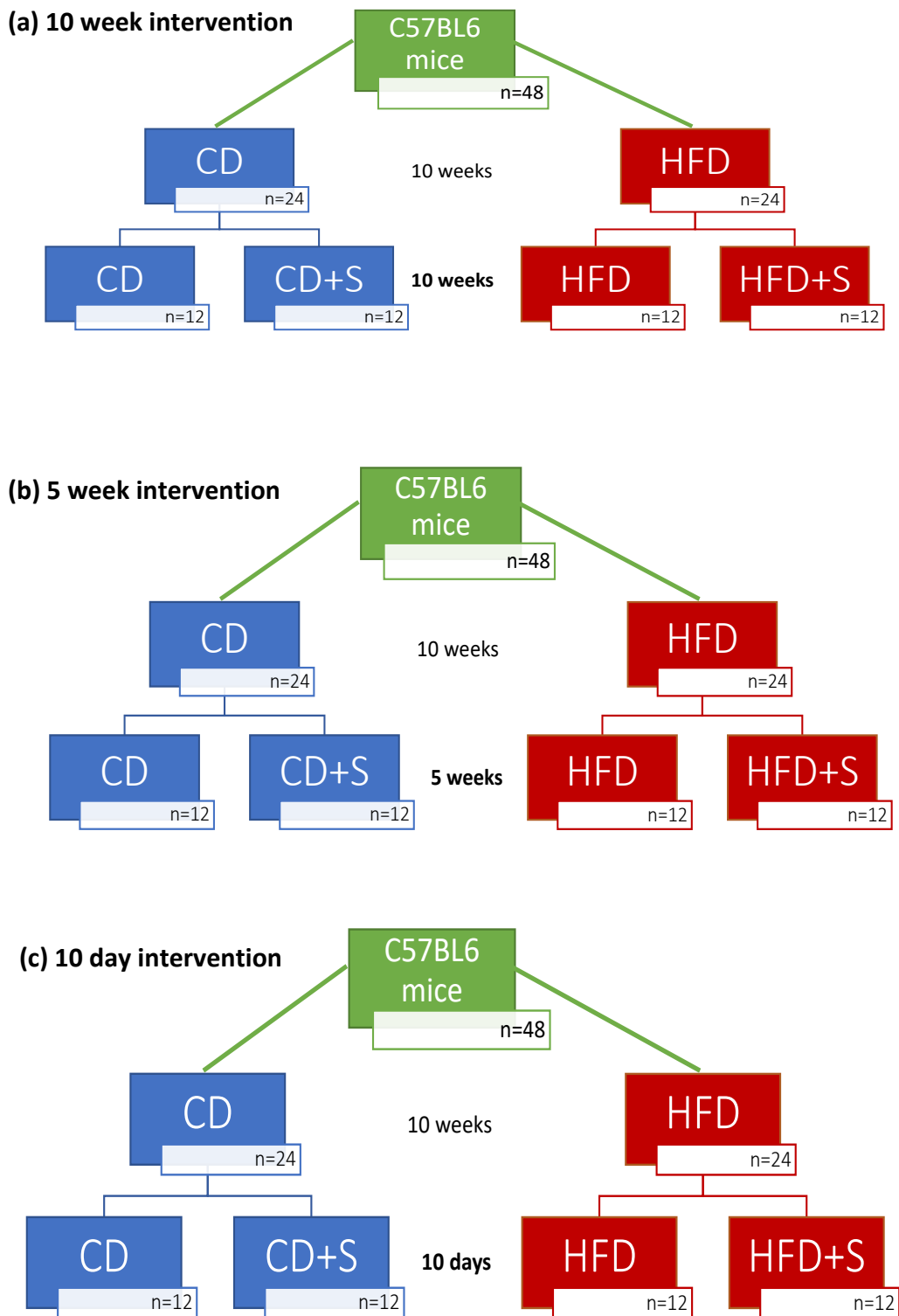
Mice were fed the second dietary regimen for one of 3 time periods:

(i) 10 weeks to approximate the duration of two rounds of spermatogenesis (Oakberg, 1957) (Figure 2.1); or



**(ii)** 5 weeks to approximate the duration of one round of spermatogenesis (Oakberg, 1957) (Figure 2.1); or

**(iii)** 10 days to approximate the duration of sperm epididymal transit (Robaire, et al., 2006) (Figure 2.1).



**Figure 2.1:** The distribution of mice to different durations of dietary intervention of micronutrient supplementation. All mice were allocated to a first dietary phase of a CD or a HFD for 10 weeks. For the second dietary phase, mice were allocated to either a no

intervention control group (CD, HFD) or a micronutrient supplementation intervention group (CD+S, HFD+S) for **(a)** 10 weeks, **(b)** 5 weeks or **(c)** 10 days. CD, control diet; HFD, high fat diet; CD+S, control diet plus micronutrient supplements; HFD+S, high fat diet plus micronutrient supplements.

**Table 2.1:** The micronutrients used to supplement the control diet and high fat diet.

<b>Vitamins, minerals and micronutrients (mg/kg)</b>	
Zinc (zinc sulphate monohydrate)	9.0
Selenium	0.14
Lycopene	0.3
Vitamin E (alpha-tocopherol acetate)	14.0
Vitamin C (ascorbic acid)	700
Folic acid	0.5
Green tea extract	0.95

## **2.1.4 Metabolic assessment**

### ***2.1.4.1 Glucose tolerance and insulin tolerance tests***

An intraperitoneal (ip) glucose tolerance test (GTT) and an ip insulin tolerance test (ITT) were performed at 9-10 weeks of the first dietary regimen and again at the end of the second dietary period (10 weeks, 5 weeks or 10 days). The GTT was conducted after 6 hours of fasting by ip injection of 2 g/kg 25% glucose solution (Sigma-Aldrich, St. Louis, MO, USA, Appendix; A1 and A2) and the ITT was conducted by ip injection of 0.75 IU/kg insulin (Actapid; Novo Nordisk, Bagsvaerd, Denmark, Appendix; A1 and A3) (Fullston, et al., 2013). Tail blood glucose concentrations were measured using a glucometer (HemoCue, Angelholm, Sweden) at 0 (pre-injection), and 15, 30, 60 and 120 minutes post-injection. Data were expressed as means of blood glucose concentration per group as area under the curve (AUC) for the GTT or area above the curve (AAC) for the ITT.

### **2.1.5 Body composition using DEXA**

At 9-10 weeks of the first dietary regimen, whole body composition including adipose tissue content, lean mass, and bone mass were measured in male mice using a dual-emission X-ray absorptiometry machine (DEXA; Piximus, Ge Lunar, Wisconsin). Male mice were fasted for 4 hours before administration of the anesthetic 0.02% Avertin (2-2-2 tribromethanol; Sigma-Aldrich, Appendix; A4) before their length was measured and then placed into the DEXA machine in the prone position. The DEXA machine provides quantitative data for fat tissue content, lean tissue content, and the total tissue mass based on the attenuation of two energy levels of X-ray absorptiometry (Nagy and Clair, 2000). One scan per mouse was performed and analyzed using the Piximus software (version

2.1, Piximus). After the analysis, each mouse was returned to their original housing and monitored until they were fully conscious.

#### **2.1.6 Body composition assessment at post-mortem**

At the end of their second dietary regimen, mice were fasted overnight, killed via cervical dislocation, and then underwent a post-mortem assessment blinded to supplementation status. Perirenal fat, retroperitoneal fat, dorsal fat, omental fat, gonadal fat, testes, seminal vesicles, soleus, vastus lateralis, liver, kidneys, and pancreas were dissected, collected, and weighed. Body organ and adipose tissue mass were expressed in absolute values and as a proportion of total body weight.

#### **2.1.7 Serum metabolite assessment**

At post-mortem, fasted blood samples were collected via a cardiac puncture and serum was isolated. For metabolites, serum was diluted 1:2 in Milli-Q water (Merck Millipore, Darmstadt, Germany) and glucose, cholesterol, high-density lipoproteins (HDLs), triglycerides, and non-esterified free fatty acids (NEFAs) concentrations were assessed using a COBAS Mira automated sample system (Roche Diagnostics, Basel, Switzerland) by an accredited pathology laboratory at the University of Adelaide.

#### **2.1.8 Serum testosterone**

Serum was diluted 1:10 in a dilution buffer (provided by the ELISA kit) and testosterone concentrations were measured using a commercially available ELISA kit (R&D Systems, Minneapolis, MN, USA) at 540nm absorbance as per the manufacturer's instructions (3.4% intra-assay and 6.2% inter-assay coefficients of variation).

### **2.1.9 Serum C-reactive Protein**

Serum C-Reactive Protein (CRP) concentrations were measured by diluting the serum 1:10 in a dilution buffer (provided by the ELISA kit) and using a commercially available ELISA kit (Abcam, Cambridge, UK) at 450nm absorbance as per the manufacturer's instructions (<10% intra-assay and inter-assay coefficients of variation).

### **2.1.10 Mature sperm isolation**

At post-mortem, mature sperm was collected from the vas deferens and caudal epididymis from each mouse. The vas deferens was cut approximately 0.5cm away from the epididymis, and the epididymis was dissected away from the gonadal fat pad and the testis and placed into a dish of warmed handling media (G-MOPS Plus; Vitrolife AB, Göteborg, Sweden). The dissected vas deferens and caudal epididymis were transferred into a dish containing 1 ml of pre-equilibrated G-IVF +10% human serum albumin (HSA; Vitrolife) whereby forceps were glided along the vas deferens to release sperm and the epididymis was punctured to release additional sperm. Sperm dishes were incubated at 37°C for 10 minutes in 5% O<sub>2</sub>, 6% CO<sub>2</sub> and 89% N<sub>2</sub> for sperm to disperse.

### **2.1.11 Conventional sperm parameters assessment**

Sperm assessments were conducted after 10 minutes of incubation in G-IVF +10% HSA (Vitrolife) at 37°C to allow for dispersion of the sperm. All sperm assessments were performed blinded to the dietary treatment groups as mice from different diet groups were assessed in a random order by giving mice random ID numbers from the post-mortem stage.

### ***2.1.11.1 Sperm motility***

Two drops of 10µl of sperm were loaded onto a slide and sperm motility was determined manually under x40 magnification by counting duplicate samples of 200 sperm for categorization as motile, non-progressive motile, or immotile (WHO, 2010). Sperm motility was expressed as a percentage of motile and non-progressive motile sperm per sample.

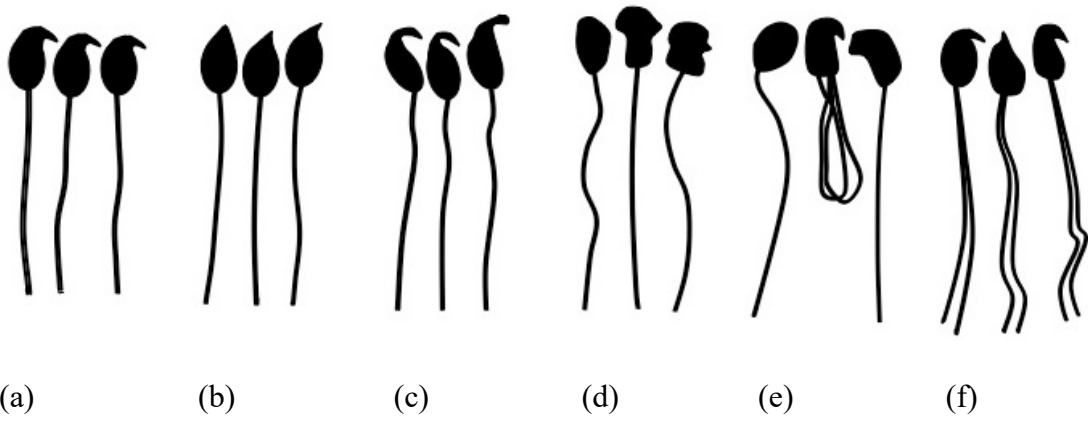
### ***2.1.11.2 Sperm concentration***

Sperm concentration was assessed using a Neubauer haemocytometer in accordance with the World Health Organization (WHO) guidelines (WHO, 2010). Sperm were immobilized by mixing 5µl of sperm with 95µl of water, and 10µl of the immobilized sperm mixture was loaded on the top and bottom chambers of the haemocytometer. Five squares of both the top and bottom haemocytometer chambers were counted and averaged to calculate the concentration of each sperm sample.

### ***2.1.11.3 Sperm morphology***

Sperm slides were fixed in 3:1 methanol:acetone (Sigma-Aldrich) for 10 minutes, air dried and stained with haematoxylin and eosin. Slides were stained in haematoxylin (Abcam) for 2-3 minutes, washed in running tap water and then stained with eosin (Abcam) for 3-5 minutes. Sperm morphology was determined by assessing 200 individual sperm per mouse and classified as normal, having an abnormal tail, or an abnormal head (Wyrobek and Bruce, 1975) (Figure 2.2).





**Figure 2.2:** Sperm morphology classifications; (a) normal, whereas (b)-(f) are classified as morphologically abnormal sperm. Sperm (b) lack the usual hook, (c) have a banana-like form, (d) are amorphous, (e) folded and (f) have two tails.

## **2.1.12 Sperm intracellular reactive oxygen species**

### ***2.1.12.1 Global measure of sperm intracellular reactive oxygen species***

Sperm intracellular reactive oxygen species (ROS), including peroxynitrite and hydroxyl radicals (Purdey, et al., 2015), were assessed by incubating progressively motile sperm with 1 $\mu$ M 2',7'-dichlorodihydrofluorescein diacetate (H<sub>2</sub>DCFDA; Molecular Probes, Eugene, USA) for 20 minutes at 37°C (Bakos, et al., 2010, Fullston, et al., 2012, Lane, et al., 2014). Sperm were then washed twice in phosphate buffered saline (PBS, Sigma-Aldrich) and imaged individually using a fluorescence microscope with a photometer attachment. Sperm samples were assessed as a ratiometric analysis to 5-(and 6-) carboxy-2',7'-dichlorofluorescein diacetate (carboxy- H<sub>2</sub>DCFDA; Molecular Probes) as a measure of sperm intracellular esterase activity, which controls variation in cellular esterase that may affect H<sub>2</sub>DCFDA as a probe (Halliwell and Whiteman, 2004). The mean relative fluorescence was measured for a minimum of 20 motile sperm per mouse and expressed in mean fluorescence units normalized to the CD (i.e. CD was set to 1.0).

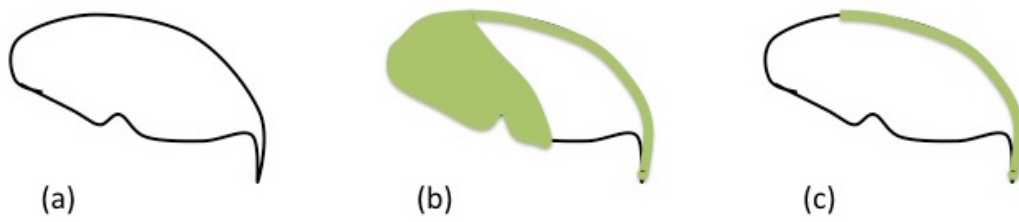
### ***2.1.12.2 Sperm intracellular superoxide***

Sperm mitochondrial superoxide production was assessed by incubating sperm in MitoSOX Red (2 $\mu$ M in dimethyl sulfoxide (DMSO); Molecular Probes) for 30 minutes at 37°C (Koppers, et al., 2008). Sperm were then centrifuged for 5 minutes at 200 g, the supernatant was removed, sperm were washed in 500 $\mu$ l of G-MOPS (Vitrolife) for 5 minutes at 200 g and finally re-suspended in 200 $\mu$ l of G-MOPS. MitoSOX Red (MSR) fluorescence was measured on a fluorescence-activated cell sorting (FACS) Canto flow cytometer (BD Bioscience, North Ryde, Australia). A sample of DMSO in G-MOPS was included as a negative control. Viable sperm were selected and 10,000 sperm cells were

examined per mouse and results were expressed as a percentage of sperm positive for MSR.

### **2.1.13 Sperm capacitation**

Sperm were incubated in G-IVF + HSA to allow spontaneous capacitation for 1 hour in 5% O<sub>2</sub>, 6% CO<sub>2</sub>, and 89% N<sub>2</sub> at 37°C. Sperm were then washed in PBS before incubation in *Arachis hypogaea* (peanut) agglutinin (Lectin PNA) Alexa 594 antibody (Molecular Probes) at 1:100 in PBS for 45 minutes and counter-stained with Hoechst to identify sperm nuclei (Baker, et al., 2004). A minimum of 200 sperm were classified as capacitated, non-capacitated, or acrosome reacted (Figure 2.3).



**Figure 2.3:** Changes in *Arachis hypogaea* (peanut) agglutinin (Lectin PNA) fluorescence with the colour green depicting fluorescent staining patterns of sperm membrane dynamics for (a) non-capacitated, (b) capacitated, and (c) acrosome reacted sperm.

## **2.1.14 Sperm binding and fertilization**

### ***2.1.14.1 Cumulus-oocyte complex collection***

C57BL/6 x CBA/ca (B6 CBAF1) female mice at 4-5 weeks old were administered an ip injection of 5 IU pregnant mare serum gonadotrophin (PMSG; Folligon, Intervet, Bendigo East, Australia), followed 48 hours later with an ip injection of 5 IU human chorionic gonadotrophin (hCG; Pregnyl, Organon, Australia) to induce ovulation (Gardner, et al., 2004).

Twelve-thirteen hours post hCG (Pregnyl) injection, mice were killed via cervical dislocation and oviducts were dissected from each female mouse and placed in warmed handling media (G-MOPS Plus; Vitrolife) immediately after dissection. The cumulus oocyte complexes (COCs), located in the oviduct, were expelled into G-IVF + 10% HSA (Vitrolife) after tearing the swollen ampulla region of the oviduct using forceps and COCs were placed in 80µl drops of pre-equilibrated G-IVF + 10% HSA in 5% O<sub>2</sub>, 6% CO<sub>2</sub> and 89% N<sub>2</sub> at 37°C.

### ***2.1.14.2 Sperm-oocyte binding and fertilization***

Sperm samples from males were collected from the vas deferens and capacitated for 1 hour in G-IVF + 10% HSA (Vitrolife) medium as described above in **2.1.10**. After 1 hour, COCs were inseminated with  $1 \times 10^4$ /ml of motile sperm and co-incubated in 5% O<sub>2</sub>, 6% CO<sub>2</sub> and 89% N<sub>2</sub> for 4 hours at 37 °C. At 4 hours post-insemination, sperm binding was assessed by counting the number of sperm bound to the zona pellucida of each oocyte using phase contrast microscopy.

Following oocyte insemination as described above in **2.1.14.2**, oocytes were washed once in both G-IVF + 10% HSA (Vitrolife) and G1 Plus (Vitrolife) media to finally be placed in G1 Plus media for culture in 5% O<sub>2</sub>, 6% CO<sub>2</sub> and 89% N<sub>2</sub> at 37°C. At 24 hours post insemination, fertilization rates were assessed by the presence of a two-cell embryo and expressed as a percentage of the total number of oocytes inseminated.

#### **2.1.15 Female mice superovulation and mating**

C57BL6 female mice at 3-4 weeks old were superovulated with an ip injection of 5 IU PMSG (Folligon), followed 48 hours later with an ip injection of 5 IU hCG (Pregnyl) to induce ovulation (Gardner, et al., 2004). Following the hCG injection, female mice were housed individually with a C57BL6 male mouse from the 10-day dietary intervention regimen overnight (Figure 2.1). Mating was assessed by the presence of a vaginal plug the following morning.

#### **2.1.16 Pronuclear-staged embryo collection**

Pronuclear-staged embryos were collected at 18-19 hours post hCG (Santos, et al., 2002) by dissecting the oviducts from each female and tearing the oviduct in G-MOPS Plus (Vitrolife) at 37°C to expel the embryos. The embryos were then denuded of cumulus cells by 1 minute incubation with 0.5 mg/ml hyaluronidase and fixed overnight in 4% paraformaldehyde at 4°C.

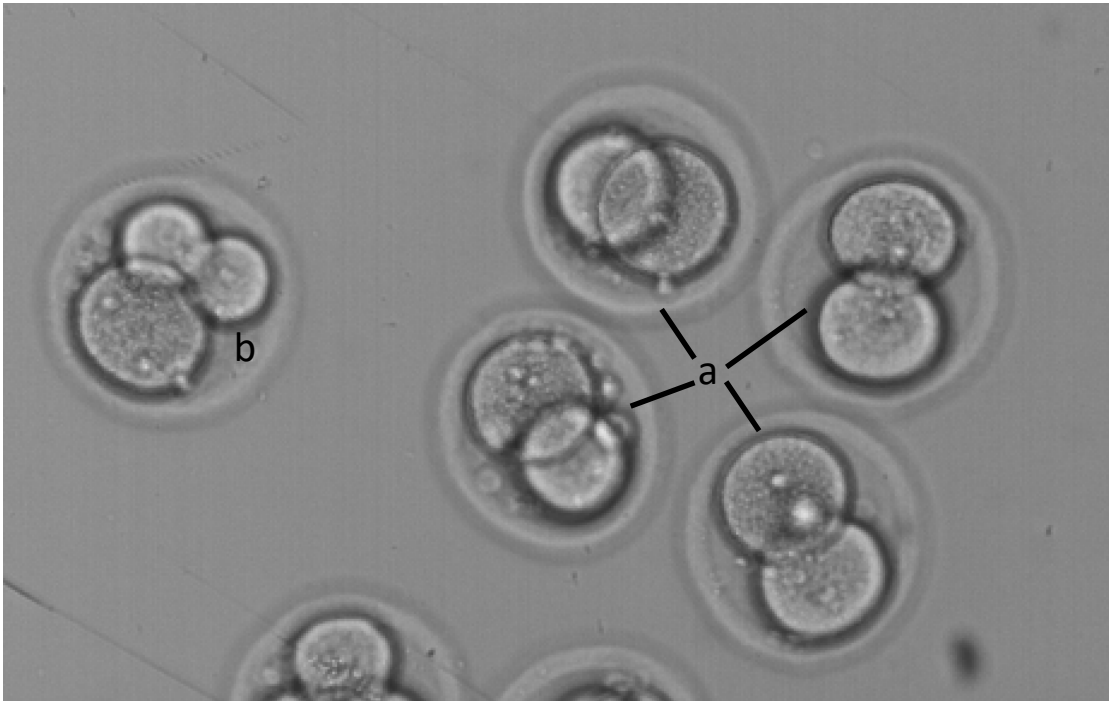
#### **2.1.17 Embryo culture and development**

Following 22–24 hours of the hCG (Pregnyl) injection, oviducts were dissected from each female mouse and placed in G-MOPS Plus (Vitrolife) at 37°C and cumulus-enclosed zygotes were isolated as above. Zygotes were washed twice in G-MOPS Plus (Vitrolife)

and once in G1 Plus medium (Vitrolife) before culture in 20µl drops (up to 10 zygotes per drop) of G1 Plus culture medium in 5% O<sub>2</sub>, 6% CO<sub>2</sub> and 89% N<sub>2</sub> at 37°C (Gardner, et al., 2004). On day 3 of embryo culture (67 hours post hCG administration), embryos were washed and cultured in G2 Plus culture medium (Vitrolife) to the blastocyst stage. All embryo culture dishes were prepared at least 4 hours prior to embryo culture to allow for gassing and temperature equilibration.

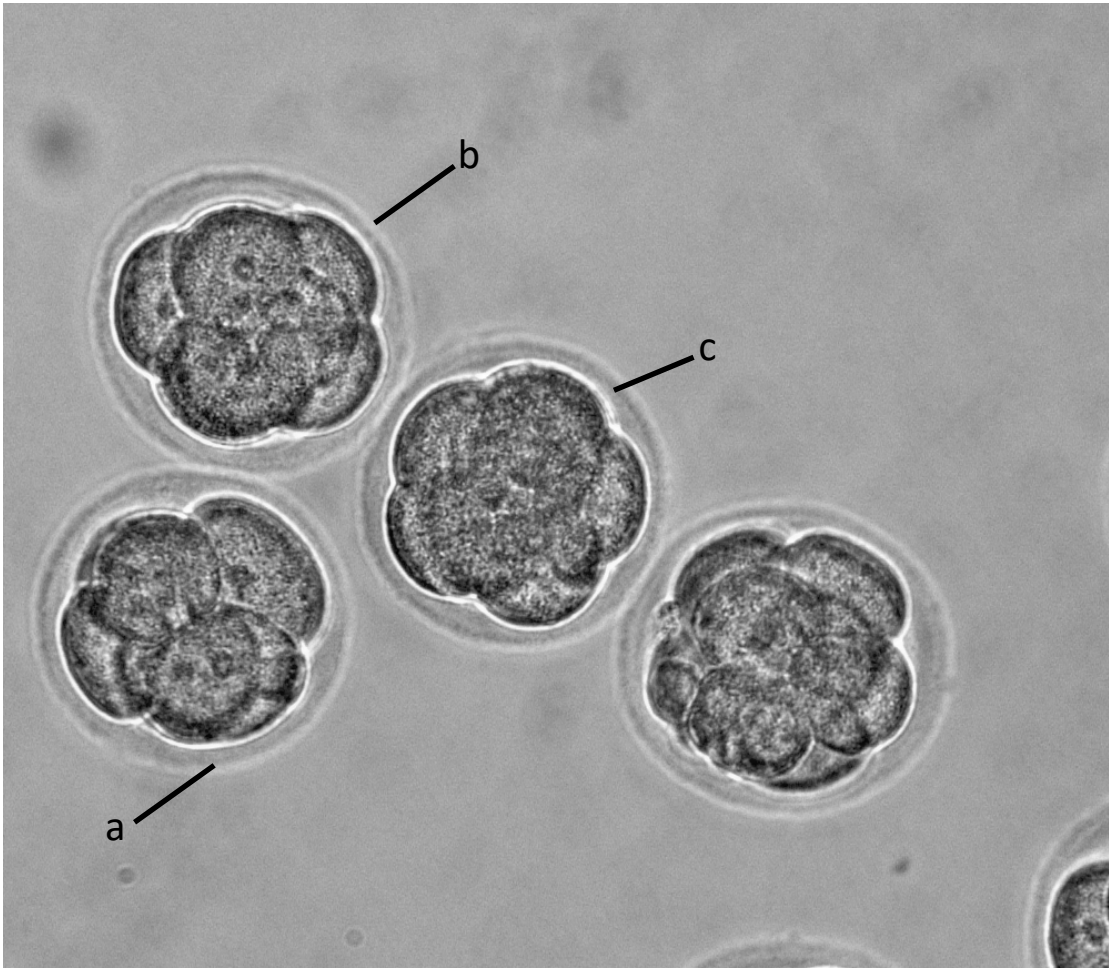
#### ***2.1.17.1 Embryo morphology***

Fertilization was denoted by day 2 cleavage to the 2-cell stage (Figure 2.4) at 43 hours post hCG. On-time embryo development assessments were evaluated for 8-cell development (Figure 2.5) at 67 hours post hCG (day 3). On-time blastocyst development (Figure 2.6) was determined at 98 hours post hCG (day 4) in which embryos containing a blastocoel cavity were classified as blastocysts. At 115 hours post hCG (day 5); blastocyst expansion and hatching blastocysts (Figure 2.7) were assessed whereby hatching blastocysts were identified by herniation of the trophectoderm through the zona pellucida of the blastocyst. Day 3, 4 and 5 of embryo development was expressed as a percentage of 2-cell cleavage.



**Figure 2.4:** A representative image of day 2 embryo development with (a) 2-cell and (b) 3-cell embryos.





**Figure 2.5:** A representative image of day 3 embryo development with (a) 6-cell, (b) 8-cell and (c) compacting embryos.



**Figure 2.6:** A representative image of day 4 embryo development with (a) early blastocysts and (b) blastocysts.



**Figure 2.7:** A representative image of day 5 embryo development with (a) expanded and (b) hatching blastocysts.

## **2.1.18 8-OHdG DNA lesion detection in mature and testicular sperm**

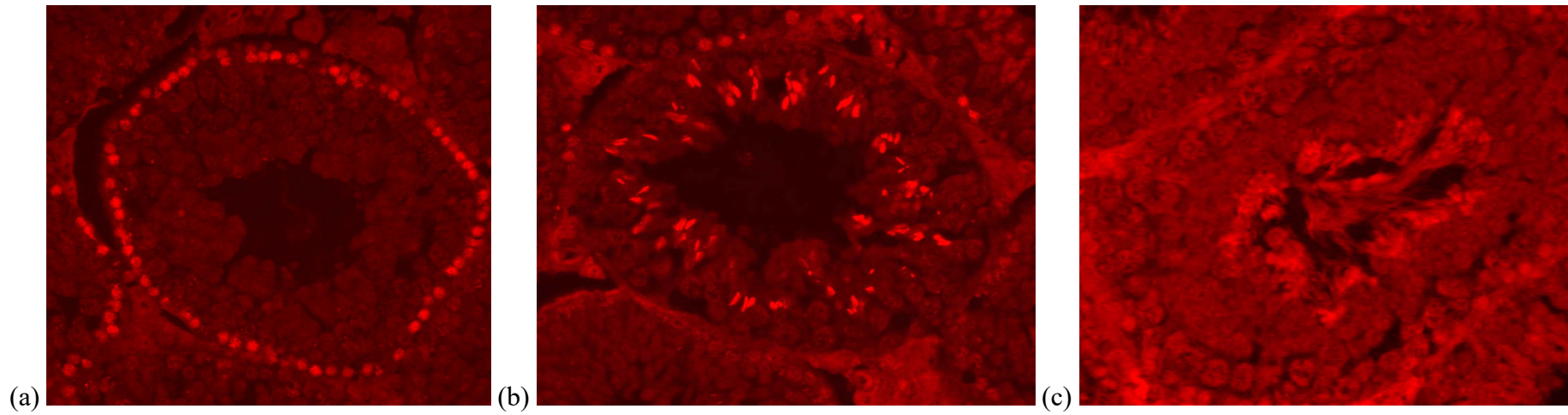
### ***2.1.18.1 Sperm 8-OHdG immunocytochemistry***

At postmortem, mature sperm collected from the vas deferens and caudal epididymis from each mouse was fixed on slides with 3:1 methanol:acetone (Sigma Aldrich) for 10 minutes. Slides were permeabilised in 0.5% Triton X-100 (Sigma Aldrich) and washed in PBS (Sigma Aldrich). Slides were incubated in decondensing buffer (1M hydrogen chloride (HCl), 10mM Tris buffer of 5mM dithiothreitol, Sigma Aldrich) for 60 minutes at 37°C. Sperm DNA was then denatured with a denaturing buffer (6M HCL, 0.1% Triton X-100 in H<sub>2</sub>O, Sigma Aldrich) at room temperature for 45 minutes and then neutralized with a neutralizing buffer (100uM Tris HCl in H<sub>2</sub>O, Sigma Aldrich) for 20 minutes. Slides were washed and then incubated overnight at 4°C in 1:100 mouse anti-8-hydroxyguanosine (8-OHdG) antibody (ab48508; Abcam) in 10% donkey serum (Abcam) whereas the negative control was incubated in 1:100 mouse serum. On the second day, slides were washed and incubated in 1:100 biotin-SP-conjugated AffiniPure donkey anti-mouse IgG (Jackson ImmunoResearch, Baltimore, PA, USA) for 2 hours at room temperature. Slides were then washed in PBS and incubated in 1:100 Cy3-conjugated streptavidin (Jackson ImmunoResearch) for 1.5 hours at room temperature followed by a nuclear counter stain with Hoechst. Finally, slides were loaded in ProLong Gold and glycerol solution (2 drops of ProLong Gold in 0.5ml glycerol; ProLong Gold, Molecular Probes; glycerol, Sigma-Aldrich) and imaged under fluorescence microscopy. Using ImageJ (Version 1.48, National Institutes of Health (NIH), USA), 30 sperm per mouse were assessed by quantifying 8-OHdG fluorescence. Results were expressed as a mean of fluorescence for 8-OHdG (minus background) and then normalized to

fluorescence from microspheres to calibrate fluorescent imaging (Molecular Probes) and the CD treatment group (i.e. CD was set to 1.0).

#### ***2.1.18.2 Testicular 8-OHdG immunohistochemistry***

At post-mortem, the testes of mice were collected and fixed overnight in Bouin's fixative (Sigma-Aldrich). After being washed with PBS, testes were stored in 100% ethanol (Sigma-Aldrich) until embedding in paraffin. The paraffin-embedded samples were sectioned at 4 $\mu$ m, de-waxed and re-hydrated before being blocked in 10% donkey serum (Abcam) in PBS overnight at 4°C. On the second day, slides were incubated in 1:500 mouse anti-8-OHdG antibody (ab48508; Abcam; antibody validation (positive control, Appendix; Figure A5) for 1 hour at 37°C whereas the negative control was incubated in 1:100 mouse serum. Slides were then washed in PBS and incubated in 1:100 biotin-SP-conjugated AffiniPure donkey anti-mouse IgG (Jackson ImmunoResearch) for 2 hours at room temperature. Slides were then washed and incubated in 1:100 Cy3-conjugated streptavidin (Jackson ImmunoResearch) for 1.5 hours at room temperature followed by counter-staining with Hoechst. Finally, slides were loaded in ProLong Gold and glycerol solution and imaged under fluorescent microscopy. Using ImageJ (Version 1.48, NIH), 20 seminiferous tubules per mouse were assessed by quantifying 8-OHdG fluorescence and each tubule staged (early, middle, or late; I-V, VI-VIII or IX-XII, respectively, Figure 2.8) (Meistrich and Hess, 2013). Results were expressed as mean fluorescence intensities for 8-OHdG (minus background) normalized to fluorescence from microspheres (Molecular Probes) for each cell type (Figure 2.8).

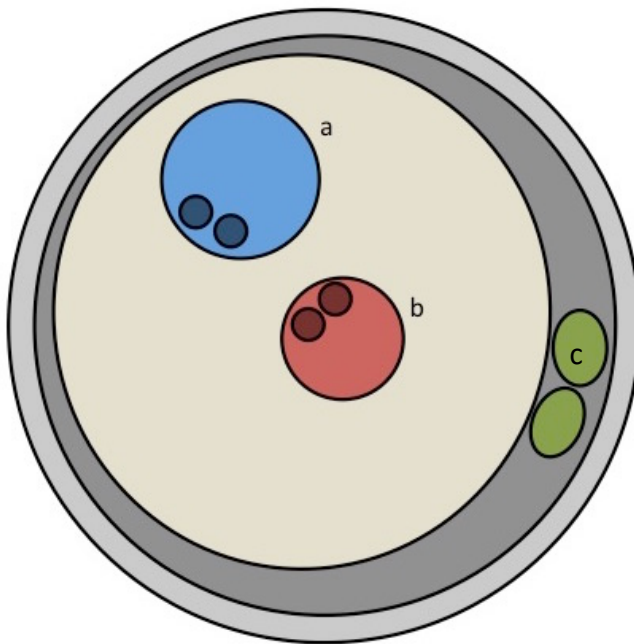


**Figure 2.8:** Representative images of seminiferous tubules stained with 8-hydroxyguanosine (8-OHdG) of (a) spermatogonia, (b) spermatocytes and (c) elongated spermatids from early, middle and late stages of spermatogenesis respectively, for fluorescence intensity quantification.

### **2.1.19 Pronuclear-staged embryo 8-OHdG immunocytochemistry**

Following fixation in 4% paraformaldehyde, pronuclear-staged embryos were stored in PBS containing 3mg/ml PVP (Sigma-Aldrich) at 4°C. Levels of 8-OHdG were assessed using an OxyDNA (Merck Millipore) test. Pronuclear-staged embryos were permeabilized in 0.25% Triton-X in PBS for 10 minutes at room temperature followed by incubation in 1:100 OxyDNA reagent for 1 hour at 37°C (as per the manufacturer's instructions) (Lord and Aitken, 2015). Finally, embryos were counterstained with Hoescht (Sigma-Aldrich) for 5 min, mounted on glass slides and imaged using a confocal microscope. Z-stacks of 10µm sections were collected of each pronucleus from each embryo (1024x1024 pixel size). Using ImageJ (Version 1.48, NIH), confocal image stacks were reconstructed and regions of interest (ROI) were defined around both the maternal and paternal pronucleus for fluorescence intensity quantification reflecting 8-OHdG levels for each pronucleus. The size of the ROI (pronucleus) was also computed by ImageJ and the smaller of the two pronuclei for each embryo was determined as the maternal pronucleus and confirmed with its closer proximity to the polar body than the paternal pronucleus (Edwards and Sirlin, 1956, Sirlin and Edwards, 1959) (Figure 2.9). Paternal pronucleus 8-OHdG fluorescence intensity quantification was normalized to the maternal pronucleus for each embryo and expressed as an average for each diet group.

**A**

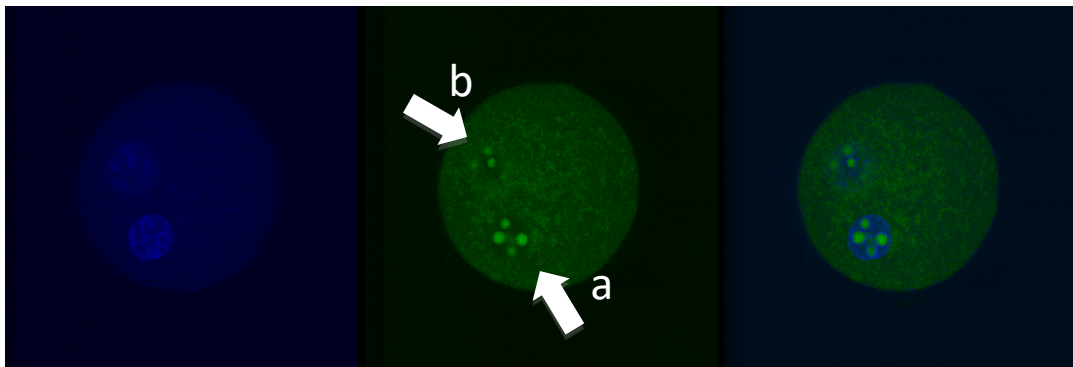


**B**

**Hoescht**

**oxyDNA**

**Merged**



**Figure 2.9:** Pronuclear-staged embryo depicting paternal and maternal pronuclei.

**A** Pronuclear-staged embryo with the (a) paternal pronucleus larger in size compared with the (b) maternal pronucleus. The (b) maternal pronucleus is also in closer in proximity to the (c) polar bodies.

**B** Immunofluorescence of a pronuclear-staged embryo representing a (a) paternal and (b) maternal pronucleus.



### **2.1.20 Blastocyst 8-OHdG detection**

On day 5 of embryo development, blastocysts were fixed overnight in 4% paraformaldehyde at 4°C. The following day, blastocysts (full, expanded and hatching blastocysts) were stored in PBS containing 3mg/ml polyvinylpyrrolidone (PVP; Sigma-Aldrich) at 4°C until immunostaining was conducted. Blastocysts were permeabilized in 0.5% Triton-X in PBS for 10 minutes at room temperature followed by blocking overnight in 5% bovine serum albumin (BSA, Sigma-Aldrich) in PBS at 4°C. The following day, blastocysts were incubated in 1:100 OxyDNA reagent for 1 hour at 37°C (as per the manufacturer's instructions). Finally, blastocysts were counterstained with Hoescht for 5 minutes, mounted on glass slides and imaged using a confocal microscope. Z-stacks of 10µm sections were collected of each blastocyst (1024x1024 pixel size). Using ImageJ (Version 1.48, NIH), confocal image stacks were reconstructed and ROI were defined around cells positive for oxyDNA and 8-OHdG fluorescence intensity was quantified. Also, total cell number (TCN) for each blastocyst was recorded by counting the number of cells positive for Hoescht. Results were expressed in fluorescent units reflecting quantified 8-OHdG fluorescence intensity and as a proportion of cells positive for 8-OHdG relative to TCN with an average for each diet group.

### **2.1.21 Natural mating and fetal development**

C57BL6 naturally cycling female mice at 9-10 weeks old were housed with a C57BL6 male mouse from the 10-day dietary intervention regimen (Figure 2.1) at 8-12 days post intake of the second dietary phase. Each male was allocated a maximum of two females for a maximum of four days. Mating was assessed by the presence of a vaginal plug the following morning and female mice were maintained on an *ad libitum* standard chow diet until day 18 of pregnancy. On day 18 of pregnancy, mothers were killed by cervical

dislocation. The number of fetuses and resorption sites per uterine horn were recorded. Fetuses were dissected and removed of connective tissue and umbilical cords, weighed and crown rump length was measured. Placentas were dissected and removed of connective tissue and maternal arterial space, weighed and dimensions were measured.

#### **2.1.22 Statistical analysis**

All data are presented as mean  $\pm$  SEM. An independent-samples Student's T Test was performed for DEXA body composition. For GTT and ITT data; AUC and AAC, a general linear model was performed between treatment by diet and micronutrient supplementation status with post hoc comparisons using the Bonferroni test. Weight gain, serum metabolite, testosterone, and CRP concentrations, postmortem body compositions, conventional sperm parameters, sperm oxidative stress, sperm function measures and testes, pronuclear and blastocyst immunofluorescence, embryo, fetal and placental development measures were determined using a general linear model between treatment by diet and micronutrient supplementation status with a least significant difference (LSD) or Dunnett's post hoc test as appropriate. All statistical analyses were performed in SPSS (SPSS version 22; IBM, Chicago, IL). Differences were considered significant at  $P < 0.05$ .

## **| 2.2 HUMAN EXPERIMENTAL METHODS**

### **2.2.1 Ethics approval**

The retrospective study, using non-identifiable information, was confirmed as negligible risk research by the University of Adelaide and subsequently classified as research exempt from Human Research Ethics Committee review. Existing collections of data containing non-identifiable information from the fertility clinic, Repromed (Dulwich, South Australia) was used in this study with approval from Repromed's Scientific Advisory Committee.

The prospective study was approved by the Women's and Children's Hospital Human Research Ethics Committee (project number HREC/14/WCHN/15) in addition to approval from Repromed's Scientific Advisory Committee. Written informed consent was obtained from all participants. No identifying information was used in this study.

### **2.2.2 Study population**

The participants for both the retrospective and prospective study were non-smoking patients undergoing intracytoplasmic sperm injection (ICSI) and/or standard insemination (IVF) as part of their assisted reproduction technology (ART) treatment at the fertility clinic, Repromed (Dulwich, South Australia). The female partner was less than 38 years of age and the male partner was less than 45 years of age at the time of oocyte collection. Couples participating in the donor program, undergoing surgical sperm retrieval, or using frozen sperm were excluded. Male patients who had pathological conditions capable of affecting sperm quality were also excluded.

The retrospective study included couples undergoing IVF/ICSI treatment between 2013-2014. The prospective study included couples undergoing IVF/ICSI treatment between 2015-2016.

### **2.2.3 Paternal and maternal body mass index**

Both paternal and maternal height and weight were measured at the clinic before the couple's treatment cycle and converted into BMI ( $\text{kg}/\text{m}^2$ ). Patients were classified according to the following BMI ranges; normal weight: 20-24.9  $\text{kg}/\text{m}^2$ , overweight: 25-29.9  $\text{kg}/\text{m}^2$ , and obese: 30  $\text{kg}/\text{m}^2$  and above. Couples with an obese maternal BMI were excluded. Couples were stratified by paternal BMI into the three BMI categories: normal weight, overweight and obese.

### **2.2.4 Patient recruitment for prospective study**

Couples undergoing treatment were approached 1-4 days before their oocyte retrieval whereby the prospective study was explained and the couples were provided with a patient information sheet regarding the study (Appendix; Figure A6). On the day of the couple's oocyte retrieval, the patients were approached again and if the couple consented, informed written consent was obtained (Appendix; Figure A7). A unique trial number was allocated to each couple after consent to ensure the subsequent experiments and data collection were de-identified and blinded.

### **2.2.5 Lifestyle and micronutrient supplement intake assessment**

Male patients completed a questionnaire after producing their semen sample (Appendix; Figure A8). The questionnaire provided information about their lifestyle factors including medication, illness and the intake of micronutrient and antioxidant supplements. Men that reported the intake of Menevit, Swisse Men's Multivitamin, or Nature's Own Men's Multivitamin were included in the study and categorized as men positive for the intake of micronutrient supplementation (Appendix; Table A9). Men that reported the intake of a single micronutrient e.g. folic acid were not included in the study.

### **2.2.6 Conventional semen analysis**

After a recommended abstinence period of 2 to 5 days, semen samples were collected by masturbation in sterile containers and were analyzed at room temperature within 1 hour of collection. Conventional sperm parameters including sperm motility, concentration, and morphology were measured according to the World Health Organization (WHO) 2010 Laboratory Manual for the Examination of Human Semen and Sperm-Cervical Mucus Interaction Methods and Criteria (WHO, 2010).

#### ***2.2.6.1 Sperm motility***

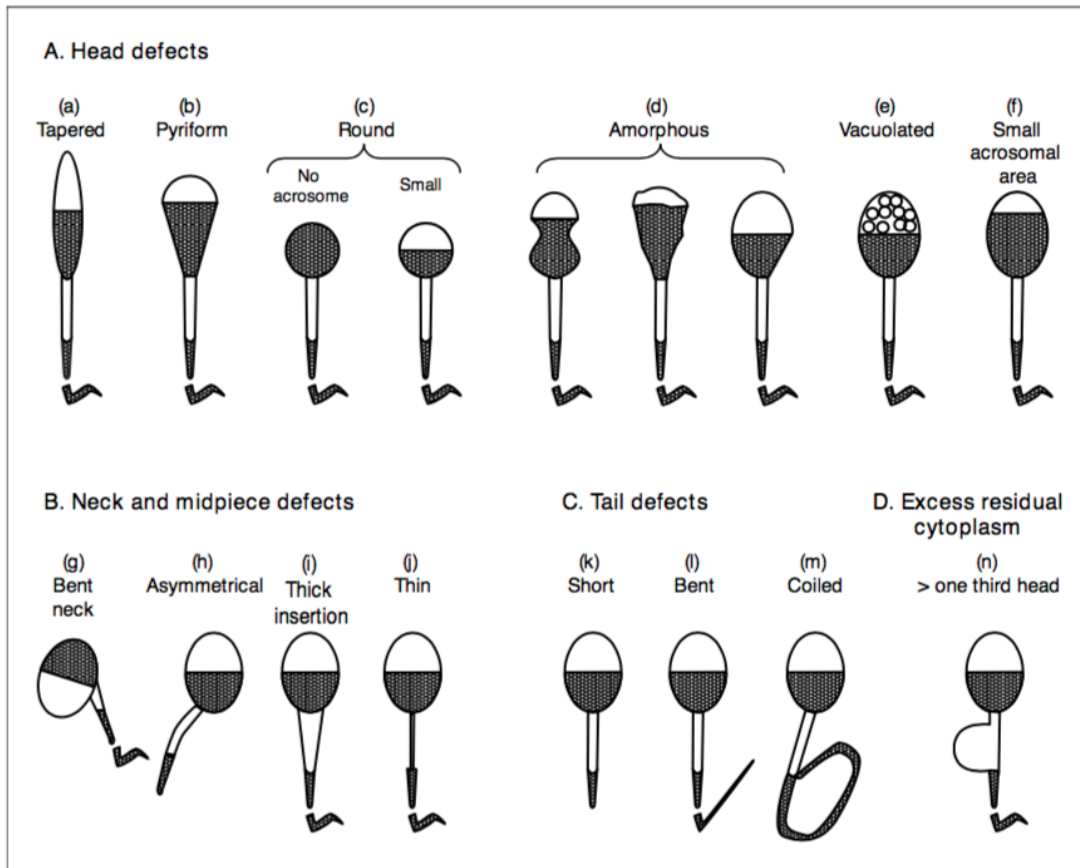
Sperm motility was determined manually under  $\times 40$  magnification with duplicate measures of 200 sperm and expressed as a percentage of motile sperm in a given sample (WHO, 2010).

#### ***2.2.6.2 Sperm concentration***

A Neubauer hemocytometer was used to determine sperm concentration. The appropriate dilution factor was applied to each sample and the number of sperm in the five squares of both the top and bottom haemocytometer chambers were counted. This number was then averaged to calculate the concentration of each sperm sample according to the WHO guidelines (WHO, 2010).

#### ***2.2.6.3 Sperm morphology***

The percentage of morphologically normal sperm was calculated by assessing 200 sperm per sample using the Diff-Quik staining method as described by the 2010 WHO manual (WHO, 2010) (Figure 2.10).



**Figure 2.10:** Human sperm morphology. A schematic adapted from the World Health Organization (WHO) laboratory manual for the examination and processing of human semen representing abnormal sperm morphology characteristics including (A) head, (B) neck and midpiece, (C) tail and (D) cytoplasmic defects.

### **2.2.7 Sperm intracellular reactive oxygen species**

The excess sperm sample, after it was used for insemination and/or injection (depending on the couple's ART procedure), was used for the detection of intracellular ROS. Sperm samples were washed in PBS containing 3mg/ml polyvinylpyrrolidone (PVP; Sigma-Aldrich, St. Louis, MO, USA) to remove the G-IVF Plus (Vitrolife) preparation medium. An Olympus BX51 fluorescent microscope in conjunction with AnalySIS LS Research Imaging software (version 3.1; Olympus Soft Imaging Solutions, Munster, Germany) was used to take images of sperm stained with fluorescent ROS probes.

#### ***2.2.7.1 MitoSOX Red***

Sperm were incubated in MitoSOX Red (2 $\mu$ M; Molecular Probes, Eugene, USA) in the dark for 15 minutes at 37°C (Aitken, et al., 2013, Koppers, et al., 2008). The sample was then washed in PBS/PVP for 5 minutes at 500g before incubation in Sytox Green (5 $\mu$ M; Molecular Probes), a vitality stain, in the dark for 15 minutes at 37°C. Sperm were washed again in PBS/PVP and then centrifuged for 5 minutes at 500g, loaded onto slides in 10 $\mu$ l drops and imaged under fluorescent microscopy. At least 100 live sperm (negative for Sytox Green) were imaged per male patient blinded. Sperm fluorescence was quantified using ImageJ software (Version 1.48, National Institutes of Health (NIH), USA) and results were expressed as a mean of fluorescence for MitoSox Red (minus background).

#### ***2.2.7.2 Dichlorodihydrofluorescein diacetate***

Sperm were incubated in 1 $\mu$ M 2',7'-dichlorodihydrofluorescein diacetate (H<sub>2</sub>DCFDA; Molecular Probes) in the dark for 1 hour at 37°C (Aitken, et al., 2013). Sperm were then



washed twice in PBS/PVP and centrifuged at 500g for 5 minutes before incubation in 1 $\mu$ M propidium iodide (PI; Molecular Probes), a vitality stain, in the dark for 5 minutes at room temperature. Sperm were washed again in PBS/PVP and centrifuged for 5 minutes at 500g, loaded onto slides in 10 $\mu$ l drops and imaged under fluorescent microscopy. At least 100 live sperm (negative for PI) were imaged per male patient blinded. Sperm fluorescence was quantified using ImageJ software (Version 1.48, NIH) and results were expressed as a mean of fluorescence for H<sub>2</sub>DCFDA (minus background). Sperm intracellular esterase activity was measured using carboxy-H<sub>2</sub>DCFDA (Molecular Probes) and was not different between men from the different BMI categories (Appendix; Table A10).

### **2.2.8 Sperm 8-OHdG DNA lesion immunocytochemistry**

Sperm were fixed on three polylysine-coated slides labeled with the couple's study number (allocated at the time of consent) and date of consent. Two squares were marked on each slide with a DAKO pen (DAKO, Glostrup, Denmark) and 20 $\mu$ l of washed sperm sample was spread into each square. Slides were then air-dried, fixed in 3:1 methanol:acetone (Sigma-Aldrich) for 40 minutes and stored in 4°C until immunocytochemistry was conducted.

Slides were permeabilised in 0.5% Triton X-100/PBS (Sigma Aldrich) for 45 minutes at room temperature. Slides were then washed in PBS before incubation in decondensing buffer (1M HCl, 10mM Tris buffer of 5mM dithiothreitol, Sigma-Aldrich) for 60 minutes at 37°C. Sperm DNA was then denatured with a denaturing buffer (6M HCL, 0.1% Triton X-100 in H<sub>2</sub>O, Sigma-Aldrich) for 45 minutes at room temperature and then neutralized with a neutralizing buffer (100uM Tris HCl in H<sub>2</sub>O, Sigma-Aldrich) for 20 minutes. Slides were washed and then incubated overnight at 4°C in 1:100 primary anti-

8-hydroxyguanosine (8-OHdG) antibody (Abcam, Cambridge, UK) in 10% donkey serum (Abcam) whereas the negative control was incubated in 1:100 mouse serum. The following day, slides were washed and incubated in 1:100 biotin-SP-conjugated AffiniPure donkey anti-mouse IgG (Jackson ImmunoResearch, Baltimore, PA, USA) for 2 hours at room temperature. Slides were then washed in PBS and incubated in 1:100 Cy3-conjugated streptavidin (Jackson ImmunoResearch) for 1.5 hours at room temperature followed by a nuclear counter stain with Hoechst. Finally, slides were loaded in ProLong Gold and glycerol solution (2 drops of ProLong Gold in 0.5ml glycerol; ProLong Gold, Molecular Probes; glycerol, Sigma-Aldrich) and imaged under fluorescent microscopy. At least 100 sperm were imaged per male patient blinded. Sperm fluorescence was quantified using ImageJ software (Version 1.48, NIH) and results were expressed as a mean of fluorescence for 8-OHdG (minus background) and then normalized to fluorescence from microspheres (Molecular Probes).

### **2.2.9 Data records**

Treatment cycle details were recorded for every couple including female anti-mullerian hormone (AMH) and baseline follicle-stimulating hormone (FSH) serum concentrations, ART cycle outcomes that included the number of eggs collected, number of eggs inseminated and fertilized, embryo grading on days 3-5 of development, number of embryos transferred and/or frozen, biochemical and ongoing pregnancy, live birth outcomes, and if the embryos underwent pre-implantation genetic testing for aneuploidy (PGT-A). Potential co-factors were also recorded including the number of previous cycles, the couple's infertility diagnosis such as unexplained infertility, polycystic ovarian syndrome (PCOS), endometriosis, tubal blockage, anovulation, and diminished ovarian reserve.

### **2.2.10 Data analysis**

Couples were stratified initially into three groups based on the male partner's BMI: normal weight <24.9 kg/m<sup>2</sup>, overweight 25-29.9 kg/m<sup>2</sup>, and obese >30 kg m<sup>2</sup>. Each BMI group was then divided into two groups: those who were consuming micronutrient supplementation and those who were not. This stratification ultimately generated six groups: couples with a male partner that was (i) normal weight without micronutrient supplementation, (ii) normal weight with micronutrient supplementation, (iii) overweight without micronutrient supplementation, (iv) overweight with micronutrient supplementation, (v) obese without micronutrient supplementation, and (vi) obese with micronutrient supplementation. The primary outcome measures were biochemical pregnancy, implantation rates, ongoing pregnancy, and live birth rates. Semen volume, sperm motility, sperm concentration, fertilization rates, embryo development, and embryo utilization were also assessed as secondary measures.

### **2.2.11 Protocols conducted by clinical staff**

#### ***2.2.11.1 Ovarian stimulation***

Controlled ovarian stimulation was conducted with the use of recombinant FSH (Puregon; Merck Sharp and Dohme, NJ, USA or Gonal-F; Merck Serono, Darmstadt, Germany) and a gonadotropin-releasing hormone (GnRH) antagonist. Follicle development was monitored by ultrasound and serum estradiol. Ovulation was induced with hCG administration (5000 IU Pregnyl; Merck Sharp and Dohme; 250 gm Ovidrel; Serono) when two or more follicles  $\geq 17$  mm in diameter were present.

Oocytes were collected by transvaginal oocyte retrieval performed 36 hours after hCG administration (Wang, et al., 2011).

#### ***2.2.11.2 Sperm preparation for IVF/ICSI cycles***

Based on sperm motility and concentration of the neat semen sample, sperm were prepared using the swim-up sperm preparation method as per standard clinical protocol. 0.2-0.6ml of sperm sample was added to the bottom of 1-4 round bottom tubes (depending on initial sperm concentration and motility) containing 1ml of pre-equilibrated G-IVF Plus (Vitrolife AB, Göteborg, Sweden) at 37°C and 6% CO<sub>2</sub>. The sample was then incubated for 30-60 minutes at 37°C before 0.3ml of sperm was gently aspirated from the upper interface of the media and transferred to a clean tube for oocyte insemination or injection with a final concentration of  $1 \times 10^6$ /ml for standard insemination and less than  $3 \times 10^6$ /ml for ICSI.

#### ***2.2.11.3 Oocyte insemination***

Insemination was conducted 4-6 hours post oocyte retrieval. Oocytes were inseminated with use of conventional IVF or injected via the ICSI procedure or a combination of both procedures. For conventional IVF, up to 2 oocytes were placed in 50µL drops of G-IVF Plus (Vitrolife) with a concentration of 1 million sperm/ml. For ICSI, a single motile sperm was injected into the oocyte and oocytes were cultured in groups of up to 4 oocytes in 50µL drops of G1 Plus medium (Vitrolife).

#### ***2.2.11.4 Fertilization***

Fertilization was assessed 16–18 hours after insemination and was confirmed by the presence of two pronuclei. A medium change from G-IVF Plus (Vitrolife) to G1 Plus medium (Vitrolife) was conducted for oocytes inseminated via conventional IVF.

#### ***2.2.11.5 Embryo cleavage***

As per standard clinical protocol, embryo cleavage and morphology were assessed based on cell number and degree of fragmentation. Embryos were assessed on day 3 at 66 hours  $\pm$  2 hours after insemination, with on-time development considered for an embryo with 7-10 cells and <10% fragmentation. An in-house scoring system was applied for day 4 embryo development as previously described (Feil, et al., 2008). Some embryos were not assessed on day 4 of development as the patient was having a day 5 embryo transfer. On day 5 of embryo development, blastocyst quality was assessed by the degree of blastocyst expansion and the quality of the inner cell mass and trophoctoderm as previously described by Gardner and Schoolcraft (Gardner and Schoolcraft, 1999). Day 5 embryos that were assessed on day 4 and had embryos that were frozen or transferred on day 4 were not included in the day 5 embryo development analysis to eliminate skewing of data.

#### ***2.2.11.6 Embryo transfer***

Embryos selected for transfer were based on morphology and grading with the highest graded embryos usually selected for transfer.

#### ***2.2.11.7 Embryo vitrification***

Embryos that were not transferred and were considered suitable for cryopreservation were vitrified using the Rapid-I vitrification system (Vitrolife).

#### ***2.2.11.8 Pregnancy determination and clinical definitions***

***2.2.11.8.1 Biochemical pregnancy:*** A pregnancy was classified as a positive biochemical pregnancy when the serum  $\beta$ -hCG level was >5 IU from day 14 after embryo transfer.

#### ***2.2.11.8.2 Implantation***

Implantation was determined by the detection of a fetal sac (or sacs) in the womb on ultrasound examination at 4 to 6 weeks after embryo transfer.

#### ***2.2.11.8.3 Ongoing pregnancy***

A pregnancy was classified as a positive ongoing pregnancy when a viable fetal heartbeat was detected on ultrasound examination at 4 to 6 weeks after embryo transfer.

#### ***2.2.11.8.4 Live birth***

Live birth outcomes were recorded including length of gestation, number of infants born, and the weight and gender of the infant(s) born.

#### ***2.2.11.8.5 Pregnancy loss***

Pregnancy loss was defined as a loss that occurred between a positive  $\beta$ -hCG test and live birth.

### **2.2.12 Statistical Analysis**

The Kolmogorov–Smirnov test was used to evaluate the normal distribution of all variables. For quantitative outcomes with mean values, a generalized linear model was performed with BMI and the use of micronutrient supplements as independent factors and Bonferroni, Least Significant Difference (LSD) and Dunnett's post hoc comparisons where appropriate. For binary reproductive outcome measures, odds ratio with 95% confidence intervals were estimated using binary logistic regression analysis. For association analyses, a Pearson or Spearman correlation coefficient was used based on normal distribution. Statistical analysis was performed using the SPSS software package (Statistical Package for Social Sciences version 23.0, SPSS Inc., Chicago, IL, USA) with  $P < 0.05$  considered as statistically significant.

## 2.3 References

Aitken RJ, Smith TB, Lord T, Kuczera L, Koppers AJ, Naumovski N, Connaughton H, Baker MA, De Iuliis GN. On methods for the detection of reactive oxygen species generation by human spermatozoa: analysis of the cellular responses to catechol oestrogen, lipid aldehyde, menadione and arachidonic acid. *Andrology* 2013;**1**: 192-205.

Baker SS, Thomas M, Thaler CD. Sperm membrane dynamics assessed by changes in lectin fluorescence before and after capacitation. *J Androl* 2004;**25**: 744-751.

Bakos HW, Mitchell M, Setchell BP, Lane M. The effect of paternal diet-induced obesity on sperm function and fertilization in a mouse model. *Int J Androl* 2010;**34**: 402-410.

Edwards RG, Sirlin JL. Labelled pronuclei in mouse eggs fertilized by labelled sperm. *Nature* 1956;**177**: 429.

Feil D, Henshaw RC, Lane M. Day 4 embryo selection is equal to Day 5 using a new embryo scoring system validated in single embryo transfers. *Hum Reprod* 2008;**23**: 1505-1510.

Fullston T, Ohlsson Teague EM, Palmer NO, DeBlasio MJ, Mitchell M, Corbett M, Print CG, Owens JA, Lane M. Paternal obesity initiates metabolic disturbances in two generations of mice with incomplete penetrance to the F2 generation and alters the transcriptional profile of testis and sperm microRNA content. *FASEB J* 2013;**27**: 4226-4243.

Fullston T, Palmer NO, Owens JA, Mitchell M, Bakos HW, Lane M. Diet-induced paternal obesity in the absence of diabetes diminishes the reproductive health of two subsequent generations of mice. *Hum Reprod* 2012;**27**: 1391-1400.

Gardner DK, Lane M, Watson AJ. *A laboratory guide to the mammalian embryo*, 2004. Oxford University Press, Oxford ; New York.

Gardner DK, Schoolcraft WB. In vitro culture of human blastocysts. *Towards Reproductive Certainty* 1999: 378-388.

Halliwell B, Whiteman M. Measuring reactive species and oxidative damage in vivo and in cell culture: how should you do it and what do the results mean? *Br J Pharmacol* 2004;**142**: 231-255.

Koppers AJ, De Iuliis GN, Finnie JM, McLaughlin EA, Aitken RJ. Significance of mitochondrial reactive oxygen species in the generation of oxidative stress in spermatozoa. *J Clin Endocr Metab* 2008;**93**: 3199-3207.

Lane M, McPherson NO, Fullston T, Spillane M, Sandeman L, Kang WX, Zander-Fox DL. Oxidative stress in mouse sperm impairs embryo development, fetal growth and alters adiposity and glucose regulation in female offspring. *PLoS One* 2014;**9**: e100832.

Lord T, Aitken RJ. Fertilization stimulates 8-hydroxy-2'-deoxyguanosine repair and antioxidant activity to prevent mutagenesis in the embryo. *Developmental biology* 2015;**406**: 1-13.



Meistrich ML, Hess RA. Assessment of spermatogenesis through staging of seminiferous tubules. *Methods Mol Biol* 2013;**927**: 299-307.

Nagy TR, Clair AL. Precision and accuracy of dual-energy X-ray absorptiometry for determining in vivo body composition of mice. *Obes Res* 2000;**8**: 392-398.

NHMRC. Australian Code for the Care and Use of Animals for Scientific Purposes. In Council NHaMR (ed). 2013. Australian Government, Australia.

Oakberg EF. Duration of spermatogenesis in the mouse. *Nature* 1957;**180**: 1137-1138.

Purdey MS, Connaughton HS, Whiting S, Schartner EP, Monro TM, Thompson JG, Aitken RJ, Abell AD. Boronate probes for the detection of hydrogen peroxide release from human spermatozoa. *Free radical biology & medicine* 2015;**81**: 69-76.

Robaire B, Hinton BT, Orgebin-Crist M. The Epididymis. In Neill JD (ed) *Knobil and Neill's Physiology of Reproduction*. 2006. Elsevier.

Santos F, Hendrich B, Reik W, Dean W. Dynamic reprogramming of DNA methylation in the early mouse embryo. *Developmental biology* 2002;**241**: 172-182.

Sirlin JL, Edwards RG. Timing of DNA synthesis in ovarian oocyte nuclei and pronuclei of the mouse. *Experimental cell research* 1959;**18**: 190-194.

Wang W, Zhang XH, Wang WH, Liu YL, Zhao LH, Xue SL, Yang KH. The time interval between hCG priming and oocyte retrieval in ART program: a meta-analysis. *J Assist Reprod Genet* 2011;**28**: 901-910.

WHO. *WHO laboratory manual for the examination and processing of human semen*. Fifth Edition edn, 2010.

Wyrobek AJ, Bruce WR. Chemical induction of sperm abnormalities in mice. *Proceedings of the National Academy of Sciences of the United States of America* 1975;**72**: 4425-4429.

Chapter 3 (publication format)

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**Dietary Micronutrient Supplementation Reduces Sperm Oxidative  
Stress and Improves Fertilization Rates in a Mouse Model of Diet-  
Induced Obesity**

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### 3.1 Statement of Authorship

## Statement of Authorship

Title of Paper	Dietary micronutrient supplementation reduces sperm oxidative stress and improves fertilization rates in a mouse model of diet induced obesity
Publication Status	<input type="checkbox"/> Published <input type="checkbox"/> Accepted for Publication <input type="checkbox"/> Submitted for Publication <input checked="" type="checkbox"/> Unpublished and Unsubmitted work written in manuscript style
Publication Details	Unpublished and unsubmitted work written in manuscript style for the journal Reproduction, Fertility and Development.

### Principal Author

Name of Principal Author (Candidate)	Helena Shahadeh		
Contribution to the Paper	Study design, performed experiments, conducted data analysis and interpretation, wrote and edited the manuscript.		
Overall percentage (%)	80%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	28/8/18

### Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:  
 the candidate's stated contribution to the publication is accurate (as detailed above);  
 permission is granted for the candidate to include the publication in the thesis; and  
 the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

Name of Co-Author	Tad Fullston		
Contribution to the Paper	Helped to evaluate and interpret the data, and edit the manuscript.		
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Contribution to the Paper	Study design, Data analysis, evaluation and interpretation, Manuscript edits.		
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### **3.2 Abstract**

Male obesity can lead to increased sperm oxidative stress and impaired sperm function, which is associated with reduced fertilization, delayed embryo development and compromised offspring health in both humans and mouse models. This study investigated the impact of dietary micronutrient supplementation of two different durations (5 weeks and 10 weeks reflecting one and two rounds of spermatogenesis respectively) on sperm quality and function in a mouse model of diet-induced obesity. Initially, C57BL/6 male mice were fed a control diet (CD) or a high fat diet (HFD) for 10 weeks to increase adiposity. Mice were then allocated to one of four diets: continuation of the initial CD or HFD or the same base diet (CD or HFD) supplemented with micronutrients (CD+S or HFD+S) for either 5 or 10 weeks – durations chosen to cover approximately one and two rounds of spermatogenesis, respectively. Males fed the HFD supplemented with micronutrients for 5 or 10 weeks displayed reduced sperm intracellular reactive oxygen species (ROS) and oxidative DNA lesions (8-OHdG), which translated into improvements in sperm function with increased sperm binding and fertilization rates. Additionally, mice fed a HFD supplemented with micronutrients for 10 weeks also displayed improvements in sperm morphology, increased sperm capacitation, and reduced testicular 8-OHdG lesions. These results suggest that consuming a combination of micronutrients in conjunction with a HFD protects sperm quality from HFD-induced impairments, with additional benefits when consumed for a duration that spans two rounds of spermatogenesis.

#### ***Key words***

DNA damage, micronutrients, obesity, oxidative stress, ROS, spermatogenesis, sperm function, sperm quality, testis

### 3.3 Introduction

The substantial increase in prevalence of overweight and obese men is a global health issue, with reports estimating that 37% are overweight or obese worldwide (Ng *et al.* 2014). This increase has occurred with a concomitant increase in subfertility in men of reproductive age (Sharlip *et al.* 2002). Overweight and obesity in men can lead to impaired reproductive health, often characterized by altered serum hormone profiles (Aggerholm *et al.* 2008; Chavarro *et al.* 2010; Hofny *et al.* 2010) and impaired sperm quality as measured by reduced sperm concentration, motility and normal morphology (Bakos *et al.* 2011; Campbell *et al.* 2015; Hammiche *et al.* 2012; Hammoud *et al.* 2008; Hofny *et al.* 2010; Jensen *et al.* 2004; Magnusdottir *et al.* 2005; Shayeb *et al.* 2011).

Animal studies further support the link between obesity and reduced sperm quality and function. Similar to human studies, diet-induced obesity in rodent models is associated with reduced sperm motility, concentration and normal morphology (Bakos *et al.* 2010; Fernandez-Gonzalez *et al.* 2008; Fullston *et al.* 2012; Ghanayem *et al.* 2010; Palmer *et al.* 2012). In addition, HFD-fed males also have increased sperm oxidative stress and DNA damage (Bakos *et al.* 2010; Chen *et al.* 2013; Duale *et al.* 2014; Palmer *et al.* 2012; Vendramini *et al.* 2014; Zhao *et al.* 2014). Elevated concentrations of reactive oxygen species (ROS) are one of the main contributors to sperm DNA damage (Aitken *et al.* 2003; De Iuliis *et al.* 2009), which has been linked to impaired reproductive health via reduced fertilization rates, delayed embryo development and reduced pregnancy rates (Larson *et al.* 2000; Lopes *et al.* 1998; Seli *et al.* 2004).

Moreover, obesity is frequently linked to micronutrient deficiencies resulting from a calorie-rich and micronutrient-poor diet (Gillis and Gillis 2005). Serum concentrations of essential vitamins and minerals with anti-oxidative properties including vitamin C, vitamin E, selenium, folate and zinc are often reduced with increased adiposity (Aasheim *et al.* 2008; Ernst *et al.* 2009; Garcia *et al.* 2012; Gobato *et al.* 2014). Micronutrient deficiencies can impair male fertility, for instance, selenium deficiency is associated with increased structural defects in sperm (Krol *et al.* 2012), and reduced sperm motility and concentration, (Hardy and Hardy 2004; Olson *et al.* 2004; Sanchez-Gutierrez *et al.* 2008) and increased sperm oxidative damage (Sanchez-Gutierrez *et al.* 2008). Men deficient for vitamin C show increased sperm oxidative DNA lesions, which is reduced upon vitamin C supplementation (Fraga *et al.* 1991). Vitamin E supplementation has been shown to reduce sperm lipid peroxidation, and increase sperm motility and pregnancy rates compared to placebo treated patients in asthenospermic men (Suleiman *et al.* 1996). While lycopene supplementation increases the proportion of sperm with normal morphology and reduces sperm lipid peroxidation in normozoospermic men (Zareba *et al.* 2013) and rodents (Ceribasi *et al.* 2010). Combined folic acid and zinc supplementation in fertile and subfertile men increased sperm count, motility and normal morphology (Wong *et al.* 2002). Whereas zinc supplementation on its own reduced sperm oxidative stress, DNA damage and apoptosis in asthenospermic and fertile men (Omu *et al.* 2008; Wong *et al.* 2002). In rats, green tea extract supplementation has been shown to increase sperm motility and normal morphology (Mosbah *et al.* 2015) and reduce sperm oxidative stress (Awoniyi *et al.* 2012).

The effects of a combination of dietary micronutrient supplementation on sperm oxidative stress, sperm oxidative DNA lesions and essential endpoints of sperm function

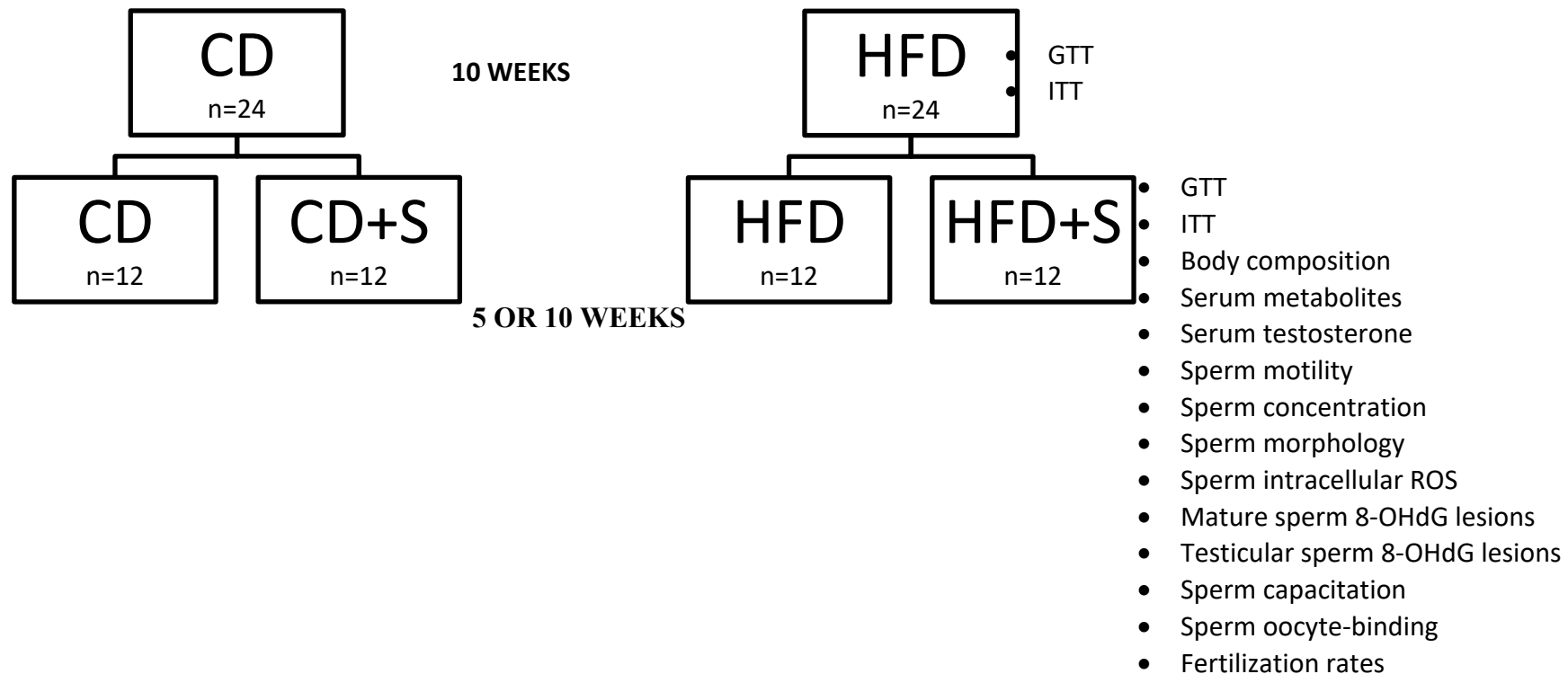
(e.g. fertilization) in a diet-induced obese model have not been investigated. Moreover, no studies to date have examined an optimal duration of supplement intake; specifically the efficacy of short or longer-term treatment durations that span one or more rounds of spermatogenesis.

Therefore, the aim of this duration based study was to investigate the effects of dietary micronutrients, which have previously been shown to have a role in reducing sperm oxidative stress in normal weight males, and the impact of duration of intake over one (5 weeks) or two rounds (10 weeks) of spermatogenesis (Oakberg 1957) on conventional sperm parameters, sperm oxidative stress, oxidative DNA damage in both epididymal and testicular sperm, sperm function and subsequent fertilization rates in a mouse model of male obesity.



### **3.4 Materials and methods**

The experimental design is outlined below in Figure 3.1. Please refer to Chapter 2, section 2.1 for experimental methods.



**Fig. 3.1** Experimental design. All mice were allocated to a first dietary phase of a CD or a HFD for 10 weeks and underwent both a glucose tolerance test (GTT) and an insulin tolerance test (ITT). For the second dietary phase, mice were allocated to either a no intervention control group (CD, HFD) or a micronutrient supplementation intervention group (CD+S, HFD+S) for 5 or 10 weeks. After 5 or 10 weeks, mice underwent a GTT, ITT, body composition at post-mortem, serum metabolites and testosterone, conventional sperm analysis, sperm intracellular ROS, mature

and testicular sperm 8-OHdG lesions, sperm capacitation, oocyte binding and fertilization rates. CD, control diet; HFD, high fat diet; CD+S, control diet plus micronutrient supplements; HFD+S, high fat diet plus micronutrient supplements.

## 3.5 Results

### ***3.5.1 Effect of diet and micronutrient supplementation on body composition, weight gain, glucose tolerance and insulin tolerance***

Consistent with previous reports, feeding mice a HFD for 10 weeks increased body fat in both absolute values (g) and relative to total body weight (%); and reduced lean mass relative to total body weight (DEXA;  $P < 0.001$ , Table S.3.1) (Ghanayem *et al.* 2010), with no change in fasting blood glucose levels, glucose tolerance or insulin tolerance ( $P > 0.05$ , Fig. S.3.1 and S.3.2) compared to CD fed mice (Bakos *et al.* 2010; Fullston *et al.* 2013; Fullston *et al.* 2012).

Micronutrient supplementation of the CD or HFD for 5 or 10 weeks of the second dietary period did not have an effect on total body weight or weight gain (Fig. S.3.3). The increase in adiposity in male mice fed a HFD during the first dietary period continued to increase when mice continued to consume the HFD for a further 5 or 10 weeks observed by increases in peri-renal, retroperitoneal, omental, dorsal and gonadal fat relative to total body weight compared to mice fed a CD ( $P < 0.05$ , Tables S.3.2 and S.3.3). Furthermore, mice fed the HFD second dietary period for 10 weeks had increased liver weights relative to total body weight compared to those fed a CD for the same time period ( $P < 0.05$ , Table S.3.3), while other organs were not affected. Micronutrient supplementation for 5 weeks in males fed a HFD (HFD+S) did not alter body composition ( $P > 0.05$ , Table S.3.2). However, mice fed the HFD+S for 10 weeks had a reduced sum of adipose tissues relative to total body weight compared to mice fed the un-supplemented HFD ( $P < 0.05$ , Table S.3.3), although still greater than either CD group.

There was no effect of diet or micronutrient supplementation for 5 or 10 weeks on glucose and insulin tolerance, fasting baseline blood glucose levels, glucose tolerance and insulin tolerance compared to the controls ( $P>0.05$ , Fig. S.3.4 and S.3.5).

### ***3.5.2 Effect of diet and micronutrient supplementation on serum metabolites, testosterone and inflammation***

Serum parameters including cholesterol, HDLs, triglycerides, NEFAs, glucose, testosterone and CRP (an inflammatory marker) were measured to provide more information about their metabolic, hormonal and inflammatory state. Mice fed a HFD had increased serum cholesterol and HDL concentrations ( $P<0.05$ , Table S.3.4) compared to CD fed animals, with an additional increase in fasted glucose levels in mice fed the 10-week HFD secondary diet regimen ( $P<0.05$ , Table S.3.5). Micronutrient supplementation for 5 or 10 weeks in either the CD or HFD did not have an effect on serum metabolites. Moreover, there was no difference in serum testosterone or CRP concentrations between mice fed a CD and mice fed a HFD; and again this was not influenced by micronutrient supplementation ( $P>0.05$ , Tables S.3.4 and S.3.5).

### ***3.5.3 Effect of diet and micronutrient supplementation on sperm parameters***

There was no effect of diet, or duration of micronutrient supplementation (5 or 10 weeks) on sperm concentration ( $P>0.05$ , Tables S.3.6 and S.3.7). Consumption of a HFD for the secondary diet period of 5 weeks did not affect sperm motility, however mice fed the 10 week HFD secondary diet regimen had a reduced percentage of total motile sperm compared to CD fed mice ( $P<0.05$ , Table S.3.7), which was not improved by micronutrient supplementation for this duration of HFD feeding ( $P>0.05$ , Table S.3.7).

The percentage of morphologically normal sperm was reduced in mice fed a HFD compared to mice fed a CD for the respective time periods. When CD fed mice consumed supplements (CD+S) for 5 or 10 weeks, the percentage of morphologically normal sperm was increased compared to mice fed the CD ( $P<0.0001$ ), resulting from improvements in sperm head morphology ( $P<0.01$ , Fig. 3.2). Furthermore, when HFD fed mice consumed supplements (HFD+S) for 10 weeks, the percentage of morphologically normal sperm increased compared to the mice fed a HFD without supplements ( $P<0.001$ ) resulting from improvements in sperm tail morphology ( $P<0.05$ , Fig. 3.2).

#### ***3.5.4 Effect of diet and micronutrient supplementation on sperm oxidative stress***

To determine the effect of diet and micronutrient supplementation on sperm oxidative stress, a global measure of sperm intracellular ROS levels was initially examined. Mice from the 5 and 10 week secondary diet regimen had increased sperm intracellular ROS levels compared to CD fed mice (+1.5-fold and +2.5-fold respectively;  $P<0.01$ , Fig. 3.3). When mice were fed HFD+S for 5 weeks, intracellular sperm ROS levels were reduced compared to mice fed the HFD and to a level equivalent to sperm from CD fed mice ( $P<0.01$ , Fig. 3.3). This reduction in sperm intracellular ROS was also evident in the mice undergoing 10 weeks of micronutrient supplementation in conjunction with a HFD ( $P<0.01$ , Fig. 3.3).

Relative MSR fluorescence intensity reflecting superoxide production in sperm was measured, as the superoxide anion is known to contribute to various pathways of secondary ROS production (Droge 2002). While, consumption of a HFD did not alter the percentage of sperm positive for MSR compared to CD fed mice ( $P>0.05$ ), supplementation with micronutrients in a HFD for 5 weeks (HFD+S) reduced sperm

superoxide production compared to mice consuming the HFD ( $P < 0.05$ , Fig. 3.4). However, this reduction was not observed for 10 weeks of supplementation in conjunction with HFD ( $P > 0.05$ , Fig. 3.4).

Sperm 8-OHdG lesions are a marker of oxidative DNA lesions, formed by the modification of a guanine base resulting from oxidative attack by ROS (Oger *et al.* 2003; Valavanidis *et al.* 2009). Sperm 8-OHdG fluorescence was increased in mice fed the 5 and 10 week HFD secondary diet regimen compared to CD fed mice (+2.3-fold and +2.6-fold, respectively;  $P < 0.01$ , Fig. 3.5). However, when mice were fed the HFD+S for 5 or 10 weeks, sperm 8-OHdG fluorescence was reduced compared to HFD fed mice, equivalent to that of CD fed mice ( $P < 0.01$ , Fig. 3.5). Micronutrient supplementation of the CD (CD+S) did not affect 8-OHdG fluorescence.

### ***3.5.5 Effect of diet and micronutrient supplementation on testicular oxidative DNA damage***

Given that HFD feeding increased oxidative DNA lesions in mature sperm compared to CD, CD+S and HFD+S fed mice, immunofluorescence for 8-OHdG in seminiferous tubules was measured to investigate whether diet and micronutrient supplementation had an effect on 8-OHdG generation in developing sperm cells during various stages of spermatogenesis. In early stages of spermatogenesis (I-V), increased 8-OHdG fluorescence was found in spermatogonia from mice fed the 10 week HFD secondary diet regimen compared to CD fed mice, with no effect of micronutrient supplementation ( $P < 0.05$ , Table 3.1). Elongated and elongating spermatids from middle (VI-VIII) and late stages (IX-XII) of spermatogenesis, respectively, from mice fed the 10 week HFD secondary diet regimen, had increased 8-OHdG fluorescence compared to mice fed a CD

( $P < 0.05$ , Table 3.1). 8-OHdG fluorescence was reduced with 10 weeks of micronutrient supplementation (HFD+S) in elongating and elongated spermatids from the early (I-V) and middle (VI-VIII) stages of spermatogenesis compared to spermatids from mice fed the un-supplemented HFD ( $P < 0.05$ , Table 3.1). There was no effect of diet or 5 weeks supplementation on testicular 8-OHdG fluorescence in spermatogonia, spermatocytes and spermatids (Table S.3.8).

### ***3.5.6 Effect of diet and micronutrient supplementation on sperm function***

While mice fed the 5 week HFD secondary diet regimen did not display changes in sperm capacitation ( $P > 0.05$ , Table 3.2), those fed the 10 week HFD secondary diet regimen had a reduced percentage of capacitated sperm compared to CD fed mice ( $P < 0.05$ , Table 3.3). Five weeks of micronutrient supplementation to either the CD or HFD did not affect sperm capacitation ( $P > 0.05$ , Table 3.2). However, when mice were fed the HFD+S for 10 weeks, the proportion of capacitated sperm increased compared to HFD fed mice, similar to CD fed mice ( $P < 0.05$ , Table 3.3).

The study then investigated whether this had an effect on the ability of sperm to bind to an oocyte. The mean number of sperm bound to an oocyte was reduced in mice from the 5 or 10 week HFD secondary diet regimen compared to those that were fed a CD ( $P < 0.05$ , Tables 3.1 and 3.2). Micronutrient supplementation for 5 or 10 weeks increased the mean number of sperm bound to an oocyte in HFD+S fed mice compared to mice fed the un-supplemented HFD ( $P < 0.05$ , Tables 3.1 and 3.2). In contrast, mice fed the CD+S for 5 or 10 weeks had no effect on sperm capacitation or sperm binding ( $P > 0.05$ , Tables 3.1 and 3.2).



Mice fed the 5 or 10 week HFD secondary diet regimen had a reduced capacity to fertilize oocytes compared to CD fed mice ( $P < 0.05$ , Tables 3.1 and 3.2). Interestingly, mice fed the HFD+S for 5 or 10 weeks had an increased fertilization capacity compared to HFD fed mice ( $P < 0.05$ , Tables 3.1 and 3.2). Furthermore, after 10 weeks of supplementation to both the CD and HFD (CD+S and HFD+S), the percentage of fertilized oocytes was increased compared to mice fed the CD or HFD, respectively ( $P < 0.05$ , Table 3.2).

### 3.6 Discussion

This study demonstrates that a combination of dietary micronutrient supplementation (vitamin E, vitamin C, lycopene, zinc, folic acid, selenium and green tea extract) ameliorates the detrimental effects that diet induced obesity has on sperm parameters beyond count, motility and morphology in the mouse. However, the length of time for the supplementation did modify the degree of improvement. Specifically, supplementing a HFD with micronutrients in obese male mice for 5 or 10 weeks improved sperm intracellular ROS, sperm oxidative DNA lesions, sperm morphology, sperm-oocyte binding and fertilization rates that are compromised by the consumption of a HFD. Furthermore, this study demonstrated that a HFD supplemented with micronutrients for two rounds of spermatogenesis (10 weeks) reduced oxidative DNA lesions in the germ cells of the testis.

Mice fed a HFD for a total of 15 or 20 weeks (5 and 10 week HFD second dietary period) had increased adiposity without overt changes to their glucose homeostasis, as evidenced by no changes in glucose and insulin tolerance compared to CD fed mice, consistent with previous studies (Fullston *et al.* 2013; Fullston *et al.* 2012). Interestingly, mice fed a second period of a 10-week HFD had increased liver weight and increased serum triglyceride concentrations compared to CD fed mice, potentially indicative of hepatic steatosis (DeAngelis *et al.* 2005). Micronutrient supplementation in the HFD for 10 weeks reduced adiposity compared to HFD fed mice. According to previous studies which separately assessed the intake of selenium, vitamin C, folic acid, zinc or green tea extract (all included in this dietary supplementation), this unexpected reduction in adiposity may be due to the anti-oxidative functions of the micronutrients to inhibit

adipocyte growth (Carillon *et al.* 2014; Kim *et al.* 2012; Stewart *et al.* 2009; Thornton *et al.* 2011; Wolfram *et al.* 2005). Lipid metabolism is regulated by oxidative stress; and micronutrients with anti-oxidative properties may reduce adipose tissue mass via the activation of adipocyte lipolysis pathways (Carillon *et al.* 2014; Thornton *et al.* 2011; Wolfram *et al.* 2005).

Rodent models of HFD supplementation with probiotics, selenium-enriched probiotics or astaxanthin and vitamin E for durations varying between 6-12 weeks demonstrated improvements in conventional sperm parameters including motility, concentration and morphology compared to the HFD controls (Chen *et al.* 2013; Ibrahim *et al.* 2012; Mortazavi *et al.* 2014). However, this study only demonstrated an improvement in sperm morphology. This inconsistency may be due to the different animal species used whereby two of the three previous studies were rat models (Chen *et al.* 2013; Mortazavi *et al.* 2014), different HFD compositions in which one study used a HFD composed of 60% fat, compared to 21% in this study (Mortazavi *et al.* 2014), potential metabolic differences and different impacts on serum testosterone seen between the studies, which regulates spermatogenesis (Ibrahim *et al.* 2012). In this study, 5 weeks of CD+S intake increased the percentage of morphologically normal sperm compared to CD fed mice. When supplements were administered for 10 weeks, normal sperm morphology increased in mice fed both the CD+S and HFD+S. These increases in normal sperm morphology were due to improvements in sperm head and tail morphology, which may be primarily due to selenium supplementation (Watanabe and Endo 1991). Although the pathways involved are unknown, selenium intake has been shown to enhance the supply of selenium to the testis (Hill *et al.* 2007), where it is required for spermatid development (Kehr *et al.* 2009) and microtubule organization to maintain proper head and tail

morphogenesis (O'Donnell and O'Bryan 2014). Furthermore, lycopene was included in the supplemented diet used in this study, and although the mechanism remains unknown, lycopene intake is associated with improved sperm morphology in both humans and rats (Ceribasi *et al.* 2010; Zareba *et al.* 2013).

ROS in sperm are key mediators of sperm dysfunction (Agarwal *et al.* 2014) and are also suggested to be a mediator of reduced fertility and poorer subsequent embryo development from an obese male. In this study, micronutrient supplementation (HFD+S) to obese males fed a HFD reduced the increased intracellular ROS levels associated with HFD feeding (Bakos *et al.* 2010; Fullston *et al.* 2012; Palmer *et al.* 2012; Zhao *et al.* 2014). These results align with previous reports from rat models where supplementation of a HFD with probiotics, which have antimicrobial and antioxidant properties, reduced sperm ROS, specifically nitric oxide, and increased enzymatic antioxidants (Chen *et al.* 2013). The combination of micronutrients with anti-oxidative functions used in the present study may scavenge and reduce ROS from various sources within sperm, which most likely contribute to the observed reduction in intracellular ROS (Greco *et al.* 2005; Joshi *et al.* 2001; Suleiman *et al.* 1996).

One of the sources of ROS in sperm are mitochondria whereby mitochondrial ROS generation, mainly superoxide anions (Koppers *et al.* 2008; Koppers *et al.* 2010), is associated with increased DNA damage and impaired sperm function in men (Aitken *et al.* 2012; Said *et al.* 2004). The percentage of sperm positive for MSR, an indicator of superoxide production, in HFD-induced obese mice was not elevated compared to the CD fed mice despite an observed reduction with 5 weeks of micronutrient supplementation (HFD+S) compared to sperm from mice fed the un-supplemented HFD.

Interestingly, the protective effect of micronutrient supplementation (HFD+S) did not persist when supplementation was extended until 10 weeks possibly due to the conversion of superoxide to hydrogen peroxide by superoxide dismutase (SOD) (Koppers *et al.* 2008; Li *et al.* 2011).

8-OHdG is a modified guanine base formed in DNA by the oxidative attack of the guanine nucleobase by ROS (Noblanc *et al.* 2013; Valavanidis *et al.* 2009), and in sperm it is highly correlated with impaired chromatin protamination and DNA fragmentation (De Iuliis *et al.* 2009). For the first time, this study shows that micronutrient supplementation (both 5 and 10 week durations) reduces sperm 8-OHdG lesions associated with HFD feeding. This may be due to the protective effects of vitamin C supplementation, as vitamin C deficiency increases the incidence of 8-OHdG lesions in human sperm (Fraga *et al.* 1991). This is likely a consequence of the micronutrients crossing the blood testis barrier and/or the blood epididymal barrier during spermatogenesis and sperm maturation to reduce the susceptibility of maturing sperm to ROS and subsequent oxidative DNA modifications (Aitken and Roman 2008). The HFD fed animals also exhibited oxidative stress in the germ cells of the testes with increased 8-OHdG lesions in elongating and elongated spermatids, which was reduced by 10 weeks of micronutrient supplementation. The observed oxidative damage to the nucleus of the maturing and elongating spermatids during spermatogenesis may be due to atrophy in seminiferous tubules, lack of cell adhesion between spermatogenic and Sertoli cells, and a damaged blood testis barrier, as found previously in HFD fed mice (Fan *et al.* 2015; Ibrahim *et al.* 2012). Collectively, these factors may contribute to the demonstrated elevated oxidative stress and impaired sperm function in mature sperm.

Human and animal studies support the link between obesity and reduced sperm-oocyte binding (Bakos *et al.* 2010; Palmer *et al.* 2012; Wegner *et al.* 2010), and reduced fertilization rates (Bakos *et al.* 2011; Mitchell *et al.* 2011). Sperm undergo capacitation to increase their oocyte binding affinity for successful fertilization to occur (Wassarman 1999). In this study, 10 weeks of micronutrient supplementation improved sperm capacitation, which was associated with increased sperm-oocyte binding and fertilization of the inseminated oocytes. Selenium deficiency in mice reduced the *in vitro* fertilization capability of sperm (Sanchez-Gutierrez *et al.* 2008) and vitamin E supplementation in men increased fertilization rates (Geva *et al.* 1996) suggesting that these agents may contribute to the improvement in sperm function and subsequent fertilization rates. This may be due to the inhibition of lipid peroxidation of the sperm plasma membrane by vitamin E, which is a lipophilic antioxidant (Keskes-Ammar *et al.* 2003). Further, the anti-oxidative capacity of selenium, which is required for the synthesis of antioxidant enzymes (Ahmadi *et al.* 2016) may also contribute to the reduction in lipid peroxidation to maintain the sperm plasma membrane's fluidity and integrity for successful sperm-oocyte binding and fertilization.

When comparing outcomes from the 5- and 10-week micronutrient supplementation durations, the longer-term treatment duration may be considered better due to the observed reduction in 8-OHdG lesions in testicular germ cells from obese male mice. In contrast, no effect was found with 5 weeks of micronutrient supplementation. However, it may be reasoned that both treatment durations were effective as reductions in 8-OHdG modifications from mature sperm were demonstrated in similar patterns in both micronutrient supplement durations. These outcomes may be considered clinically whereby micronutrient supplementation intake for a duration that spans one round of

spermatogenesis may reduce oxidative DNA lesions in mature sperm from obese men. Also, a longer-term treatment duration of micronutrient supplements may improve testicular germ cell DNA integrity in obese men in addition to maintaining DNA integrity in mature sperm, which could be investigated with other fertility treatment outcomes in an ART setting in future studies.

In conclusion, this study shows that impaired sperm quality and function resulting from male obesity can be improved by micronutrient supplementation. This study also demonstrates that there are additional benefits of supplement intake over two rounds of spermatogenesis to improve fertilization rates in both a CD and HFD. This study is the first to establish a spermatogenic effect from the intake of a combination of micronutrients, and illustrates that a reduction in sperm intracellular ROS and 8-OHdG lesions with micronutrient supplementation may be a result of reduced oxidative DNA modifications during spermatogenesis. In turn, these improvements in mature sperm quality can increase sperm-oocyte binding through enhanced capacitation and ultimately improve chances of fertilization. Further investigations beyond fertilization and into embryo development and offspring health are warranted.

### **3.7 Acknowledgements**

The authors acknowledge the technical assistance of M. Spillane and statistical assistance of N. Briggs.

### 3.8 TABLES

**Table 3.1: The effect of diet and 10 weeks of micronutrient supplementation on testicular 8-OHdG immunofluorescence from CD, CD+S, HFD and HFD+S fed male mice.**

CD: control diet, CD+S: control diet plus micronutrient supplementation, HFD: high fat diet, HFD+S: high fat diet plus micronutrient supplementation. Data are presented as mean fluorescence units  $\pm$  SEM. Different letters denote significantly distinct groups at  $P < 0.05$ .

Stage	Cell type	CD	CD+S	HFD	HFD+S
I-V	Spermatogonia	3.1 $\pm$ 1.5 <sup>a</sup>	3.1 $\pm$ 1.5 <sup>a</sup>	8.9 $\pm$ 2.1 <sup>b</sup>	4.8 $\pm$ 2.2 <sup>ab</sup>
	Spermatocytes	2.1 $\pm$ 1.4 <sup>a</sup>	0.7 $\pm$ 0.5 <sup>a</sup>	3.2 $\pm$ 1.6 <sup>a</sup>	0.9 $\pm$ 0.3 <sup>a</sup>
	Spermatids	6.8 $\pm$ 2.2 <sup>ab</sup>	11.2 $\pm$ 2.4 <sup>ab</sup>	12.1 $\pm$ 2.2 <sup>a</sup>	6.5 $\pm$ 3.1 <sup>b</sup>
VI-VIII	Spermatogonia	7.3 $\pm$ 1.3 <sup>a</sup>	9.8 $\pm$ 2.7 <sup>a</sup>	10.6 $\pm$ 2.7 <sup>a</sup>	7.4 $\pm$ 2.4 <sup>a</sup>
	Spermatocytes	0.0 $\pm$ 0.0 <sup>a</sup>	0.0 $\pm$ 0.0 <sup>a</sup>	2.1 $\pm$ 1.5 <sup>a</sup>	0.0 $\pm$ 0.0 <sup>a</sup>
	Spermatids	9.0 $\pm$ 2.7 <sup>a</sup>	10.9 $\pm$ 1.8 <sup>a</sup>	19.2 $\pm$ 2.3 <sup>b</sup>	10.1 $\pm$ 2.3 <sup>a</sup>
IX-XII	Spermatogonia	5.9 $\pm$ 2.4 <sup>a</sup>	9.4 $\pm$ 2.1 <sup>a</sup>	7.8 $\pm$ 2.2 <sup>a</sup>	7.1 $\pm$ 2.3 <sup>a</sup>
	Spermatocytes	5.7 $\pm$ 1.3 <sup>a</sup>	5.9 $\pm$ 2.3 <sup>a</sup>	6.9 $\pm$ 2.3 <sup>a</sup>	5.6 $\pm$ 1.4 <sup>a</sup>
	Spermatids	6.3 $\pm$ 1.7 <sup>a</sup>	6.9 $\pm$ 3.2 <sup>ab</sup>	13.4 $\pm$ 2.8 <sup>b</sup>	7.2 $\pm$ 2.1 <sup>ab</sup>



**Table 3.2: The effect of diet and 5 weeks of micronutrient supplementation on capacitation, sperm binding and fertilization rates from CD, CD+S, HFD and HFD+S fed mice.**

CD: control diet, CD+S: control diet plus micronutrient supplementation, HFD: high fat diet, HFD+S: high fat diet plus micronutrient supplementation. Data are presented as mean  $\pm$  SEM. Different letters denote significantly distinct groups at  $P < 0.05$ .

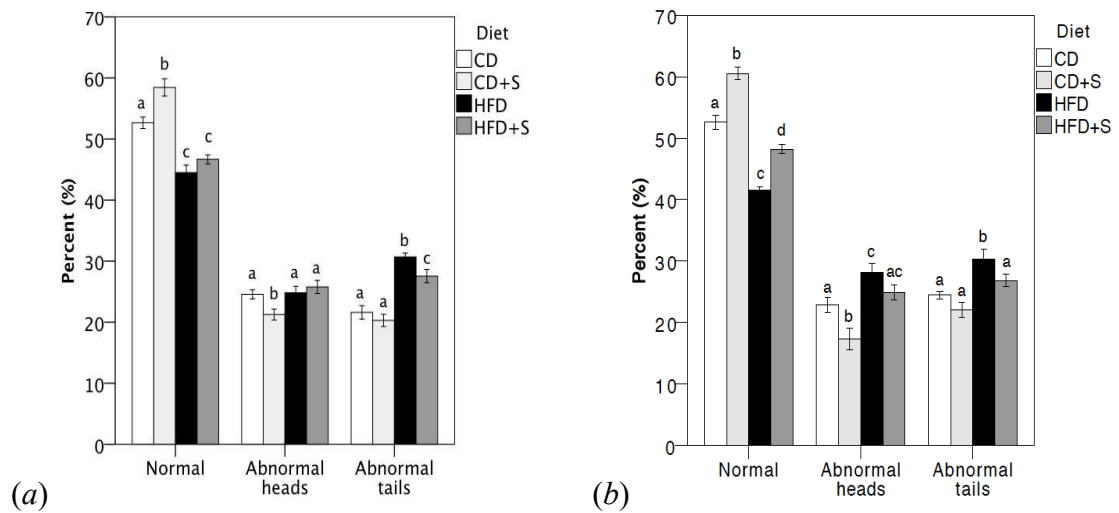
	<b>CD</b>	<b>CD+S</b>	<b>HFD</b>	<b>HFD+S</b>
Capacitated sperm (%)	91.8 $\pm$ 0.8 <sup>a</sup>	88.5 $\pm$ 1.8 <sup>a</sup>	86.8 $\pm$ 6.5 <sup>a</sup>	84.3 $\pm$ 2.4 <sup>a</sup>
Non-capacitated sperm (%)	4.6 $\pm$ 0.3 <sup>a</sup>	5.1 $\pm$ 0.9 <sup>a</sup>	7.0 $\pm$ 2.5 <sup>a</sup>	7.2 $\pm$ 1.1 <sup>a</sup>
Acrosome reacted (%)	4.3 $\pm$ 0.6 <sup>a</sup>	6.3 $\pm$ 1.1 <sup>a</sup>	6.7 $\pm$ 3.6 <sup>a</sup>	8.5 $\pm$ 1.5 <sup>a</sup>
Mean number of sperm bound	15.1 $\pm$ 1.1 <sup>a</sup>	17.5 $\pm$ 0.7 <sup>a</sup>	9.6 $\pm$ 0.3 <sup>b</sup>	15.7 $\pm$ 1.6 <sup>a</sup>
Fertilization rates (%)	54.6 $\pm$ 1.1 <sup>a</sup>	53.2 $\pm$ 1.4 <sup>a</sup>	47.5 $\pm$ 1.9 <sup>b</sup>	58.7 $\pm$ 1.6 <sup>a</sup>

**Table 3.3: The effect of diet and 10 weeks of micronutrient supplementation on capacitation, sperm binding and fertilization rates from CD, CD+S, HFD and HFD+S fed mice.**

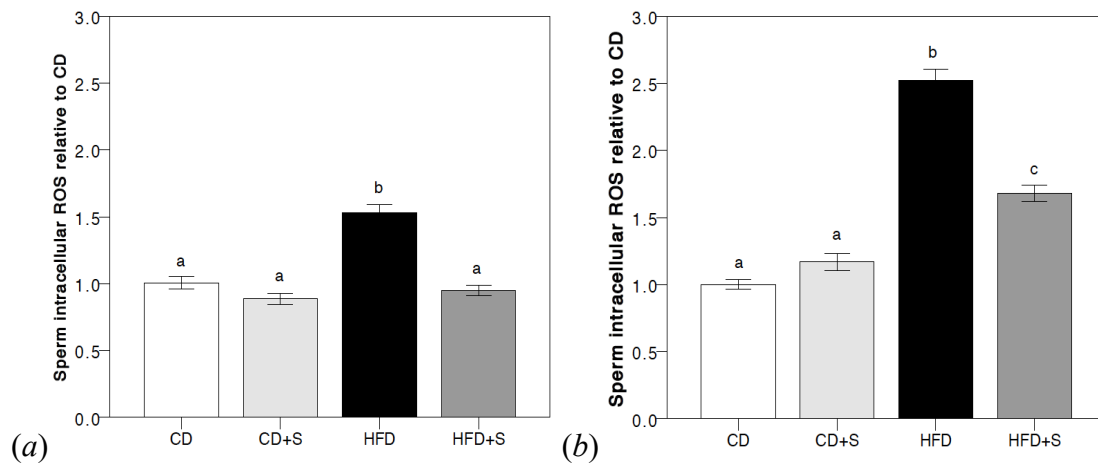
CD: control diet, CD+S: control diet plus micronutrient supplementation, HFD: high fat diet, HFD+S: high fat diet plus micronutrient supplementation. Data are presented as mean  $\pm$  SEM. Different letters denote significantly distinct groups at  $P < 0.05$ .

	<b>CD</b>	<b>CD+S</b>	<b>HFD</b>	<b>HFD+S</b>
Capacitated sperm (%)	88.7 $\pm$ 1.8 <sup>a</sup>	88.9 $\pm$ 1.3 <sup>a</sup>	81.1 $\pm$ 2.1 <sup>b</sup>	87.0 $\pm$ 1.2 <sup>a</sup>
Non-capacitated sperm (%)	6.0 $\pm$ 1.1 <sup>a</sup>	5.6 $\pm$ 0.5 <sup>a</sup>	9.0 $\pm$ 0.6 <sup>a</sup>	6.8 $\pm$ 0.8 <sup>a</sup>
Acrosome reacted (%)	5.3 $\pm$ 1.0 <sup>a</sup>	4.9 $\pm$ 0.6 <sup>a</sup>	7.6 $\pm$ 1.2 <sup>a</sup>	6.2 $\pm$ 0.5 <sup>a</sup>
Mean number of sperm bound	15.7 $\pm$ 1.1 <sup>a</sup>	15.8 $\pm$ 1.2 <sup>a</sup>	10.5 $\pm$ 0.6 <sup>b</sup>	15.0 $\pm$ 1.5 <sup>a</sup>
Fertilization rates (%)	41.1 $\pm$ 1.8 <sup>a</sup>	50.9 $\pm$ 2.0 <sup>b</sup>	32.6 $\pm$ 1.3 <sup>c</sup>	49.7 $\pm$ 1.8 <sup>b</sup>

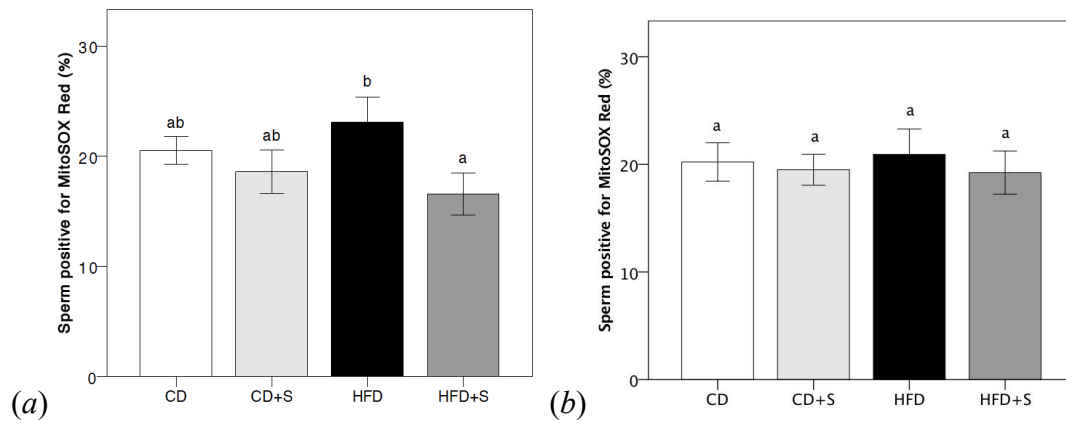
### 3.9 FIGURES



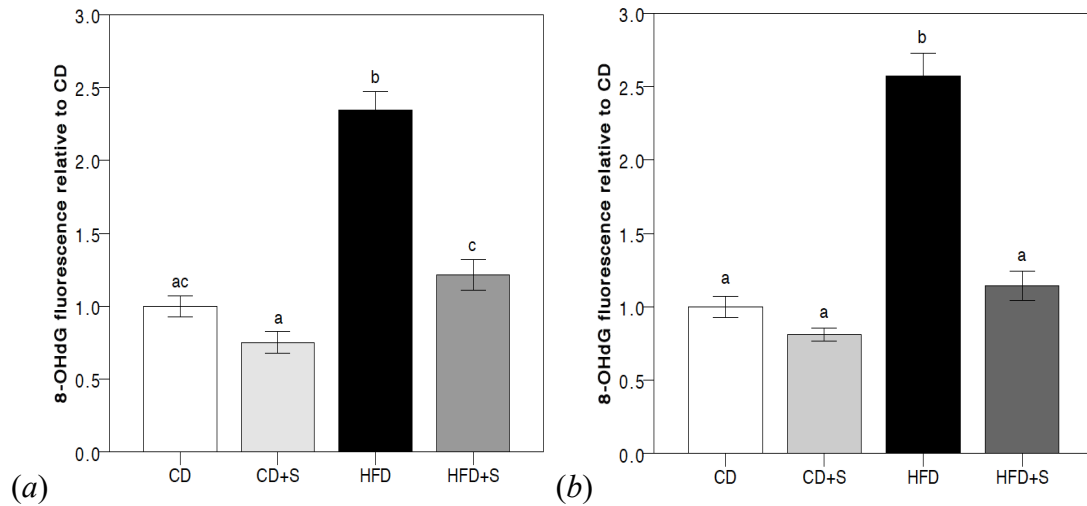
**Fig. 3.2.** Sperm morphology at (a) 5 weeks of diet and micronutrient supplementation; and (b) at 10 weeks of diet and micronutrient supplementation from CD, CD+S, HFD and HFD+S fed mice. Data are presented as mean  $\pm$  SEM. Different letters denote significantly distinct groups at  $P < 0.05$ . CD, control diet; HFD, high fat diet; CD+S, control diet plus micronutrient supplements; HFD+S, high fat diet plus micronutrient supplements.



**Fig. 3.3.** Sperm intracellular ROS levels relative to the CD at (a) 5 weeks of diet and micronutrient supplementation; and (b) 10 weeks of diet and micronutrient supplementation from CD, CD+S, HFD and HFD+S fed mice. Data are presented as mean  $\pm$  SEM. Different letters denote significantly distinct groups at  $P < 0.05$ . CD, control diet; HFD, high fat diet; CD+S, control diet plus micronutrient supplements; HFD+S, high fat diet plus micronutrient supplements.



**Fig. 3.4.** Percentage of sperm positive for MitoSOX Red at (a) 5 weeks of diet and micronutrient supplementation; and (b) 10 weeks of diet and micronutrient supplementation from CD, CD+S, HFD and HFD+S fed mice. Data are mean  $\pm$  SEM. Different letters denote significantly distinct groups at  $P < 0.05$  and an absence of the superscript letters denotes no statistical difference. CD, control diet; HFD, high fat diet; CD+S, control diet plus micronutrient supplements; HFD+S, high fat diet plus micronutrient supplements.



**Fig. 3.5.** Sperm 8-OHdG fluorescence relative to the CD at (a) 5 weeks of diet and micronutrient supplementation; and (b) 10 weeks of diet and micronutrient supplementation from CD, CD+S, HFD and HFD+S fed mice. Data are presented as mean  $\pm$  SEM. Different letters denote significantly distinct groups at  $P < 0.01$ . CD, control diet; HFD, high fat diet; CD+S, control diet plus micronutrient supplements; HFD+S, high fat diet plus micronutrient supplements.

### 3.10 SUPPLEMENTARY TABLES

**Table S.3.1. Body fat and lean mass in both mass and relative to total body weight (%) of CD and HFD fed mice at 9-10 weeks of the first dietary phase, as measured by dual-emission X-ray absorptiometry (DEXA).**

Data are mean  $\pm$  SEM. \*\*\*P<0.001. CD, control diet; HFD, high fat diet.

<b>Body measure</b>	<b>CD</b>	<b>HFD</b>
Body Fat (g)	4.4 $\pm$ 0.1	8.1 $\pm$ 0.3***
Body Fat (%)	17.7 $\pm$ 0.3	27.3 $\pm$ 0.5***
Lean Mass (g)	20.3 $\pm$ 0.1	21.3 $\pm$ 0.2***
Lean Mass (%)	75.7 $\pm$ 0.5	67.3 $\pm$ 0.6***

**Table S.3.2. The effect of diet and 5 weeks of micronutrient supplementation on body composition from CD, CD+S, HFD and HFD+S fed male mice.**

Data are mean  $\pm$  SEM. Different letters denote significantly distinct groups at  $P < 0.05$ . CD, control diet; HFD, high fat diet; CD+S, control diet plus micronutrient supplements; HFD+S, high fat diet plus micronutrient supplements.

	<b>CD</b>	<b>CD+S</b>	<b>HFD</b>	<b>HFD+S</b>
Total body weight (g)	28.8 $\pm$ 0.8 <sup>a</sup>	28.5 $\pm$ 0.5 <sup>a</sup>	36.2 $\pm$ 1.4 <sup>b</sup>	33.8 $\pm$ 0.7 <sup>b</sup>
Adipose tissues (% of body weight)				
Peri-renal fat	0.06 $\pm$ 0.03 <sup>a</sup>	0.07 $\pm$ 0.02 <sup>ab</sup>	0.10 $\pm$ 0.02 <sup>b</sup>	0.09 $\pm$ 0.02 <sup>b</sup>
Retroperitoneal fat	0.64 $\pm$ 0.05 <sup>a</sup>	0.60 $\pm$ 0.06 <sup>a</sup>	1.09 $\pm$ 0.10 <sup>b</sup>	1.26 $\pm$ 0.04 <sup>b</sup>
Omental fat	1.18 $\pm$ 0.10 <sup>a</sup>	1.10 $\pm$ 0.05 <sup>a</sup>	1.53 $\pm$ 0.12 <sup>b</sup>	1.75 $\pm$ 0.06 <sup>b</sup>
Dorsal fat	0.59 $\pm$ 0.06 <sup>a</sup>	0.49 $\pm$ 0.02 <sup>a</sup>	0.86 $\pm$ 0.07 <sup>b</sup>	0.64 $\pm$ 0.04 <sup>ab</sup>
Gonadal fat	2.69 $\pm$ 0.13 <sup>a</sup>	2.39 $\pm$ 0.10 <sup>a</sup>	4.69 $\pm$ 0.10 <sup>b</sup>	4.34 $\pm$ 0.14 <sup>b</sup>
Sum of adipose tissues	4.95 $\pm$ 0.24 <sup>a</sup>	4.57 $\pm$ 0.13 <sup>a</sup>	8.87 $\pm$ 0.35 <sup>b</sup>	8.25 $\pm$ 0.22 <sup>b</sup>
Organs (% of body weight)				



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Testis	$0.32 \pm 0.01^a$	$0.30 \pm 0.01^a$	$0.25 \pm 0.01^a$	$0.25 \pm 0.01^a$
Seminal Vesicles	$0.99 \pm 0.03^a$	$1.05 \pm 0.03^a$	$0.90 \pm 0.03^a$	$0.91 \pm 0.04^a$
Vastus Lateralis	$0.31 \pm 0.07^a$	$0.32 \pm 0.06^a$	$0.21 \pm 0.04^a$	$0.33 \pm 0.05^a$
Soleus	$0.02 \pm 0.00^a$	$0.02 \pm 0.00^a$	$0.01 \pm 0.00^a$	$0.01 \pm 0.00^a$
Liver	$4.80 \pm 0.08^a$	$4.59 \pm 0.07^a$	$4.73 \pm 0.20^a$	$4.72 \pm 0.13^a$
Pancreas	$0.41 \pm 0.02^a$	$0.40 \pm 0.02^a$	$0.32 \pm 0.01^a$	$0.39 \pm 0.03^a$
Kidney	$0.72 \pm 0.01^a$	$0.71 \pm 0.01^a$	$0.62 \pm 0.01^a$	$0.65 \pm 0.01^a$

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**Table S.3.3 The effect of diet and 10 weeks of micronutrient supplementation on body composition from CD, CD+S, HFD and HFD+S fed male mice.**

Data are mean  $\pm$  SEM. Different letters denote significantly distinct groups at  $P < 0.05$ . CD, control diet; HFD, high fat diet; CD+S, control diet plus micronutrient supplements; HFD+S, high fat diet plus micronutrient supplements.

	<b>CD</b>	<b>CD+S</b>	<b>HFD</b>	<b>HFD+S</b>
Total body weight (g)	31.5 $\pm$ 0.7 <sup>a</sup>	30.1 $\pm$ 1.0 <sup>a</sup>	35.7 $\pm$ 0.9 <sup>b</sup>	34.0 $\pm$ 0.9 <sup>b</sup>
Adipose tissues (% of body weight)				
Peri-renal fat	0.06 $\pm$ 0.01 <sup>a</sup>	0.05 $\pm$ 0.01 <sup>a</sup>	0.10 $\pm$ 0.01 <sup>b</sup>	0.10 $\pm$ 0.01 <sup>b</sup>
Retroperitoneal fat	0.95 $\pm$ 0.08 <sup>a</sup>	0.94 $\pm$ 0.01 <sup>a</sup>	1.63 $\pm$ 0.06 <sup>b</sup>	1.31 $\pm$ 0.05 <sup>ab</sup>
Omental fat	1.27 $\pm$ 0.10 <sup>a</sup>	1.44 $\pm$ 0.09 <sup>ab</sup>	2.00 $\pm$ 0.42 <sup>b</sup>	1.43 $\pm$ 0.04 <sup>ab</sup>
Dorsal fat	0.65 $\pm$ 0.03 <sup>a</sup>	0.70 $\pm$ 0.01 <sup>a</sup>	0.92 $\pm$ 0.03 <sup>b</sup>	0.82 $\pm$ 0.04 <sup>b</sup>
Gonadal fat	3.11 $\pm$ 0.11 <sup>a</sup>	2.99 $\pm$ 0.18 <sup>a</sup>	5.13 $\pm$ 0.08 <sup>b</sup>	4.89 $\pm$ 0.14 <sup>b</sup>
Sum of adipose tissues	6.30 $\pm$ 0.28 <sup>a</sup>	6.12 $\pm$ 0.32 <sup>a</sup>	9.11 $\pm$ 0.15 <sup>b</sup>	8.37 $\pm$ 0.29 <sup>c</sup>
Organs (% of body weight)				

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Testis	$0.27 \pm 0.01^a$	$0.29 \pm 0.01^a$	$0.24 \pm 0.01^a$	$0.26 \pm 0.01^a$
Seminal Vesicles	$1.08 \pm 0.02^a$	$1.09 \pm 0.04^a$	$0.88 \pm 0.03^a$	$0.90 \pm 0.03^a$
Vastus Lateralis	$0.21 \pm 0.01^a$	$0.21 \pm 0.01^a$	$0.17 \pm 0.01^a$	$0.21 \pm 0.03^a$
Soleus	$0.02 \pm 0.00^a$	$0.02 \pm 0.00^a$	$0.02 \pm 0.00^a$	$0.02 \pm 0.00^a$
Liver	$4.38 \pm 0.10^a$	$4.60 \pm 0.18^a$	$5.32 \pm 0.24^b$	$5.61 \pm 0.22^b$
Pancreas	$0.35 \pm 0.02^a$	$0.39 \pm 0.02^a$	$0.31 \pm 0.02^a$	$0.37 \pm 0.02^a$
Kidney	$0.74 \pm 0.03^a$	$0.76 \pm 0.02^a$	$0.64 \pm 0.01^a$	$0.67 \pm 0.02^a$

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**Table S.3.4. The effect of diet and 5 weeks of micronutrient supplementation on serum metabolites, testosterone and C-Reactive Protein concentrations from CD, CD+S, HFD and HFD+S fed male mice.**

Data are mean  $\pm$  SEM. Different letters denote significantly distinct groups at  $P < 0.05$ . CD, control diet; HFD, high fat diet; CD+S, control diet plus micronutrient supplements; HFD+S, high fat diet plus micronutrient supplements.

	<b>CD</b>	<b>CD+S</b>	<b>HFD</b>	<b>HFD+S</b>
Cholesterol (mmol/L <sup>-1</sup> )	1.74 $\pm$ 0.08 <sup>a</sup>	1.72 $\pm$ 0.11 <sup>a</sup>	2.91 $\pm$ 0.18 <sup>b</sup>	2.92 $\pm$ 0.11 <sup>b</sup>
Glucose (mmol/L <sup>-1</sup> )	6.59 $\pm$ 0.39 <sup>a</sup>	6.97 $\pm$ 0.39 <sup>a</sup>	7.45 $\pm$ 0.50 <sup>a</sup>	7.14 $\pm$ 0.34 <sup>a</sup>
HDL (mmol/L <sup>-1</sup> )	1.49 $\pm$ 0.08 <sup>a</sup>	1.48 $\pm$ 0.08 <sup>a</sup>	2.47 $\pm$ 0.11 <sup>b</sup>	2.51 $\pm$ 0.07 <sup>b</sup>
Triglycerides (mmol/L <sup>-1</sup> )	0.26 $\pm$ 0.02 <sup>a</sup>	0.26 $\pm$ 0.01 <sup>a</sup>	0.36 $\pm$ 0.03 <sup>b</sup>	0.32 $\pm$ 0.03 <sup>b</sup>
NEFA (mmol/L <sup>-1</sup> )	0.34 $\pm$ 0.02 <sup>a</sup>	0.31 $\pm$ 0.02 <sup>a</sup>	0.32 $\pm$ 0.01 <sup>a</sup>	0.31 $\pm$ 0.01 <sup>a</sup>
Testosterone (nmol/L <sup>-1</sup> )	0.07 $\pm$ 0.04 <sup>a</sup>	0.13 $\pm$ 0.06 <sup>a</sup>	0.05 $\pm$ 0.03 <sup>a</sup>	0.08 $\pm$ 0.07 <sup>a</sup>
C-Reactive Protein (ng/mL)	14.49 $\pm$ 1.47 <sup>a</sup>	12.06 $\pm$ 0.64 <sup>a</sup>	12.59 $\pm$ 1.06 <sup>a</sup>	13.20 $\pm$ 1.42 <sup>a</sup>

**Table S.3.5. The effect of diet and 10 weeks of micronutrient supplementation on serum metabolites, testosterone and C-Reactive Protein concentrations from CD, CD+S, HFD and HFD+S fed male mice.**

Data are mean  $\pm$  SEM. Different letters denote significantly distinct groups at  $P < 0.05$ . CD, control diet; HFD, high fat diet; CD+S, control diet plus micronutrient supplements; HFD+S, high fat diet plus micronutrient supplements.

	<b>CD</b>	<b>CD+S</b>	<b>HFD</b>	<b>HFD+S</b>
Cholesterol (mmol/L <sup>-1</sup> )	1.48 $\pm$ 0.07 <sup>a</sup>	1.47 $\pm$ 0.13 <sup>a</sup>	2.75 $\pm$ 0.13 <sup>b</sup>	2.63 $\pm$ 0.18 <sup>b</sup>
Glucose (mmol/L <sup>-1</sup> )	6.94 $\pm$ 0.52 <sup>a</sup>	6.29 $\pm$ 0.43 <sup>a</sup>	7.94 $\pm$ 0.35 <sup>b</sup>	7.73 $\pm$ 0.24 <sup>b</sup>
HDL (mmol/L <sup>-1</sup> )	1.36 $\pm$ 0.07 <sup>a</sup>	1.22 $\pm$ 0.15 <sup>a</sup>	2.45 $\pm$ 0.10 <sup>b</sup>	2.55 $\pm$ 0.09 <sup>b</sup>
Triglycerides (mmol/L <sup>-1</sup> )	0.24 $\pm$ 0.01 <sup>a</sup>	0.23 $\pm$ 0.01 <sup>a</sup>	0.30 $\pm$ 0.02 <sup>a</sup>	0.24 $\pm$ 0.01 <sup>a</sup>
NEFA (mmol/L <sup>-1</sup> )	0.44 $\pm$ 0.03 <sup>a</sup>	0.46 $\pm$ 0.03 <sup>a</sup>	0.50 $\pm$ 0.04 <sup>a</sup>	0.46 $\pm$ 0.02 <sup>a</sup>
Testosterone (nmol/L <sup>-1</sup> )	0.06 $\pm$ 0.02 <sup>a</sup>	0.06 $\pm$ 0.02 <sup>a</sup>	0.05 $\pm$ 0.02 <sup>a</sup>	0.11 $\pm$ 0.06 <sup>a</sup>
C-Reactive Protein (ng/mL)	13.93 $\pm$ 0.64 <sup>a</sup>	17.90 $\pm$ 2.86 <sup>a</sup>	12.71 $\pm$ 1.38 <sup>a</sup>	15.61 $\pm$ 1.19 <sup>a</sup>

**Table S.3.6. The effect of diet and 5 weeks of micronutrient supplementation on sperm concentration and motility from CD, CD+S, HFD and HFD+S fed male mice.**

Data are presented as mean fluorescence units  $\pm$  SEM. No differences were found between diet groups. CD, control diet; HFD, high fat diet; CD+S, control diet plus micronutrient supplements; HFD+S, high fat diet plus micronutrient supplements.

	<b>CD</b>	<b>CD+S</b>	<b>HFD</b>	<b>HFD+S</b>
Sperm motility (%)	53.33 $\pm$ 2.29	60.00 $\pm$ 3.86	47.33 $\pm$ 2.86	49.62 $\pm$ 2.79
Sperm concentration ( $\times 10^6$ )	34.82 $\pm$ 2.11	36.81 $\pm$ 2.86	35.66 $\pm$ 2.46	34.88 $\pm$ 2.83

**Table S.3.7. The effect of diet and 10 weeks of micronutrient supplementation on sperm concentration and motility from CD, CD+S, HFD and HFD+S fed male mice.**

Data are presented as mean fluorescence units  $\pm$  SEM. Different letters denote significantly distinct groups at  $P < 0.05$ . CD, control diet; HFD, high fat diet; CD+S, control diet plus micronutrient supplements; HFD+S, high fat diet plus micronutrient supplements.

	<b>CD</b>	<b>CD+S</b>	<b>HFD</b>	<b>HFD+S</b>
Sperm motility (%)	65.00 $\pm$ 2.12 <sup>a</sup>	61.25 $\pm$ 1.87 <sup>a</sup>	50.14 $\pm$ 2.36 <sup>b</sup>	49.37 $\pm$ 2.31 <sup>b</sup>
Sperm concentration ( $\times 10^6$ )	41.91 $\pm$ 3.72 <sup>a</sup>	34.48 $\pm$ 1.56 <sup>a</sup>	39.64 $\pm$ 4.70 <sup>a</sup>	38.47 $\pm$ 4.50 <sup>a</sup>

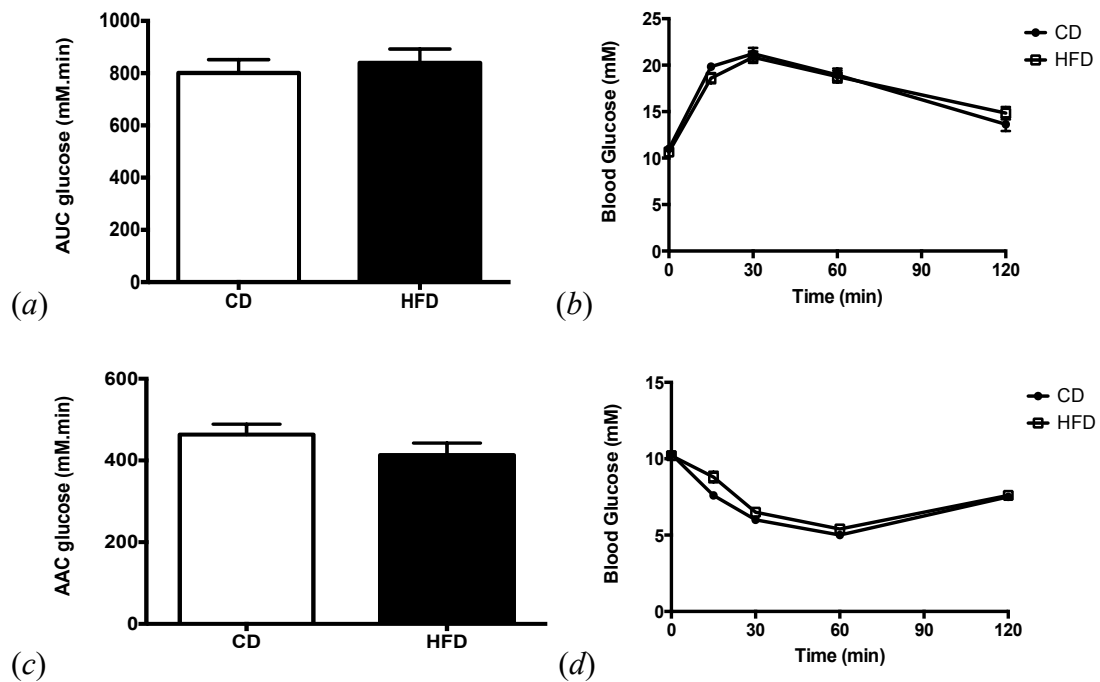
**Table S.3.8. The effect of diet and 5 weeks of micronutrient supplementation on testicular 8-OHdG immunofluorescence from CD, CD+S, HFD and HFD+S fed male mice.**

Data are presented as mean fluorescence units  $\pm$  SEM. No differences were found between diet groups. CD, control diet; HFD, high fat diet; CD+S, control diet plus micronutrient supplements; HFD+S, high fat diet plus micronutrient supplements.

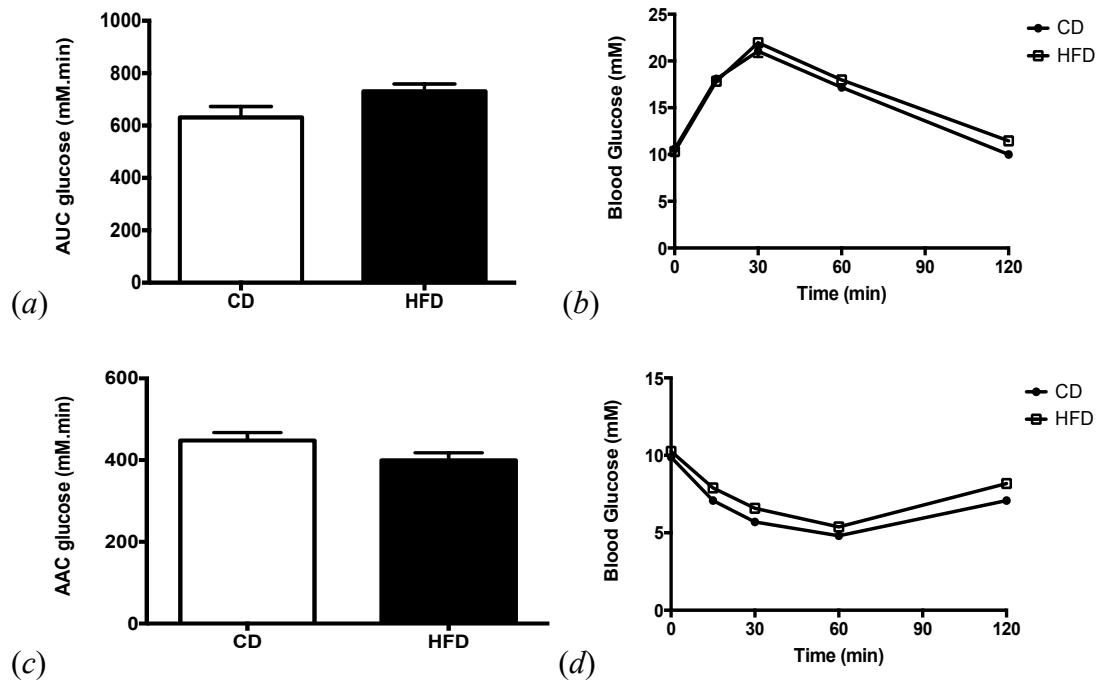
Stage	Cell type	CD	CD+S	HFD	HFD+S
I-V	Spermatogonia	3.5 $\pm$ 1.7	4.9 $\pm$ 1.6	7.6 $\pm$ 2.2	3.2 $\pm$ 1.4
	Spermatocytes	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	1.3 $\pm$ 0.3	0.0 $\pm$ 0.0
	Spermatids	3.0 $\pm$ 1.1	3.9 $\pm$ 1.6	7.4 $\pm$ 2.3	4.1 $\pm$ 1.5
VI-VIII	Spermatogonia	7.8 $\pm$ 2.4	8.0 $\pm$ 1.5	10.8 $\pm$ 3.9	10.2 $\pm$ 3.1
	Spermatocytes	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0
	Spermatids	8.2 $\pm$ 1.1	9.4 $\pm$ 0.7	13.0 $\pm$ 2.1	11.4 $\pm$ 1.4
IX-XII	Spermatogonia	9.0 $\pm$ 1.9	9.9 $\pm$ 1.8	8.4 $\pm$ 2.4	9.7 $\pm$ 1.9
	Spermatocytes	5.0 $\pm$ 1.7	5.7 $\pm$ 1.1	6.4 $\pm$ 1.9	7.2 $\pm$ 2.2
	Spermatids	7.6 $\pm$ 1.8	8.5 $\pm$ 2.0	8.6 $\pm$ 2.5	10.8 $\pm$ 2.5



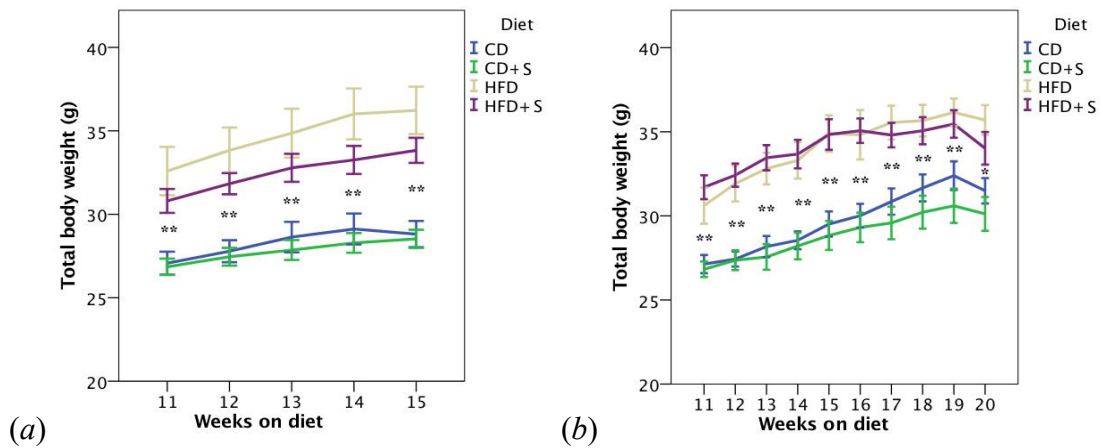
### 3.11 SUPPLEMENTARY FIGURES



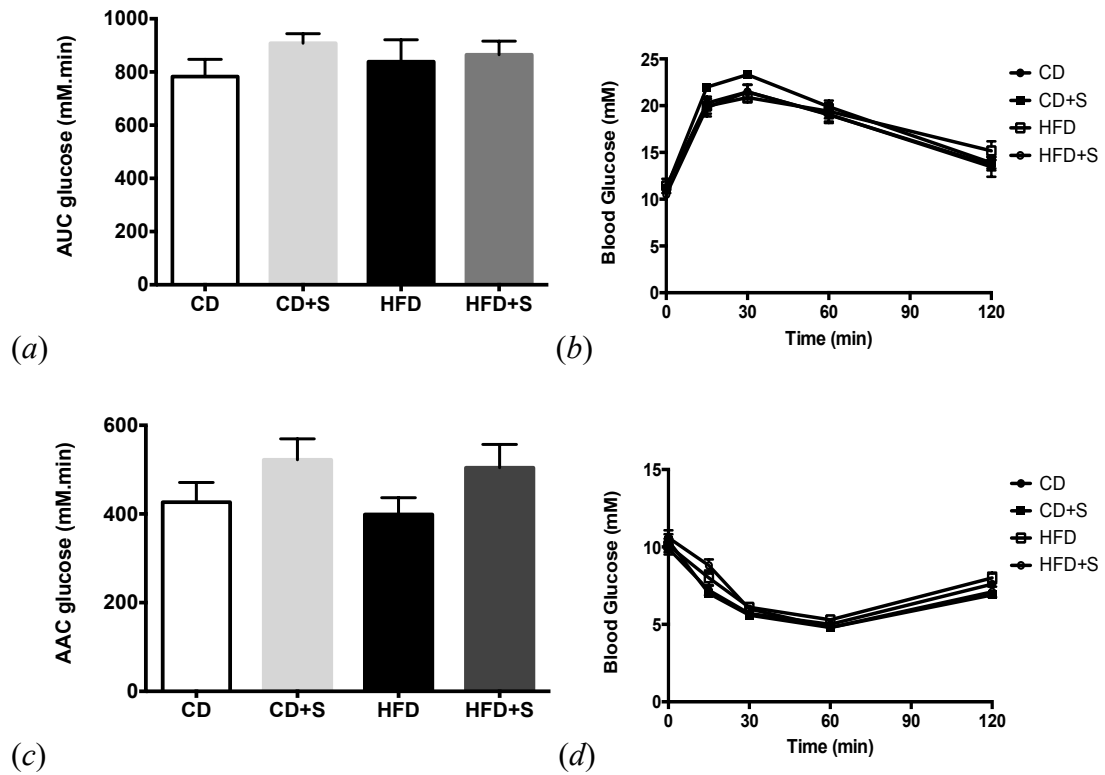
**Fig. S.3.1.** Glucose tolerance test (GTT) and insulin tolerance test (ITT) performed at 9-10 weeks of the first dietary phase from mice allocated to the 5-week micronutrient supplementation. GTT (a) Area Under the Curve (AUC) values and (b) blood glucose levels over time. ITT (c) Area Above the Curve (AAC) values and (d) blood glucose levels over time. Data are mean  $\pm$  SEM. No difference was found between the diet groups. CD, control diet; HFD, high fat diet.



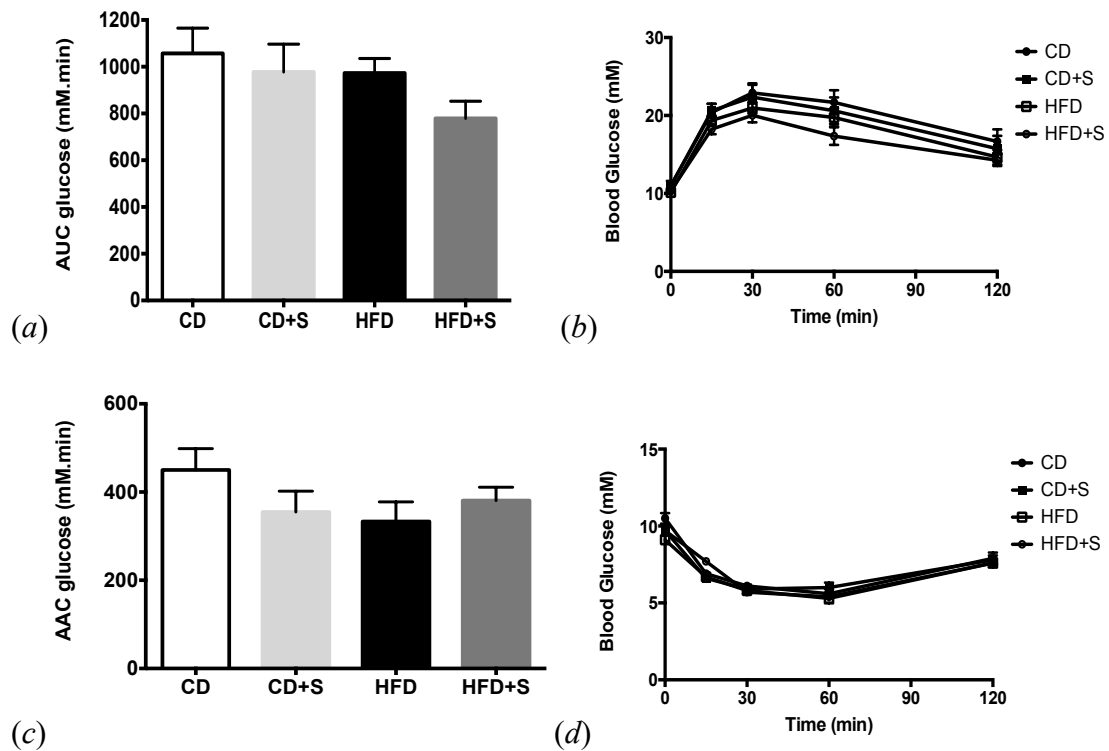
**Fig. S.3.2.** Glucose tolerance test (GTT) and insulin tolerance test (ITT) performed at 9-10 weeks of the first dietary phase from mice allocated to the 10-week micronutrient supplementation. GTT (a) Area Under the Curve (AUC) values and (b) blood glucose levels over time. ITT (c) Area Above the Curve (AAC) values and (d) blood glucose levels over time. Data are mean  $\pm$  SEM. No difference was found between the diet groups. CD, control diet; HFD, high fat diet.



**Fig. S.3.3.** The gain of total body weight over time. Micronutrient supplementation did not have an effect on total body weight from mice during the (a) 5-week second dietary phase and (b) 10-week second dietary phase. Data are mean  $\pm$  SEM. \* $P < 0.05$ , \*\* $P < 0.01$ . CD, control diet; HFD, high fat diet; CD+S, control diet plus micronutrient supplements; HFD+S, high fat diet plus micronutrient supplements.



**Fig. S.3.4.** Glucose tolerance test (GTT) and insulin tolerance test (ITT) performed during the second dietary phase at 5 weeks post micronutrient supplementation. GTT (a) Area Above the Curve (AUC) values and (b) blood glucose levels over time. ITT (c) Area Above the Curve (AAC) values and (d) blood glucose levels over time. Data are mean  $\pm$  SEM. No difference was found between the diet groups. CD, control diet; HFD, high fat diet; CD+S, control diet plus micronutrient supplements; HFD+S, high fat diet plus micronutrient supplements.



**Fig. S.3.5.** Glucose tolerance test (GTT) and insulin tolerance test (ITT) performed during the second dietary phase at 10 weeks post micronutrient supplementation. GTT (a) Area Under the Curve (AUC) values and (b) blood glucose levels over time. ITT (c) Area Above the Curve (AAC) values and (d) blood glucose levels over time. Data are mean  $\pm$  SEM. No difference was found between the diet groups. CD, control diet; HFD, high fat diet; CD+S, control diet plus micronutrient supplements; HFD+S, high fat diet plus micronutrient supplements.

### 3.12 References

- Aasheim, E.T., Hofso, D., Hjelmesaeth, J., Birkeland, K.I., and Bohmer, T. (2008) Vitamin status in morbidly obese patients: a cross-sectional study. *Am J Clin Nutr* **87**, 362-369.
- Agarwal, A., Virk, G., Ong, C., and du Plessis, S.S. (2014) Effect of oxidative stress on male reproduction. *World J Mens Health* **32**, 1-17.
- Aggerholm, A.S., Thulstrup, A.M., Toft, G., Ramlau-Hansen, C.H., and Bonde, J.P. (2008) Is overweight a risk factor for reduced semen quality and altered serum sex hormone profile? *Fertil Steril* **90**, 619-626.
- Ahmadi, S., Bashiri, R., Ghadiri-Anari, A., and Nadjarzadeh, A. (2016) Antioxidant supplements and semen parameters: An evidence based review. *Int J Reprod Biomed (Yazd)* **14**, 729-736.
- Aitken, R.J., Baker, M.A., and Sawyer, D. (2003) Oxidative stress in the male germ line and its role in the aetiology of male infertility and genetic disease. *Reprod Biomed Online* **7**, 65-70.
- Aitken, R.J., Gibb, Z., Mitchell, L.A., Lambourne, S.R., Connaughton, H.S., and De Iulii, G.N. (2012) Sperm motility is lost in vitro as a consequence of mitochondrial free radical production and the generation of electrophilic aldehydes but can be significantly rescued by the presence of nucleophilic thiols. *Biol Reprod* **87**, 110.
- Aitken, R.J., and Roman, S.D. (2008) Antioxidant systems and oxidative stress in the testes. *Oxid Med Cell Longev* **1**, 15-24.
- Awoniyi, D.O., Aboua, Y.G., Marnewick, J., and Brooks, N. (2012) The effects of rooibos (*Aspalathus linearis*), green tea (*Camellia sinensis*) and commercial rooibos and green tea supplements on epididymal sperm in oxidative stress-induced rats. *Phytother Res* **26**, 1231-1239.
- Bakos, H.W., Henshaw, R.C., Mitchell, M., and Lane, M. (2011) Paternal body mass index is associated with decreased blastocyst development and reduced live birth rates following assisted reproductive technology. *Fertil Steril* **95**, 1700-1704.
- Bakos, H.W., Mitchell, M., Setchell, B.P., and Lane, M. (2010) The effect of paternal diet-induced obesity on sperm function and fertilization in a mouse model. *Int J Androl* **34**, 402-410.
- Campbell, J.M., Lane, M., Owens, J.A., and Bakos, H.W. (2015) Paternal obesity negatively affects male fertility and assisted reproduction outcomes: a systematic review and meta-analysis. *Reproductive BioMedicine Online* **31**, 593-604.
- Carillon, J., Knabe, L., Montalban, A., Stevant, M., Keophiphath, M., Lacan, D., Cristol, J.P., and Rouanet, J.M. (2014) Curative diet supplementation with a melon

superoxide dismutase reduces adipose tissue in obese hamsters by improving insulin sensitivity. *Mol Nutr Food Res* **58**, 842-850.

Ceribasi, A.O., Turk, G., Sonmez, M., Sakin, F., and Atessahin, A. (2010) Toxic effect of cyclophosphamide on sperm morphology, testicular histology and blood oxidant-antioxidant balance, and protective roles of lycopene and ellagic acid. *Basic Clin Pharmacol Toxicol* **107**, 730-736.

Chavarro, J.E., Toth, T.L., Wright, D.L., Meeker, J.D., and Hauser, R. (2010) Body mass index in relation to semen quality, sperm DNA integrity, and serum reproductive hormone levels among men attending an infertility clinic. *Fertil Steril* **93**, 2222-2231.

Chen, X.L., Gong, L.Z., and Xu, J.X. (2013) Antioxidative activity and protective effect of probiotics against high-fat diet-induced sperm damage in rats. *Animal* **7**, 287-292.

De Iuliis, G.N., Thomson, L.K., Mitchell, L.A., Finnie, J.M., Koppers, A.J., Hedges, A., Nixon, B., and Aitken, R.J. (2009) DNA damage in human spermatozoa is highly correlated with the efficiency of chromatin remodeling and the formation of 8-hydroxy-2'-deoxyguanosine, a marker of oxidative stress. *Biol Reprod* **81**, 517-524.

DeAngelis, R.A., Markiewski, M.M., Taub, R., and Lambris, J.D. (2005) A high-fat diet impairs liver regeneration in C57BL/6 mice through overexpression of the NF-kappaB inhibitor, IkappaBalpha. *Hepatology* **42**, 1148-1157.

Droge, W. (2002) Free radicals in the physiological control of cell function. *Physiol Rev* **82**, 47-95.

Duale, N., Steffensen, I.L., Andersen, J., Brevik, A., Brunborg, G., and Lindeman, B. (2014) Impaired sperm chromatin integrity in obese mice. *Andrology* **2**, 234-243.

Ernst, B., Thurnheer, M., Schmid, S.M., and Schultes, B. (2009) Evidence for the necessity to systematically assess micronutrient status prior to bariatric surgery. *Obes Surg* **19**, 66-73.

Fan, Y., Liu, Y., Xue, K., Gu, G., Fan, W., Xu, Y., and Ding, Z. (2015) Diet-induced obesity in male C57BL/6 mice decreases fertility as a consequence of disrupted blood-testis barrier. *PLoS One* **10**, e0120775.

Fernandez-Gonzalez, R., Moreira, P.N., Perez-Crespo, M., Sanchez-Martin, M., Ramirez, M.A., Pericuesta, E., Bilbao, A., Bermejo-Alvarez, P., de Dios Hourcade, J., de Fonseca, F.R., *et al.* (2008) Long-term effects of mouse intracytoplasmic sperm injection with DNA-fragmented sperm on health and behavior of adult offspring. *Biol Reprod* **78**, 761-772.

Fraga, C.G., Motchnik, P.A., Shigenaga, M.K., Helbock, H.J., Jacob, R.A., and Ames, B.N. (1991) Ascorbic acid protects against endogenous oxidative DNA damage in human sperm. *Proc Natl Acad Sci U S A* **88**, 11003-11006.

- Fullston, T., Ohlsson Teague, E.M., Palmer, N.O., DeBlasio, M.J., Mitchell, M., Corbett, M., Print, C.G., Owens, J.A., and Lane, M. (2013) Paternal obesity initiates metabolic disturbances in two generations of mice with incomplete penetrance to the F2 generation and alters the transcriptional profile of testis and sperm microRNA content. *FASEB J* **27**, 4226-4243.
- Fullston, T., Palmer, N.O., Owens, J.A., Mitchell, M., Bakos, H.W., and Lane, M. (2012) Diet-induced paternal obesity in the absence of diabetes diminishes the reproductive health of two subsequent generations of mice. *Hum Reprod* **27**, 1391-1400.
- Garcia, O.P., Ronquillo, D., Caamano Mdel, C., Camacho, M., Long, K.Z., and Rosado, J.L. (2012) Zinc, vitamin A, and vitamin C status are associated with leptin concentrations and obesity in Mexican women: results from a cross-sectional study. *Nutr Metab (Lond)* **9**, 59.
- Geva, E., Bartoov, B., Zabludovsky, N., Lessing, J.B., Lerner-Geva, L., and Amit, A. (1996) The effect of antioxidant treatment on human spermatozoa and fertilization rate in an in vitro fertilization program. *Fertil Steril* **66**, 430-434.
- Ghanayem, B.I., Bai, R., Kissling, G.E., Travlos, G., and Hoffler, U. (2010) Diet-induced obesity in male mice is associated with reduced fertility and potentiation of acrylamide-induced reproductive toxicity. *Biol Reprod* **82**, 96-104.
- Gillis, L., and Gillis, A. (2005) Nutrient inadequacy in obese and non-obese youth. *Can J Diet Pract Res* **66**, 237-242.
- Gobato, R.C., Seixas Chaves, D.F., and Chaim, E.A. (2014) Micronutrient and physiologic parameters before and 6 months after RYGB. *Surg Obes Relat Dis* **10**, 944-951.
- Greco, E., Iacobelli, M., Rienzi, L., Ubaldi, F., Ferrero, S., and Tesarik, J. (2005) Reduction of the incidence of sperm DNA fragmentation by oral antioxidant treatment. *J Androl* **26**, 349-353.
- Hammiche, F., Laven, J.S., Twigt, J.M., Boellaard, W.P., Steegers, E.A., and Steegers-Theunissen, R.P. (2012) Body mass index and central adiposity are associated with sperm quality in men of subfertile couples. *Hum Reprod* **27**, 2365-2372.
- Hammoud, A.O., Wilde, N., Gibson, M., Parks, A., Carrell, D.T., and Meikle, A.W. (2008) Male obesity and alteration in sperm parameters. *Fertil Steril* **90**, 2222-2225.
- Hardy, G., and Hardy, I. (2004) Selenium: the Se-XY nutraceutical. *Nutrition* **20**, 590-593.
- Hill, K.E., Zhou, J., Austin, L.M., Motley, A.K., Ham, A.J., Olson, G.E., Atkins, J.F., Gesteland, R.F., and Burk, R.F. (2007) The selenium-rich C-terminal domain of



mouse selenoprotein P is necessary for the supply of selenium to brain and testis but not for the maintenance of whole body selenium. *J Biol Chem* **282**, 10972-10980.

Hofny, E.R., Ali, M.E., Abdel-Hafez, H.Z., Kamal Eel, D., Mohamed, E.E., Abd El-Azeem, H.G., and Mostafa, T. (2010) Semen parameters and hormonal profile in obese fertile and infertile males. *Fertil Steril* **94**, 581-584.

Ibrahim, H.A., Zhu, Y., Wu, C., Lu, C., Ezekwe, M.O., Liao, S.F., and Huang, K. (2012) Selenium-enriched probiotics improves murine male fertility compromised by high fat diet. *Biol Trace Elem Res* **147**, 251-260.

Jensen, T.K., Andersson, A.M., Jorgensen, N., Andersen, A.G., Carlsen, E., Petersen, J.H., and Skakkebaek, N.E. (2004) Body mass index in relation to semen quality and reproductive hormones among 1,558 Danish men. *Fertil Steril* **82**, 863-870.

Joshi, R., Adhikari, S., Patro, B.S., Chattopadhyay, S., and Mukherjee, T. (2001) Free radical scavenging behavior of folic acid: evidence for possible antioxidant activity. *Free Radic Biol Med* **30**, 1390-1399.

Kehr, S., Malinouski, M., Finney, L., Vogt, S., Labunskyy, V.M., Kasaikina, M.V., Carlson, B.A., Zhou, Y., Hatfield, D.L., and Gladyshev, V.N. (2009) X-ray fluorescence microscopy reveals the role of selenium in spermatogenesis. *J Mol Biol* **389**, 808-818.

Keskes-Ammar, L., Feki-Chakroun, N., Rebai, T., Sahnoun, Z., Ghozzi, H., Hammami, S., Zghal, K., Fki, H., Damak, J., and Bahloul, A. (2003) Sperm oxidative stress and the effect of an oral vitamin E and selenium supplement on semen quality in infertile men. *Arch Androl* **49**, 83-94.

Kim, J.E., Choi, S.I., Lee, H.R., Hwang, I.S., Lee, Y.J., An, B.S., Lee, S.H., Kim, H.J., Kang, B.C., and Hwang, D.Y. (2012) Selenium significantly inhibits adipocyte hypertrophy and abdominal fat accumulation in OLETF rats via induction of fatty acid beta-oxidation. *Biol Trace Elem Res* **150**, 360-370.

Koppers, A.J., De Iuliis, G.N., Finnie, J.M., McLaughlin, E.A., and Aitken, R.J. (2008) Significance of mitochondrial reactive oxygen species in the generation of oxidative stress in spermatozoa. *Journal of Clinical Endocrinology & Metabolism* **93**, 3199-3207.

Koppers, A.J., Garg, M.L., and Aitken, R.J. (2010) Stimulation of mitochondrial reactive oxygen species production by unesterified, unsaturated fatty acids in defective human spermatozoa. *Free Radic Biol Med* **48**, 112-119.

Krol, M.B., Gromadzinska, J., and Wasowicz, W. (2012) SeP, ApoER2 and megalin as necessary factors to maintain Se homeostasis in mammals. *J Trace Elem Med Biol* **26**, 262-266.

Larson, K.L., DeJonge, C.J., Barnes, A.M., Jost, L.K., and Evenson, D.P. (2000) Sperm chromatin structure assay parameters as predictors of failed pregnancy following assisted reproductive techniques. *Hum Reprod* **15**, 1717-1722.

Li, R., Jen, N., Yu, F., and Hsiai, T.K. (2011) Assessing mitochondrial redox status by flow cytometric methods: vascular response to fluid shear stress. *Curr Protoc Cytom* **Chapter 9**, Unit9 37.

Lopes, S., Sun, J.G., Jurisicova, A., Meriano, J., and Casper, R.F. (1998) Sperm deoxyribonucleic acid fragmentation is increased in poor-quality semen samples and correlates with failed fertilization in intracytoplasmic sperm injection. *Fertil Steril* **69**, 528-532.

Magnusdottir, E.V., Thorsteinsson, T., Thorsteinsdottir, S., Heimisdottir, M., and Olafsdottir, K. (2005) Persistent organochlorines, sedentary occupation, obesity and human male subfertility. *Hum Reprod* **20**, 208-215.

Mitchell, M., Bakos, H.W., and Lane, M. (2011) Paternal diet-induced obesity impairs embryo development and implantation in the mouse. *Fertil Steril* **95**, 1349-1353.

Mortazavi, M., Salehi, I., Alizadeh, Z., Vahabian, M., and Roushandeh, A.M. (2014) Protective Effects of Antioxidants on Sperm Parameters and Seminiferous Tubules Epithelium in High Fat-fed Rats. *J Reprod Infertil* **15**, 22-28.

Mosbah, R., Yousef, M.I., and Mantovani, A. (2015) Nicotine-induced reproductive toxicity, oxidative damage, histological changes and haematotoxicity in male rats: the protective effects of green tea extract. *Exp Toxicol Pathol* **67**, 253-259.

Ng, M., Fleming, T., Robinson, M., Thomson, B., Graetz, N., Margono, C., Mullany, E.C., Biryukov, S., Abbafati, C., Abera, S.F., *et al.* (2014) Global, regional, and national prevalence of overweight and obesity in children and adults during 1980-2013: a systematic analysis for the Global Burden of Disease Study 2013. *Lancet* **384**, 766-781.

Noblanc, A., Damon-Soubeyrand, C., Karrich, B., Henry-Berger, J., Cadet, R., Saez, F., Guiton, R., Janny, L., Pons-Rejraji, H., Alvarez, J.G., *et al.* (2013) DNA oxidative damage in mammalian spermatozoa: where and why is the male nucleus affected? *Free Radic Biol Med* **65**, 719-723.

O'Donnell, L., and O'Bryan, M.K. (2014) Microtubules and spermatogenesis. *Semin Cell Dev Biol* **30**, 45-54.

Oakberg, E.F. (1957) Duration of spermatogenesis in the mouse. *Nature* **180**, 1137-1138.

Oger, I., Da Cruz, C., Panteix, G., and Menezo, Y. (2003) Evaluating human sperm DNA integrity: relationship between 8-hydroxydeoxyguanosine quantification and the sperm chromatin structure assay. *Zygote* **11**, 367-371.

- Olson, G.E., Winfrey, V.P., Hill, K.E., and Burk, R.F. (2004) Sequential development of flagellar defects in spermatids and epididymal spermatozoa of selenium-deficient rats. *Reproduction* **127**, 335-342.
- Omu, A.E., Al-Azemi, M.K., Kehinde, E.O., Anim, J.T., Oriowo, M.A., and Mathew, T.C. (2008) Indications of the mechanisms involved in improved sperm parameters by zinc therapy. *Med Princ Pract* **17**, 108-116.
- Palmer, N.O., Bakos, H.W., Owens, J.A., Setchell, B.P., and Lane, M. (2012) Diet and exercise in an obese mouse fed a high-fat diet improve metabolic health and reverse perturbed sperm function. *Am J Physiol Endocrinol Metab* **302**, E768-780.
- Said, T.M., Agarwal, A., Sharma, R.K., Mascha, E., Sikka, S.C., and Thomas, A.J., Jr. (2004) Human sperm superoxide anion generation and correlation with semen quality in patients with male infertility. *Fertil Steril* **82**, 871-877.
- Sanchez-Gutierrez, M., Garcia-Montalvo, E.A., Izquierdo-Vega, J.A., and Del Razo, L.M. (2008) Effect of dietary selenium deficiency on the in vitro fertilizing ability of mice spermatozoa. *Cell Biol Toxicol* **24**, 321-329.
- Seli, E., Gardner, D.K., Schoolcraft, W.B., Moffatt, O., and Sakkas, D. (2004) Extent of nuclear DNA damage in ejaculated spermatozoa impacts on blastocyst development after in vitro fertilization. *Fertil Steril* **82**, 378-383.
- Sharlip, I.D., Jarow, J.P., Belker, A.M., Lipshultz, L.I., Sigman, M., Thomas, A.J., Schlegel, P.N., Howards, S.S., Nehra, A., Damewood, M.D., *et al.* (2002) Best practice policies for male infertility. *Fertil Steril* **77**, 873-882.
- Shayeb, A.G., Harrild, K., Mathers, E., and Bhattacharya, S. (2011) An exploration of the association between male body mass index and semen quality. *Reprod Biomed Online* **23**, 717-723.
- Stewart, C.P., Christian, P., LeClerq, S.C., West, K.P., Jr., and Khatry, S.K. (2009) Antenatal supplementation with folic acid + iron + zinc improves linear growth and reduces peripheral adiposity in school-age children in rural Nepal. *Am J Clin Nutr* **90**, 132-140.
- Suleiman, S.A., Ali, M.E., Zaki, Z.M., el-Malik, E.M., and Nasr, M.A. (1996) Lipid peroxidation and human sperm motility: protective role of vitamin E. *J Androl* **17**, 530-537.
- Thornton, S.J., Wong, I.T., Neumann, R., Kozlowski, P., and Wasan, K.M. (2011) Dietary supplementation with phytosterol and ascorbic acid reduces body mass accumulation and alters food transit time in a diet-induced obesity mouse model. *Lipids Health Dis* **10**, 107.

- Valavanidis, A., Vlachogianni, T., and Fiotakis, C. (2009) 8-hydroxy-2' - deoxyguanosine (8-OHdG): A critical biomarker of oxidative stress and carcinogenesis. *J Environ Sci Health C Environ Carcinog Ecotoxicol Rev* **27**, 120-139.
- Vendramini, V., Cedenho, A.P., Miraglia, S.M., and Spaine, D.M. (2014) Reproductive function of the male obese Zucker rats: alteration in sperm production and sperm DNA damage. *Reprod Sci* **21**, 221-229.
- Wassarman, P.M. (1999) Fertilization in animals. *Dev Genet* **25**, 83-86.
- Watanabe, T., and Endo, A. (1991) Effects of selenium deficiency on sperm morphology and spermatocyte chromosomes in mice. *Mutat Res* **262**, 93-99.
- Wegner, C.C., Clifford, A.L., Jilbert, P.M., Henry, M.A., and Gentry, W.L. (2010) Abnormally high body mass index and tobacco use are associated with poor sperm quality as revealed by reduced sperm binding to hyaluronan-coated slides. *Fertil Steril* **93**, 332-334.
- Wolfram, S., Raederstorff, D., Wang, Y., Teixeira, S.R., Elste, V., and Weber, P. (2005) TEAVIGO (epigallocatechin gallate) supplementation prevents obesity in rodents by reducing adipose tissue mass. *Ann Nutr Metab* **49**, 54-63.
- Wong, W.Y., Merkus, H.M., Thomas, C.M., Menkveld, R., Zielhuis, G.A., and Steegers-Theunissen, R.P. (2002) Effects of folic acid and zinc sulfate on male factor subfertility: a double-blind, randomized, placebo-controlled trial. *Fertil Steril* **77**, 491-498.
- Zareba, P., Colaci, D.S., Afeiche, M., Gaskins, A.J., Jorgensen, N., Mendiola, J., Swan, S.H., and Chavarro, J.E. (2013) Semen quality in relation to antioxidant intake in a healthy male population. *Fertil Steril* **100**, 1572-1579.
- Zhao, J., Zhai, L., Liu, Z., Wu, S., and Xu, L. (2014) Leptin level and oxidative stress contribute to obesity-induced low testosterone in murine testicular tissue. *Oxid Med Cell Longev* **2014**, 190945.

Chapter 4 (publication format)

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**Short-Term Dietary Micronutrient Supplementation Reduces Sperm  
Oxidative Stress and Paternal Pronuclear Oxidative DNA Lesions of the  
Subsequent Embryo in a Mouse Model of Obesity**

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## 4.1 Chapter Link

The duration-based study in Chapter 3, which investigated dietary micronutrient interventions that spanned one or two rounds of spermatogenesis in diet-induced obese male mice, showed improvements in both immature (testicular) and mature (epididymal) sperm oxidative stress, sperm function and fertilization rates. This indicates that micronutrient intake may have a spermatogenic/ testicular effect in improving the quality of sperm compromised by a high fat diet and obesity. Given that spermatogenesis is a complex process composed of multiple stages that occur in different locations of the male reproductive tract, primarily the testis and epididymis, the next step was to investigate whether short term micronutrient supplementation may act on the epididymis resulting in improved sperm oxidative stress, sperm function, embryo development and pregnancy outcome.

## 4.2 Statement of Authorship

### Statement of Authorship

Title of Paper	Short-term dietary micronutrient supplementation reduces sperm oxidative stress and paternal pronuclear oxidative DNA lesions of the subsequent embryo in a mouse model of obesity	
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Name of Principal Author (Candidate)	Helana Shehadeh		
Contribution to the Paper	Study design, performed experiments, conducted data analysis and interpretation, wrote and edited the manuscript.		
Overall percentage (%)	80%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	28/8/18

#### Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:  
 the candidate's stated contribution to the publication is accurate (as detailed above);  
 permission is granted for the candidate to include the publication in the thesis; and  
 the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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### 4.3 Abstract

The increased prevalence of male obesity worldwide occurs in parallel with a decline in fertility rates. Obesity often occurs in conjunction with micronutrient deficiencies, due to a calorie-dense but nutrient-poor diet. This study investigated whether short-term dietary supplementation of micronutrients (zinc, selenium, lycopene, vitamins E and C, folic acid and green tea extract) for 10 days to span the duration of epididymal transit could improve the compromised reproductive health found in a mouse model of diet-induced obesity. Mice were subjected to a range of metabolic and reproductive assessments with results showing that mice fed a high fat diet (HFD) supplemented with micronutrients had increased normal sperm morphology and sperm-oocyte binding in addition to reduced sperm intracellular reactive oxygen species and oxidative DNA modifications (8-OHdG) compared to mice fed an un-supplemented HFD. Interestingly, increased oxidative DNA lesions in sperm from HFD fed mice persisted to the paternal pronucleus of the subsequent embryo and these 8-OHdG lesions in the paternal pronucleus were also reduced with micronutrient supplementation. After natural mating and on day 18 of gestation, fetal and placental outcomes were assessed and fetal weight was increased with micronutrient supplementation of a paternal HFD in comparison with mice fed the un-supplemented HFD. Overall, these results demonstrate that micronutrient supplementation for 10 days, to mimic epididymal transit, can reduce some detrimental effects of diet-induced obesity on oxidative stress in sperm, with subsequent improvements also seen in the paternal pronucleus of the embryo and during pregnancy.

#### ***Keywords***

DNA damage, embryo, epididymis, micronutrient, obesity, pronucleus, reactive oxygen species, spermiogenesis, sperm maturation



#### 4.4 Introduction

The prevalence of obesity worldwide has more than doubled in the past three decades making adult obesity more common than under-nutrition (WHO 2015). Obese individuals can present with micronutrient deficiencies, which can influence systemic physiological functions (Astrup and Bugel 2010; Garcia *et al.* 2009) and the increased prevalence of obesity worldwide is occurring in parallel with a decline in fertility rates (Hammoud *et al.* 2012; Sallmen *et al.* 2006). Male obesity is associated with subfertility, characterized by changes in hormone profiles (Haffner *et al.* 1993; Handelsman and Swerdloff 1985; Magnusdottir *et al.* 2005), reduced sperm count (Bakos *et al.* 2011; Fejes *et al.* 2005; Hammoud *et al.* 2008; Jensen *et al.* 2004), reduced sperm motility (Bakos *et al.* 2011; Hammoud *et al.* 2008; Kort *et al.* 2006; Zhang *et al.* 2015) and increased sperm DNA damage (Chavarro *et al.* 2011; Kort *et al.* 2006). Although it must be noted that not all human studies support a link between male obesity and impaired conventional sperm parameters suggesting that there may be other contributing lifestyle factors such as metabolic syndrome (Aggerholm *et al.* 2008; Pauli *et al.* 2008; Rybar *et al.* 2011). To avoid the multitude of lifestyle factors that confound human studies, animal models have been devised to investigate the effects of obesity on sperm quality and function. Mouse models of diet-induced obesity have demonstrated that male obesity can lead to reductions in sperm motility (Bakos *et al.* 2010; Fullston *et al.* 2012; Ghanayem *et al.* 2010; Palmer *et al.* 2012b), sperm capacitation, sperm binding and fertilization rates (Bakos *et al.* 2010; Palmer *et al.* 2012b) concomitant with increases to sperm intracellular reactive oxygen species (ROS) (Bakos *et al.* 2010; Fullston *et al.* 2012; Palmer *et al.* 2012a), sperm DNA damage (Bakos *et al.* 2010; Fullston *et al.* 2012; Palmer *et al.* 2012a)

and delayed subsequent embryo development (Binder *et al.* 2012; McPherson *et al.* 2013; Mitchell *et al.* 2011).

Given that obesity can alter both hormonal profiles and impair sperm quality and function, modifications to the tightly regulated process of spermatogenesis has been implicated as a causative factor (Reis and Dias 2012). Spermatogenesis is comprised of many stages and the final stage, post-testicular sperm maturation, is considered as one of the most crucial stages (Dacheux and Dacheux 2014; Whitfield *et al.* 2015). This is due to the fact that during the transit of the epididymal tubule, infertile testicular sperm mature and acquire forward motility and fertilizing competence (Cooper 1996). Also, during epididymal sperm maturation, sperm continue a remodeling process that includes migration of the cytoplasmic droplet along the sperm flagellum, acrosomal reshaping, nuclear chromatin condensation and structural changes to intracellular organelles (Bedford 2004; Olson *et al.* 2002).

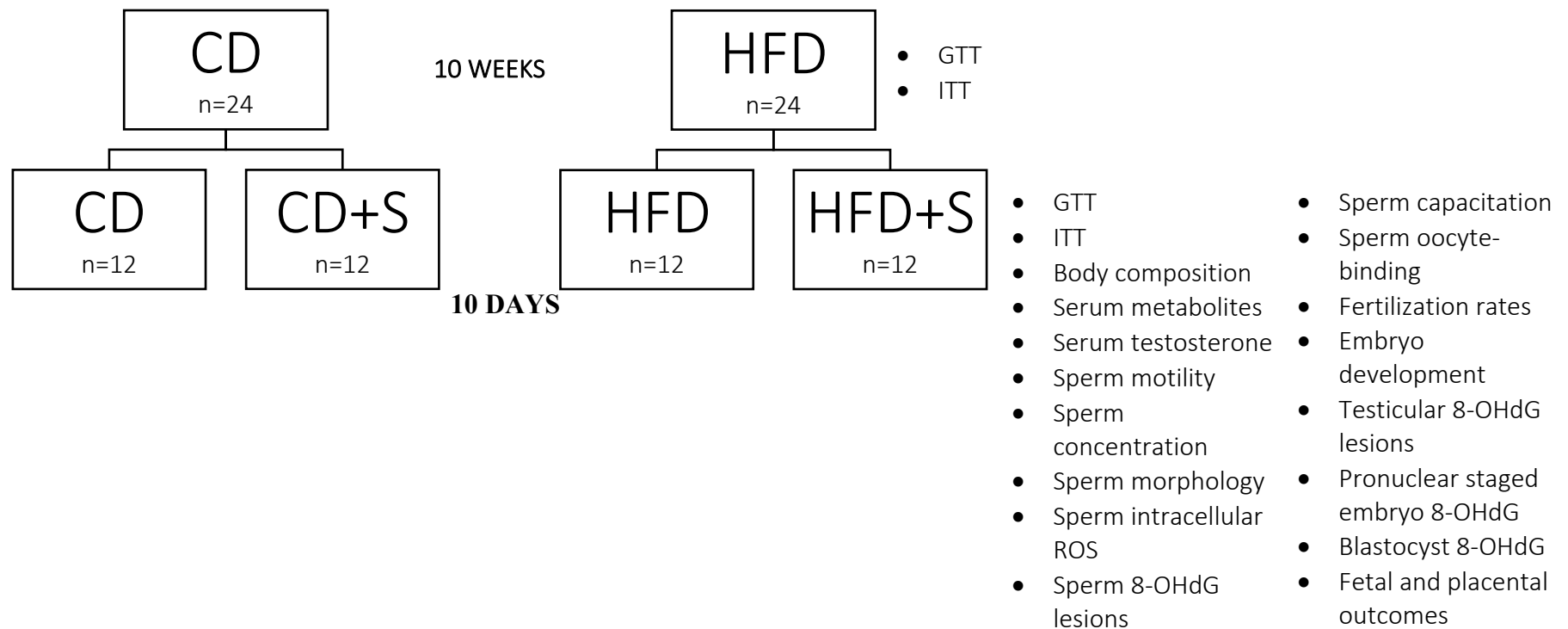
In the mouse, the process of sperm maturation during epididymal transit takes approximately 9.5 days (Oakberg 1957). The epididymis contains a semi-permeable barrier exposing developing sperm to possible damage from the physical and chemical environment (Hedger 2011). Further, during epididymal transit, sperm lack intracellular antioxidant enzyme protection due to cytoplasmic shedding, which makes epididymal sperm highly susceptible to oxidative stress and damage (Aitken and Baker 2002; Saleh and Agarwal 2002). Sperm oxidative stress, a characteristic found to be increased in obese males, has been shown to have many detrimental downstream effects including sperm oxidative DNA lesions and fragmentation (Aitken and Curry 2011; Horak *et al.* 2003), reduced fertilization rates (Aitken and Fisher 1994; Aitken 1999; Saleh and

Agarwal 2002), reduced pregnancy rates (Aitken and Curry 2011; Zribi *et al.* 2011) and impaired offspring health in both humans and rodent models (Ji *et al.* 1997; Lane *et al.* 2014; Reichenberg *et al.* 2006; Sipos *et al.* 2004). Therefore, dietary micronutrient and antioxidant supplementation has been used to mitigate sperm oxidative stress; and generally results in improved sperm parameters, reduced oxidative DNA lesions and improved pregnancy outcomes (Showell *et al.* 2011; Zini *et al.* 2009). Lycopene supplementation, which is a ROS scavenger, improves sperm morphology in both humans (Zareba *et al.* 2013) and rodents (Ceribasi *et al.* 2010). Whilst selenium deficiency is associated with abnormal sperm morphology in addition to reduced sperm motility and concentration (Hardy and Hardy 2004; Olson *et al.* 2004; Sanchez-Gutierrez *et al.* 2008). Rat sperm motility and normal morphology was increased (Mosbah *et al.* 2015), and sperm oxidative stress was reduced (Awoniyi *et al.* 2012) after two months and 10 weeks, respectively of green tea extract supplementation, a natural antioxidant. In men, sperm oxidative DNA modifications were reduced after 28 days of vitamin C supplementation (Fraga *et al.* 1991). Also, 3-6 months of zinc supplementation (both of which neutralize free radicals) reduced sperm oxidative stress, DNA damage and apoptosis (Omu *et al.* 2008; Wong *et al.* 2002). Further, combined folic acid and zinc supplementation for 6 months improved conventional sperm parameters including increased sperm count, motility and normal morphology in fertile and subfertile men (Wong *et al.* 2002). In a placebo-controlled trial, sperm motility and pregnancy rates were increased with 6 months of vitamin E supplementation, which also has antioxidant properties primarily the inhibition of lipid peroxidation, in subfertile men (Suleiman *et al.* 1996).

Although the effects of various long-term (6-12 weeks) micronutrient and antioxidant treatments on conventional sperm parameters in diet-induced obese males have been reported (Chen *et al.* 2013; Ibrahim *et al.* 2012; Mortazavi *et al.* 2014), the effects on sperm oxidative stress, oxidative DNA damage, sperm function and subsequent paternal pronuclear oxidative DNA damage have not been assessed. Furthermore, the effects on blastocyst quality and fetal and placental growth measures have not been investigated. Therefore, given that sperm maturation in the epididymis is most vulnerable to oxidative damage, a treatment duration that approximates the period of epididymal transit was investigated to assess the effects of micronutrient supplementation on compromised sperm quality, sperm function, embryo development, pronuclear DNA integrity, blastocyst cell DNA integrity and fetal growth from obese males.

## **4.5 Materials and methods**

The experimental design is outlined below in Figure 4.1. Please refer to Chapter 2, section 2.1 for experimental methods.



**Fig. 4.1** Experimental design. All mice were allocated to a first dietary phase of a CD or a HFD for 10 weeks and underwent both a glucose tolerance test (GTT) and an insulin tolerance test (ITT). For the second dietary phase, mice were allocated to either a no intervention control group (CD, HFD) or a micronutrient supplementation intervention group (CD+S, HFD+S) for 10 days. After 10 days, mice underwent a GTT, ITT, body composition at post-mortem, serum metabolites and testosterone, conventional sperm analysis, sperm intracellular ROS and 8-OHdG, sperm capacitation, oocyte binding and fertilization rates, subsequent embryo development, testicular 8-OHdG, 8-OHdG in the

pronuclear-staged embryo and blastocyst, and fetal and placental outcomes. CD, control diet; HFD, high fat diet; CD+S, control diet plus micronutrient supplements; HFD+S, high fat diet plus micronutrient supplements.

## 4.6 Results

### *4.6.1 The effect of diet and micronutrient supplementation on glucose and insulin tolerance, body mass, body composition and serum metabolites*

After 10 weeks of the dietary phase of a CD or HFD to induce increased adiposity, the mice were assessed for metabolic health. There was no difference in fasting blood glucose concentration, glucose tolerance or insulin tolerance between mice fed a CD or HFD ( $P>0.05$ , Fig. S.4.1). Glucose and insulin tolerance tests were repeated after 10 days of micronutrient supplementation and there was no effect of diet or micronutrient supplementation on fasting blood glucose concentration, glucose tolerance or insulin tolerance ( $P>0.05$ , Fig. S.4.2).

Consistent with previous studies, HFD fed mice had increased total body weight from 8 weeks of diet exposure compared to CD fed mice ( $P<0.05$ , Fig. S3). After 10 days of micronutrient supplementation, there was no effect of supplementation on weight gain ( $P>0.05$ , Fig. S3). Body composition was assessed by post mortem dissection, which revealed that mice fed a HFD had increased retroperitoneal, omental, dorsal and gonadal fat masses and increased sum of these adipose depots relative to total body weight. No effect was observed on organ mass compared to CD fed mice ( $P<0.05$ , Table 4.1). Furthermore, there was no effect of micronutrient supplementation on any body composition measures.

Mice fed a HFD had increased serum cholesterol, HDL and triglyceride concentrations compared to CD fed mice ( $P<0.05$ ) with no effect observed for the other serum metabolites tested, nor for micronutrient supplementation (Table S.4.1). Serum



testosterone concentrations were not affected by either diet or micronutrient supplementation ( $P>0.05$ , Table S.4.1).

#### ***4.6.2 The effect of diet and micronutrient supplementation on conventional sperm parameters***

Diet or micronutrient supplementation had no effect on sperm motility or sperm concentration ( $P>0.05$ , Table S.4.2). A reduction in morphologically normal sperm was found for HFD fed mice with increased abnormal tail morphology, compared to CD fed mice ( $P<0.05$ ). Micronutrient supplementation for 10 days improved sperm morphology in mice fed the CD+S and HFD+S compared to mice fed the un-supplemented CD and HFD, respectively ( $P<0.05$ , Fig. 4.2).

#### ***4.6.3 The effect of diet and micronutrient supplementation on sperm oxidative stress***

HFD fed mice had increased sperm intracellular ROS (2',7'-dichlorodihydrofluorescein diacetate; H<sub>2</sub>DCFDA) compared to CD fed mice ( $P<0.05$ , Fig. 4.3). Interestingly, HFD+S consumption reduced sperm intracellular ROS compared to mice fed an un-supplemented HFD, equivalent to that of CD fed mice ( $P<0.05$ , Fig. 4.3). There was no effect of micronutrient supplementation on sperm intracellular ROS in CD+S fed mice compared to mice fed an un-supplemented CD ( $P>0.05$ ).

8-OHdG fluorescence was measured as a marker of oxidative lesions in sperm DNA, presumably resulting from oxidative attack from the elevated sperm intracellular ROS. Sperm from HFD fed mice had increased 8-OHdG fluorescence compared to mice fed a CD ( $P<0.05$ , Fig. 4.3). Mice fed the supplemented HFD (HFD+S) had reduced sperm 8-OHdG fluorescence compared to sperm from mice fed an un-supplemented HFD

( $P < 0.05$ , Fig. 4.3). No effect was observed in mice fed a CD+S compared to CD fed mice ( $P > 0.05$ , Fig. 4.3).

#### ***4.6.4 The effect of diet and micronutrient supplementation on capacitation and sperm-oocyte binding***

Sperm capacitation was reduced in mice fed a HFD, concomitant with an increased percentage of acrosome reacted sperm, compared to CD fed mice ( $P < 0.05$ , Table 4.2). Micronutrient supplementation did not have an effect on sperm capacitation or acrosome reaction in either dietary setting ( $P > 0.05$ ). Although sperm-oocyte binding was not different between CD and HFD fed mice, sperm binding was increased with supplementation (CD+S and HFD+S) compared to mice fed the un-supplemented diets ( $P < 0.05$ , Table 4.2).

#### ***4.6.5 The effect of diet and micronutrient supplementation on fertilization rates and embryo development***

Fertilization was determined by assessing cleavage to the 2-cell stage on day 2 of embryo development with the proportion of embryos that cleaved reduced after fertilization in male mice fed a HFD compared to CD fed mice ( $P < 0.05$ , Table 4.3). However, HFD+S fed male mice produced embryos with increased day 2 cleavage rates (fertilization rates) compared to mice fed a HFD ( $P < 0.05$ , Table 4.3). A difference in embryo development rate was not observed on day 3 with the percentage of embryos at the 6-8 cell stage not different between the diet groups ( $P > 0.05$ , Table 4.3). Similarly, on day 4, the percentages of early and total blastocysts were not different between the CD and HFD groups and were not affected by micronutrient supplementation ( $P > 0.05$ , Table 4.3). By day 5 of embryo development, the percentages of expanded, hatching and total

blastocysts were not different between the CD and HFD fed mice, however male mice fed the CD with micronutrient supplementation (CD+S) had an increased rate of total blastocyst development compared to mice fed the un-supplemented CD ( $P < 0.05$ , Table 4.3). Interestingly, this increase in blastocyst development rate was not observed in mice fed the HFD+S.

#### ***4.6.6 The effect of diet and micronutrient supplementation on paternal pronuclear oxidative DNA damage in embryos***

8-OHdG fluorescence in paternal pronuclei was quantified to determine if the 8-OHdG lesions found in mature sperm DNA persisted to the paternal pronucleus following fertilization (18-19 hours post hCG injection/ 4-5 hours post fertilization). HFD fed mice produced embryos with increased paternal pronuclear 8-OHdG fluorescence compared to that from CD fed mice ( $P < 0.05$ , Fig. 4.4). Paternal pronuclear 8-OHdG fluorescence was reduced with micronutrient supplementation in the HFD (HFD+S) compared to pronuclear 8-OHdG fluorescence from mice fed a HFD ( $P < 0.05$ , Fig. 4.4). Moreover, this reduction in paternal pronuclear 8-OHdG fluorescence in embryos from mice fed a HFD supplemented with micronutrients was comparable to that from CD fed mice (Fig. 4.4).

#### ***4.6.7 The effect of diet and micronutrient supplementation on blastocyst quality***

8-OHdG fluorescence was further quantified in blastocyst cells (both trophectoderm and inner cell mass cells) to assess if the elevated 8-OHdG modifications found in the paternal pronucleus persisted in the blastocyst-stage embryo. Interestingly, there was no effect of diet or micronutrient supplementation on 8-OHdG fluorescence in blastocyst cells

( $P>0.05$ ), however the number of cells in the blastocyst was reduced in both the high fat diets (HFD, HFD+S) compared to the control diets (CD, CD+S) ( $P<0.05$ , Table 4.4).

#### ***4.6.8 The effect of diet and micronutrient supplementation on fetal and placental development***

The average number of implantations (number of fetuses or resorptions) were not different between the diet groups. Further the percentage of fetuses and resorptions as a proportion of implantations was not affected by diet ( $P>0.05$ , Table 4.5). Fetal weight was increased in those sired by males fed a HFD supplemented with micronutrients compared to that from the un-supplemented HFD ( $P<0.05$ , Table 4.5). There was no effect of diet or micronutrient supplementation on placental weight, fetal:placental weight ratio or fetal crown-rump length ( $P>0.05$ , Table 4.5).

## 4.7 Discussion

This study demonstrates that a short-term intake of dietary micronutrient supplementation (zinc, selenium, lycopene, vitamins E and C, folic acid and green tea extract) was sufficient to reduce sperm oxidative stress, evident by reduced sperm intracellular ROS and reduced oxidative DNA lesions. Furthermore, mice fed a HFD supplemented with micronutrients improved sperm morphology, increased sperm-oocyte binding capacity and fertilization rates in addition to reduced oxidative DNA lesions in the paternal pronucleus of the subsequent embryo. Further, fetal weight was increased with micronutrient supplementation of the paternal HFD compared to that fed the un-supplemented HFD.

Consistent with previous reports for diet-induced obesity of a similar duration, male mice demonstrated increased adiposity with increased serum cholesterol, HDL and triglyceride concentrations but without overt changes to glucose homeostasis as measured by GTT and ITT (Fullston *et al.* 2012; Palmer *et al.* 2012b). Serum testosterone concentrations did not differ between any of the diet groups, suggesting an unlikely or minimal contribution of testosterone driven changes in spermatogenesis (McLachlan *et al.* 2002; Walker 2011).

This study shows that micronutrient supplementation, limited to 10 days duration, improves sperm morphology in both CD and HFD fed males by most likely maintaining the integrity of sperm morphogenesis during spermatogenesis. Although previous studies suggest a spermatogenic effect of micronutrient supplementation in improving sperm morphology (Ibrahim *et al.* 2012; Mortazavi *et al.* 2014; Tufek *et al.* 2015), this study

indicates that micronutrient supplement intake may also have an epididymal effect in increasing normal sperm morphology during sperm maturation and epididymal transit. The morphogenesis of sperm begins when round spermatids are differentiated into elongating spermatids, which are then released from the lumen of the seminiferous tubules of the testis into the epididymis where further morphological changes occur. These epididymal based morphological changes include the dimension and appearance of the sperm's acrosome and flagellum to maintain normal sperm head and tail morphology, respectively (Olson *et al.* 2002). Micronutrient supplementation may support these epididymal based morphological changes by preventing the accumulation of oxidative damage and maintaining antioxidant enzyme activity in the epididymis (Olson *et al.* 2004), which ultimately may contribute to the observed improvement in normal sperm morphology.

In addition to improvements in sperm morphology, mice fed a micronutrient supplemented HFD had reductions to sperm oxidative stress, demonstrated by reduced intracellular ROS and reduced oxidative DNA lesions (8-OHdG). One possible explanation for the observed reduction in sperm intracellular ROS is an increased secretion of micronutrients and antioxidants into the epididymis resulting in the protection of maturing sperm (Nonogaki *et al.* 1992; Perry *et al.* 1993; Rejraji *et al.* 2002). Sperm are protected from ROS by antioxidant enzymes including SOD through dismutation of reactive oxygen to hydrogen peroxide, which is then rapidly converted to water by the enzyme's catalase activity or by GPx (Alvarez and Storey 1989). A key novel finding from this study is that micronutrient supplementation in mice fed a HFD reduced sperm DNA oxidative 8-OHdG modifications. Given that 8-OHdG is an oxidized base adduct resulting from the oxidative attack of ROS to the guanine nucleobase in

DNA, these results suggest that reduced sperm intracellular ROS with micronutrient supplementation may lead to reduced oxidative damage to sperm DNA. Most DNA damage is initially oxidatively induced compromising DNA integrity of the male germ line (De Iuliis *et al.* 2009; Santiso *et al.* 2010), which can perturb offspring growth and long-term health. Paternal micronutrient fortification during under-nutrition, has been reported to reduce sperm 8-OHdG lesions and subsequently prevented early growth restriction and dyslipidaemia in offspring (McPherson *et al.* 2016) illustrating the benefits of micronutrient supplementation of the paternal diet and their offspring health.

Sperm function measures were also assessed in this study and a novel finding was that micronutrient supplementation increased sperm binding to the zona pellucida of the oocyte. An increase in sperm binding with vitamin E supplement intake has been observed in clinical studies (Geva *et al.* 1996; Kessopoulou *et al.* 1995). This observed increase in sperm binding with supplementation intake may be due to the inhibitory effects of micronutrients and antioxidants on the proliferation of lipid peroxidation and the restoration of fluidity to the sperm plasma membrane, which may ultimately improve sperm-oocyte binding (Aitken *et al.* 1989; Suleiman *et al.* 1996). In this study, fertilization rates were assessed by the presence of a two-cell embryo and interestingly; the percentage of two-cell embryos were increased from mice fed a HFD supplemented with micronutrients. This increase in fertilization rates may be a reflection of the observed increase in sperm-oocyte binding. Although day 3 and day 4 of embryo development were not affected by diet or supplementation, the rate of blastocyst development on day 5 was increased from mice fed a CD with micronutrient supplementation. Curiously, this advancement in blastocyst development was not seen with micronutrient supplementation of the HFD, which may suggest a HFD's burden on the subsequent

embryo's development is beyond micronutrient-induced anti-oxidative repair (Campbell *et al.* 2015; Fan *et al.* 2015). Further, although the exact mechanisms involved in the influence of environmental conditions and/or diet on embryo development are unclear, recent studies have indicated that epididymosomes, small vesicles secreted by the epididymal epithelium that carry small molecular material to maturing sperm during epididymal transit, facilitate successful pre- and post-implantation embryo development (Conine *et al.* 2018; Sharma *et al.* 2018). Collectively, the results from this study suggest that micronutrient supplementation may implicate epididymosome function and ultimately embryo development, which could be investigated in detail in future studies.

Elevated 8-OHdG modifications were detected in the paternal pronucleus in embryos from mice fed a HFD compared to CD fed mice, suggesting the persistence of 8-OHdG lesions from mature sperm to the subsequent embryo. Although previous studies have shown that sperm DNA damage is associated with abnormal chromatin decondensation patterns during pronucleus formation (Ward 2010), this is the first study to show the oxidized base, 8-OHdG, in mature sperm from HFD-induced obese mice persists to the paternal pronucleus. Micronutrient supplementation reduced 8-OHdG fluorescence in the paternal pronucleus in embryos from HFD fed mice in line with the reduced 8-OHdG in sperm. This reduction in 8-OHdG in the paternal pronucleus may lessen or prevent the potential for mutagenesis in the embryo resulting from the oxidized paternal chromatin by activating the maternal base excision repair (BER) enzymatic pathway in the oocyte to excise 8-OHdG in the embryo following fertilization (Bruner *et al.* 2000; Lord and Aitken 2015). Although DNA 8-OHdG lesions were elevated in the paternal pronucleus of embryos produced by HFD fed males, 8-OHdG fluorescence in the cells of the subsequent blastocyst was not increased. Whilst the exact mechanism is unknown, DNA



8-OHdG lesions from the paternal pronucleus from HFD-induced obese mice may have not persisted to the subsequent blastocyst cells due to the activation of the 8-OHdG excision pathway during DNA replications in the zygote and developing embryo as DNA replication has been shown to occur in the embryo even if 50% of the paternal DNA is damaged or removed (Shaman *et al.* 2007).

Male mice were mated to assess implantation in addition to fetal and placental growth on day 18 of gestation. The number of fetuses and resorptions, placental weight and fetal crown to rump length were not affected by diet or micronutrient supplementation however, fetal weight was increased with supplementation of the HFD. An increase in fetal weight has been previously demonstrated whereby exercise in HFD-induced obese mice without or in combination with a change from a HFD to a CD was associated with an increase and restoration of fetal weight (McPherson *et al.* 2013). A theory that may explain the increase in fetal weight with supplementation of the HFD may be attributed to the observed reduction in 8-OHdG lesions in the paternal lineage. Subsequently, this may contribute to a reduced mutational load in the embryo and developing fetus (Chong *et al.* 2007) resulting in healthy fetal outcomes which is indicative of a reduced susceptibility to the onset of adult disease (Barker 2004; Johnson and Schoeni 2011).

In summary, these outcomes establish that compromised sperm quality and function, due to diet-induced obesity, can be improved with short-term intake of dietary micronutrient supplementation. Obesity-induced sperm oxidative stress and subsequent oxidative sperm DNA damage is reduced with just 10 days of micronutrient supplementation with further reductions in oxidative DNA lesions of the paternal pronucleus from the subsequent embryo in addition to improvements in fetal weight. Although these results

represent early findings toward establishing the optimal duration of intake, it highlights some potential mechanisms that underlie sperm quality and function. The potential for micronutrient treatment in obese males to improve sperm quality/function and implicate epididymal sperm maturation is a key area of investigation. Further comparative and prospective studies in obese men, whereby epididymal transit time is 6-10 days (Amann and Howards 1980; Robaire *et al.* 2006), to confirm these findings in a human setting are warranted.

#### **4.8 Acknowledgements**

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## 4.9 Tables

**Table 4.1. The effect of diet and micronutrient supplementation on body composition relative to total body weight.**

Values are a mean  $\pm$  SEM. CD, control diet; CD+S, control diet plus micronutrient supplements; HFD, high fat diet; HFD+S, high fat diet plus micronutrient supplements. Different letters denote significantly distinct groups at  $P < 0.05$ .

	CD	CD+S	HFD	HFD+S
Total body weight (g)	26.07 $\pm$ 0.61 <sup>a</sup>	24.84 $\pm$ 0.45 <sup>a</sup>	29.07 $\pm$ 0.45 <sup>b</sup>	29.71 $\pm$ 0.82 <sup>b</sup>
Adipose tissues (% of body weight)				
Peri-renal fat	0.002 $\pm$ 0.000 <sup>a</sup>	0.002 $\pm$ 0.000 <sup>a</sup>	0.003 $\pm$ 0.000 <sup>ab</sup>	0.004 $\pm$ 0.000 <sup>b</sup>
Retroperitoneal fat	0.005 $\pm$ 0.000 <sup>a</sup>	0.005 $\pm$ 0.000 <sup>a</sup>	0.009 $\pm$ 0.000 <sup>b</sup>	0.008 $\pm$ 0.000 <sup>b</sup>
Omental fat	0.011 $\pm$ 0.001 <sup>a</sup>	0.009 $\pm$ 0.000 <sup>a</sup>	0.013 $\pm$ 0.001 <sup>b</sup>	0.015 $\pm$ 0.001 <sup>b</sup>
Dorsal fat	0.006 $\pm$ 0.000 <sup>a</sup>	0.006 $\pm$ 0.000 <sup>a</sup>	0.008 $\pm$ 0.000 <sup>b</sup>	0.009 $\pm$ 0.000 <sup>b</sup>
Gonadal fat	0.026 $\pm$ 0.002 <sup>a</sup>	0.022 $\pm$ 0.001 <sup>a</sup>	0.041 $\pm$ 0.001 <sup>b</sup>	0.043 $\pm$ 0.002 <sup>b</sup>
Sum of adipose tissues	0.050 $\pm$ 0.004 <sup>a</sup>	0.044 $\pm$ 0.002 <sup>a</sup>	0.074 $\pm$ 0.002 <sup>b</sup>	0.079 $\pm$ 0.003 <sup>b</sup>
Organs (% of body weight)				

Testis	0.003 ± 0.000 <sup>a</sup>	0.004 ± 0.000 <sup>a</sup>	0.003 ± 0.000 <sup>a</sup>	0.003 ± 0.000 <sup>a</sup>
Seminal vesicles	0.011 ± 0.001 <sup>a</sup>	0.011 ± 0.001 <sup>a</sup>	0.010 ± 0.000 <sup>a</sup>	0.011 ± 0.001 <sup>a</sup>
Vastus lateralis	0.006 ± 0.000 <sup>a</sup>	0.006 ± 0.000 <sup>a</sup>	0.005 ± 0.000 <sup>a</sup>	0.005 ± 0.000 <sup>a</sup>
Soleus	0.0004 ± 0.000 <sup>a</sup>	0.0004 ± 0.000 <sup>a</sup>	0.0004 ± 0.000 <sup>a</sup>	0.0003 ± 0.000 <sup>a</sup>
Liver	0.046 ± 0.002 <sup>a</sup>	0.045 ± 0.002 <sup>a</sup>	0.041 ± 0.001 <sup>a</sup>	0.040 ± 0.002 <sup>a</sup>
Pancreas	0.005 ± 0.000 <sup>a</sup>	0.005 ± 0.000 <sup>a</sup>	0.005 ± 0.000 <sup>a</sup>	0.005 ± 0.000 <sup>a</sup>
Kidney	0.007 ± 0.000 <sup>a</sup>	0.007 ± 0.000 <sup>a</sup>	0.007 ± 0.000 <sup>a</sup>	0.006 ± 0.000 <sup>a</sup>

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**Table 4.2. The effect of diet and micronutrient supplementation on sperm capacitation and sperm-oocyte binding.**

Values are a mean  $\pm$  SEM. CD, control diet; CD+S, control diet plus micronutrient supplements; HFD, high fat diet; HFD+S, high fat diet plus micronutrient supplements.

Different letters denote significantly distinct groups at  $P < 0.05$ .

	<b>CD</b>	<b>CD+S</b>	<b>HFD</b>	<b>HFD+S</b>
Capacitated sperm (%)	90.6 $\pm$ 1.0 <sup>a</sup>	89.5 $\pm$ 1.1 <sup>ab</sup>	87.3 $\pm$ 0.9 <sup>b</sup>	86.9 $\pm$ 0.6 <sup>b</sup>
Non-capacitated sperm (%)	4.2 $\pm$ 0.6 <sup>a</sup>	5.6 $\pm$ 0.6 <sup>ab</sup>	5.3 $\pm$ 0.5 <sup>ab</sup>	6.1 $\pm$ 0.4 <sup>b</sup>
Acrosome reacted (%)	4.7 $\pm$ 0.4 <sup>a</sup>	4.9 $\pm$ 0.6 <sup>a</sup>	7.5 $\pm$ 0.6 <sup>b</sup>	7.1 $\pm$ 0.6 <sup>b</sup>
Mean number of sperm bound (%)	15.9 $\pm$ 0.6 <sup>a</sup>	19.9 $\pm$ 0.9 <sup>bc</sup>	18.2 $\pm$ 0.7 <sup>ab</sup>	21.0 $\pm$ 0.6 <sup>c</sup>

**Table 4.3. The effect of diet and micronutrient supplementation on fertilization and embryo development.**

Values are a proportion of total embryos for day 2 cleavage and a proportion of day 2 cleaved-embryos for days 3-5 of development. CD, control diet; CD+S, control diet plus micronutrient supplements; HFD, high fat diet; HFD+S, high fat diet plus micronutrient supplements. Total blastocyst (%) included early, full, expanded and hatching blastocysts. Different letters denote significantly distinct groups at  $P < 0.05$ .

	<b>CD</b>	<b>CD+S</b>	<b>HFD</b>	<b>HFD+S</b>
<b>Day 2</b>				
Cleavage (%)	85.6 <sup>a</sup>	79.2 <sup>a</sup>	39.3 <sup>b</sup>	60.2 <sup>c</sup>
<b>Day 3</b>				
6-8 cell (%)	46.5 <sup>a</sup>	51.9 <sup>a</sup>	55.2 <sup>a</sup>	46.3 <sup>a</sup>
<b>Day 4</b>				
Early blastocyst (%)	14.9 <sup>a</sup>	16.4 <sup>a</sup>	18.8 <sup>a</sup>	12.4 <sup>a</sup>
Total blastocyst (%)	53.2 <sup>ab</sup>	61.7 <sup>b</sup>	43.6 <sup>a</sup>	38.0 <sup>a</sup>
<b>Day 5</b>				
Expanded blastocyst (%)	27.7 <sup>a</sup>	31.1 <sup>a</sup>	29.2 <sup>a</sup>	23.1 <sup>a</sup>
Hatching blastocyst (%)	19.5 <sup>ab</sup>	27.3 <sup>a</sup>	15.8 <sup>ab</sup>	11.6 <sup>b</sup>
Total blastocyst (%)	72.1 <sup>a</sup>	84.2 <sup>b</sup>	62.4 <sup>a</sup>	58.7 <sup>a</sup>

**Table 4.4. The effect of diet and micronutrient supplementation on oxidative DNA lesion fluorescence in blastocyst cells.**

Values are a mean  $\pm$  SEM for fluorescent units measures and total cell number (TCN); and as proportion of TCN for the number of cells positive for oxidative DNA lesions (8-OHdG). CD, control diet; CD+S, control diet plus micronutrient supplements; HFD, high fat diet; HFD+S, high fat diet plus micronutrient supplements. Different letters denote significantly distinct groups at  $P < 0.05$ .

	<b>CD</b>	<b>CD+S</b>	<b>HFD</b>	<b>HFD+S</b>
Cells positive for 8-OHdG (fluorescent units)	11.9 $\pm$ 0.5 <sup>a</sup>	12.1 $\pm$ 0.4 <sup>a</sup>	12.9 $\pm$ 0.3 <sup>a</sup>	12.9 $\pm$ 0.3 <sup>a</sup>
Total cell number (TCN)	64.2 $\pm$ 1.1 <sup>a</sup>	64.2 $\pm$ 1.3 <sup>a</sup>	55.1 $\pm$ 1.6 <sup>b</sup>	53.1 $\pm$ 1.0 <sup>b</sup>
Number of cells positive for 8-OHdG (% of TCN)	36.1 <sup>a</sup>	33.7 <sup>a</sup>	37.9 <sup>a</sup>	37.3 <sup>a</sup>

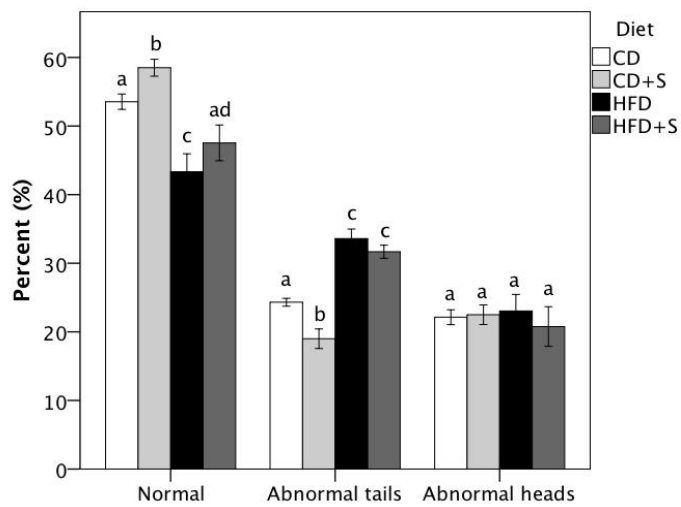
**Table 4.5. The effect of diet and micronutrient supplementation on fetal and placental development measures at day 18 of gestation.**

Values are a mean  $\pm$  SEM. CD, control diet; CD+S, control diet plus micronutrient supplements; HFD, high fat diet; HFD+S, high fat diet plus micronutrient supplements. The number of implantations reflect the number of fetuses and resorptions. The number of fetuses and resorptions are a mean and in parenthesis are the total number of implantations, fetuses and resorptions. Different letters denote significantly distinct groups at  $P < 0.05$ .

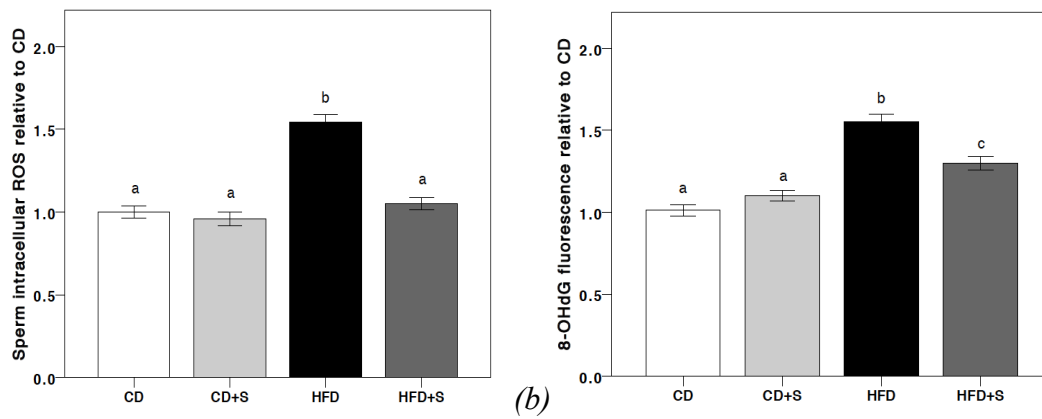
	<b>CD</b>	<b>CD+S</b>	<b>HFD</b>	<b>HFD+S</b>
Number of implantations	4.3 (86)	4.4 (87)	4.3 (26)	4.4 (44)
Number of fetuses	3.4 (68)	4.3 (86)	4.2 (25)	3.8 (38)
Number of resorptions	0.9 (18)	0.1 (1)	0.2 (1)	0.6 (6)
Fetuses/implantation (%)	80.5	99.4	96.7	89.8
Fetal weight (mg)	767 $\pm$ 27 <sup>a</sup>	787 $\pm$ 9 <sup>ab</sup>	771 $\pm$ 25 <sup>a</sup>	842 $\pm$ 34 <sup>b</sup>
Placental weight (mg)	85.7 $\pm$ 4.5 <sup>a</sup>	92.5 $\pm$ 1.5 <sup>a</sup>	98.1 $\pm$ 4.0 <sup>a</sup>	94.8 $\pm$ 5.5 <sup>a</sup>
Fetal:placental weight ratio	9.2 $\pm$ 0.5 <sup>a</sup>	8.6 $\pm$ 0.2 <sup>a</sup>	7.8 $\pm$ 0.4 <sup>a</sup>	9.1 $\pm$ 0.6 <sup>a</sup>
Crown-rump length (mm)	18.4 $\pm$ 5.8 <sup>a</sup>	18.2 $\pm$ 0.2 <sup>a</sup>	18.4 $\pm$ 0.5 <sup>a</sup>	19.0 $\pm$ 0.7 <sup>a</sup>



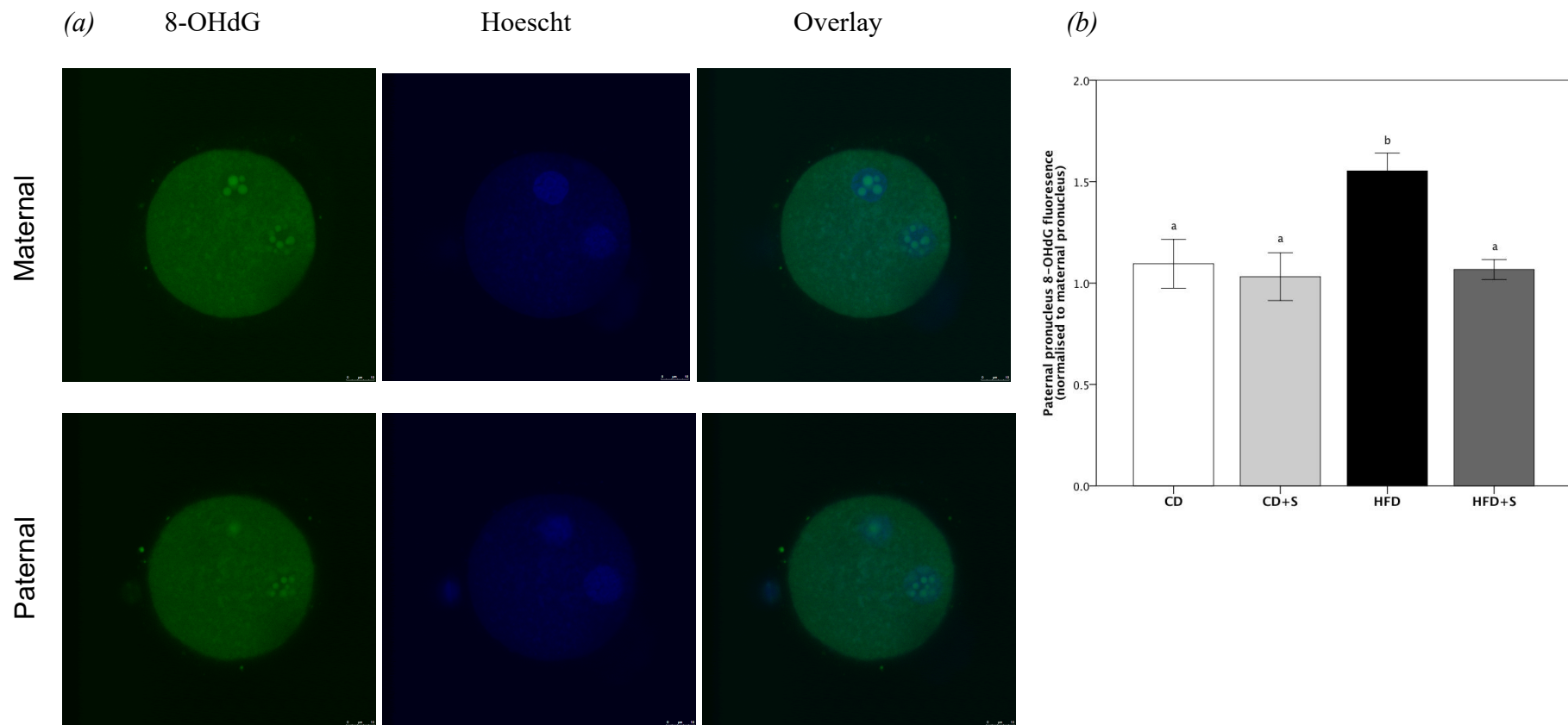
#### 4.10 Figures



**Fig. 4.2.** The effect of diet and micronutrient supplementation on sperm morphology. Values are a mean  $\pm$  SEM. CD, control diet; CD+S, control diet plus micronutrient supplements; HFD, high fat diet; HFD+S, high fat diet plus micronutrient supplements. Different letters denote significantly distinct groups at  $P < 0.05$ .



**Fig. 4.3.** The effect of diet and micronutrient supplementation on sperm oxidative stress assessed by (a) sperm intracellular ROS levels normalised to CD fed mice, and (b) sperm 8-OHdG fluorescence normalised to CD fed mice. Values are a mean  $\pm$  SEM. CD, control diet; CD+S, control diet plus micronutrient supplements; HFD, high fat diet; HFD+S, high fat diet plus micronutrient supplements. Different letters denote significantly distinct groups at  $P < 0.05$ .



**Fig. 4.4.** The effect of diet and micronutrient supplementation on pronuclear oxidative DNA lesion fluorescence. (a) Representative images of 8-OHdG and nuclear fluorescence and (b) quantified fluorescent intensity of the paternal pronucleus normalized to the maternal pronucleus in the subsequent embryo. Values are a mean  $\pm$  SEM. CD, control diet; CD+S, control diet plus micronutrient supplements; HFD, high fat diet; HFD+S, high fat diet plus micronutrient supplements. Different letters denote significantly distinct groups at  $P < 0.05$ .

#### 4.11 Supplementary Tables

**Table S.4.1. The effect of diet and micronutrient supplementation on serum metabolites and testosterone.**

Values are a mean  $\pm$  SEM. CD, control diet; CD+S, control diet plus micronutrient supplements; HFD, high fat diet; HFD+S, high fat diet plus micronutrient supplements. Different letters denote significantly distinct groups at  $P < 0.05$ .

	<b>CD</b>	<b>CD+S</b>	<b>HFD</b>	<b>HFD+S</b>
Cholesterol (mmol/L <sup>-1</sup> )	1.55 $\pm$ 0.08 <sup>a</sup>	1.49 $\pm$ 0.11 <sup>a</sup>	2.12 $\pm$ 0.11 <sup>b</sup>	2.13 $\pm$ 0.11 <sup>b</sup>
Glucose (mmol/L <sup>-1</sup> )	5.31 $\pm$ 0.30 <sup>a</sup>	4.75 $\pm$ 0.13 <sup>a</sup>	5.36 $\pm$ 0.41 <sup>a</sup>	5.40 $\pm$ 0.35 <sup>a</sup>
HDL (mmol/L <sup>-1</sup> )	1.35 $\pm$ 0.09 <sup>a</sup>	1.41 $\pm$ 0.03 <sup>a</sup>	1.88 $\pm$ 0.11 <sup>b</sup>	1.90 $\pm$ 0.14 <sup>b</sup>
Triglycerides (mmol/L <sup>-1</sup> )	0.23 $\pm$ 0.02 <sup>a</sup>	0.23 $\pm$ 0.02 <sup>a</sup>	0.30 $\pm$ 0.02 <sup>b</sup>	0.31 $\pm$ 0.03 <sup>b</sup>
NEFA (mmol/L <sup>-1</sup> )	0.37 $\pm$ 0.02 <sup>a</sup>	0.36 $\pm$ 0.03 <sup>a</sup>	0.36 $\pm$ 0.02 <sup>a</sup>	0.38 $\pm$ 0.02 <sup>a</sup>
Testosterone (nmol/L <sup>-1</sup> )	0.05 $\pm$ 0.01 <sup>a</sup>	0.07 $\pm$ 0.01 <sup>a</sup>	0.05 $\pm$ 0.01 <sup>a</sup>	0.06 $\pm$ 0.01 <sup>a</sup>

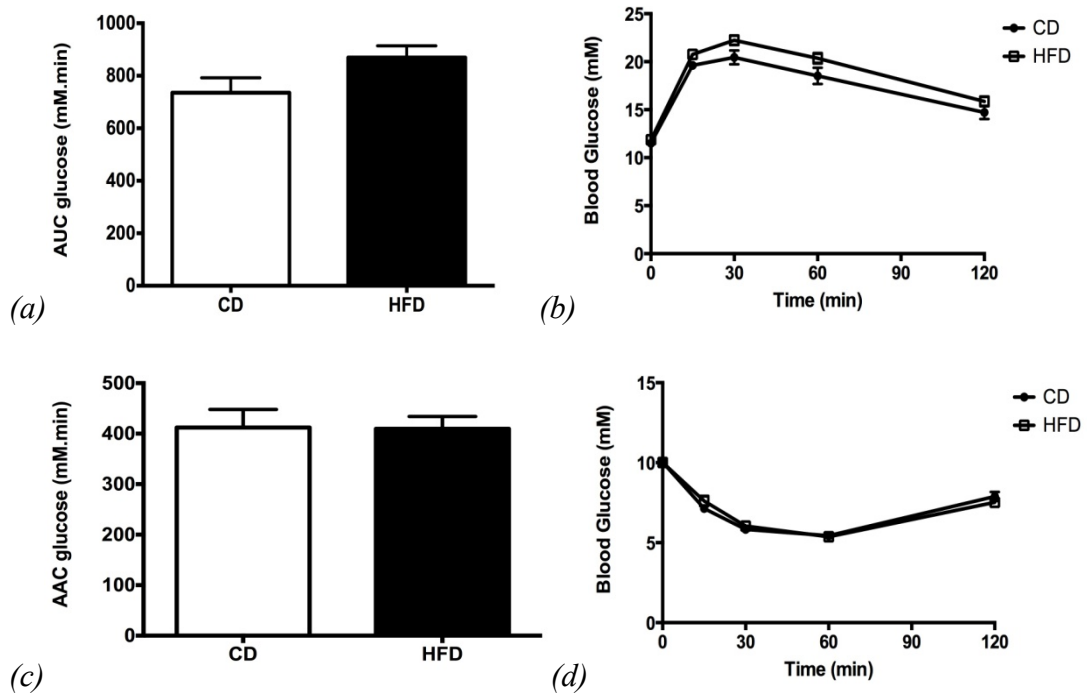
**Table S.4.2. The effect of diet and micronutrient supplementation on sperm motility and concentration.**

Values are a mean  $\pm$  SEM. CD, control diet; CD+S, control diet plus micronutrient supplements; HFD, high fat diet; HFD+S, high fat diet plus micronutrient supplements.

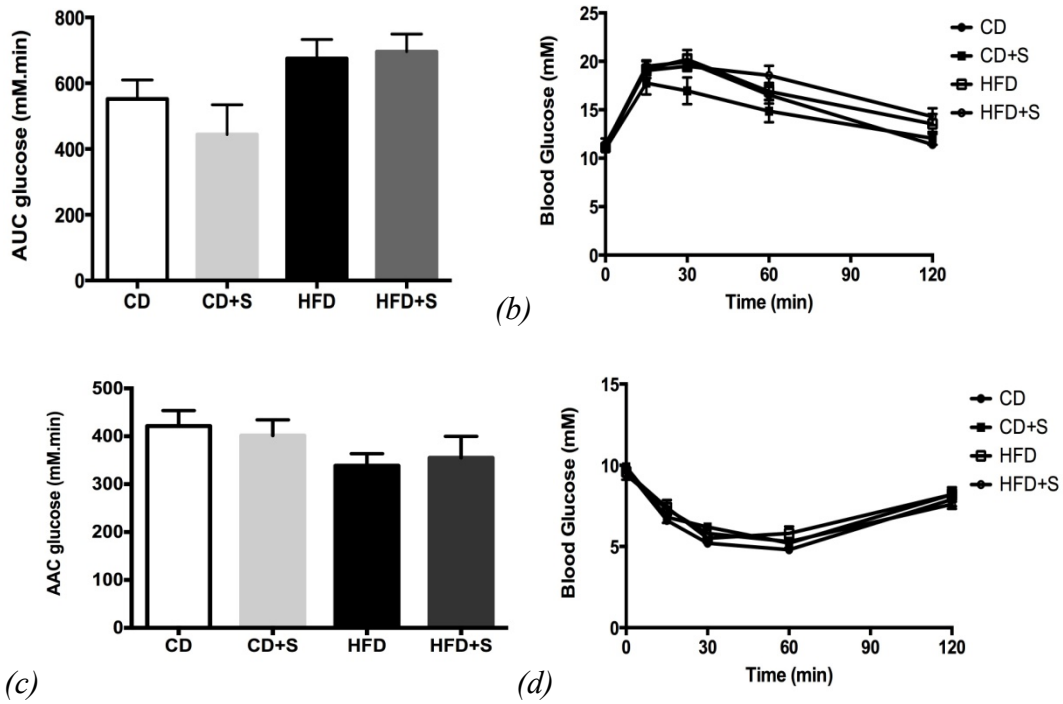
No statistical differences were detected.

	<b>CD</b>	<b>CD+S</b>	<b>HFD</b>	<b>HFD+S</b>
Motility (%)	64.5 $\pm$ 2.3	61.0 $\pm$ 1.9	59.8 $\pm$ 2.8	56.1 $\pm$ 2.9
Concentration (10 <sup>6</sup> /ml)	41.9 $\pm$ 3.7	34.5 $\pm$ 1.6	39.6 $\pm$ 4.7	38.5 $\pm$ 4.5

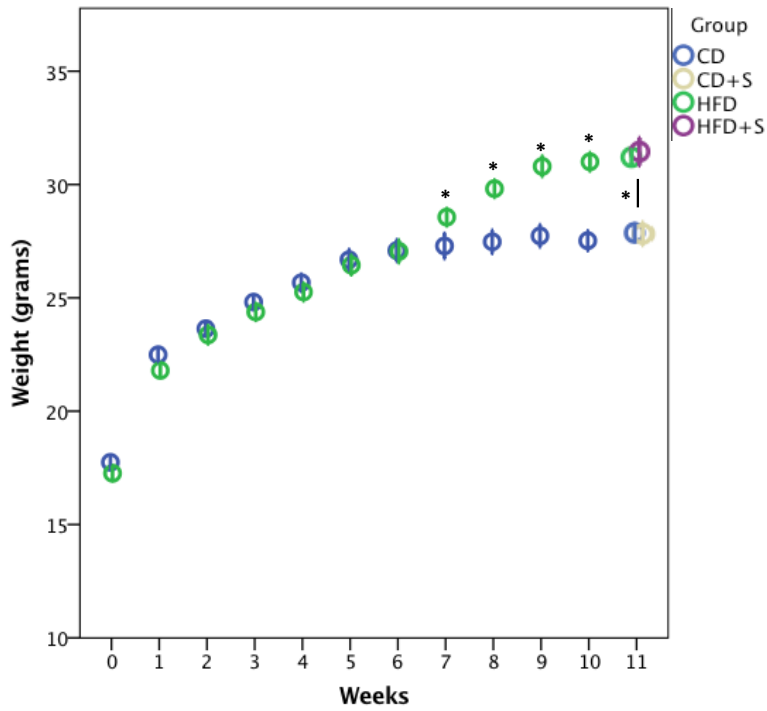
#### 4.12 Supplementary Figures



**Fig S.4.1.** An intraperitoneal (ip) glucose tolerance test (GTT) and an ip insulin tolerance test (ITT) performed at 9-10 weeks of the first dietary phase. CD, control diet; HFD, high fat diet. GTT (a) area under the curve (AUC) values, and (b) blood glucose concentration over time. ITT (c) area above the curve (AAC) values, and (d) blood glucose concentration over time. No statistical differences were detected. Values are mean  $\pm$  SEM.



**Fig. S.4.2.** An intraperitoneal (ip) glucose tolerance test (GTT) and an ip insulin tolerance test (ITT) performed after 10 days of micronutrient supplementation. CD, control diet; CD+S, control diet plus micronutrient supplements; HFD, high fat diet; HFD+S, high fat diet plus micronutrient supplements. GTT (a) area under the curve (AUC) values, and (b) blood glucose concentration over time. ITT (c) area above the curve (AAC) values, and (d) blood glucose concentration over time. No statistical differences were detected. Values are a mean  $\pm$  SEM.



**Fig. S.4.3.** Total body weight from control diet (CD) and high fat diet (HFD) fed mice following 10 weeks of diet exposure in addition to control diet plus micronutrient supplements (CD+S) and high fat diet plus micronutrient supplements (HFD+S) after one week of supplement diet exposure. Values are a mean  $\pm$  SEM. \*P<0.05.



#### 4.13 References

- Aggerholm, A.S., Thulstrup, A.M., Toft, G., Ramlau-Hansen, C.H., and Bonde, J.P. (2008) Is overweight a risk factor for reduced semen quality and altered serum sex hormone profile? *Fertil Steril* **90**, 619-626. [In eng]
- Aitken, J., and Fisher, H. (1994) Reactive oxygen species generation and human spermatozoa: the balance of benefit and risk. *Bioessays* **16**, 259-267.
- Aitken, R.J. (1999) The Amoroso Lecture - The human spermatozoon - a cell in crisis? *Journal of Reproduction and Fertility* **115**, 1-7. [In English]
- Aitken, R.J., and Baker, M.A. (2002) Reactive oxygen species generation by human spermatozoa: a continuing enigma. *Int J Androl* **25**, 191-194.
- Aitken, R.J., Clarkson, J.S., and Fishel, S. (1989) Generation of reactive oxygen species, lipid peroxidation, and human sperm function. *Biol Reprod* **41**, 183-197.
- Aitken, R.J., and Curry, B.J. (2011) Redox regulation of human sperm function: from the physiological control of sperm capacitation to the etiology of infertility and DNA damage in the germ line. *Antioxid Redox Signal* **14**, 367-381.
- Alvarez, J.G., and Storey, B.T. (1989) Role of glutathione peroxidase in protecting mammalian spermatozoa from loss of motility caused by spontaneous lipid peroxidation. *Gamete Res* **23**, 77-90.
- Amann, R.P., and Howards, S.S. (1980) Daily spermatozoal production and epididymal spermatozoal reserves of the human male. *J Urol* **124**, 211-215.
- Astrup, A., and Bugel, S. (2010) Micronutrient deficiency in the aetiology of obesity. *Int J Obes (Lond)* **34**, 947-948.
- Awoniyi, D.O., Aboua, Y.G., Marnewick, J., and Brooks, N. (2012) The effects of rooibos (*Aspalathus linearis*), green tea (*Camellia sinensis*) and commercial rooibos and green tea supplements on epididymal sperm in oxidative stress-induced rats. *Phytother Res* **26**, 1231-1239.
- Bakos, H.W., Henshaw, R.C., Mitchell, M., and Lane, M. (2011) Paternal body mass index is associated with decreased blastocyst development and reduced live birth rates following assisted reproductive technology. *Fertil Steril* **95**, 1700-1704.
- Bakos, H.W., Mitchell, M., Setchell, B.P., and Lane, M. (2010) The effect of paternal diet-induced obesity on sperm function and fertilization in a mouse model. *Int J Androl* **34**, 402-410.

Barker, D.J. (2004) The developmental origins of chronic adult disease. *Acta Paediatr Suppl* **93**, 26-33.

Bedford, J.M. (2004) Enigmas of mammalian gamete form and function. *Biol Rev Camb Philos Soc* **79**, 429-460.

Binder, N.K., Hannan, N.J., and Gardner, D.K. (2012) Paternal diet-induced obesity retards early mouse embryo development, mitochondrial activity and pregnancy health. *PLoS One* **7**, e52304.

Bruner, S.D., Norman, D.P., and Verdine, G.L. (2000) Structural basis for recognition and repair of the endogenous mutagen 8-oxoguanine in DNA. *Nature* **403**, 859-866.

Campbell, J.M., Lane, M., Owens, J.A., and Bakos, H.W. (2015) Paternal obesity negatively affects male fertility and assisted reproduction outcomes: a systematic review and meta-analysis. *Reproductive BioMedicine Online* **31**, 593-604.

Ceribasi, A.O., Turk, G., Sonmez, M., Sakin, F., and Atessahin, A. (2010) Toxic effect of cyclophosphamide on sperm morphology, testicular histology and blood oxidant-antioxidant balance, and protective roles of lycopene and ellagic acid. *Basic Clin Pharmacol Toxicol* **107**, 730-736.

Chavarro, J.E., Furtado, J., Toth, T.L., Ford, J., Keller, M., Campos, H., and Hauser, R. (2011) Trans-fatty acid levels in sperm are associated with sperm concentration among men from an infertility clinic. *Fertil Steril* **95**, 1794-1797. [In eng]

Chen, X.L., Gong, L.Z., and Xu, J.X. (2013) Antioxidative activity and protective effect of probiotics against high-fat diet-induced sperm damage in rats. *Animal* **7**, 287-292.

Chong, S., Vickaryous, N., Ashe, A., Zamudio, N., Youngson, N., Hemley, S., Stopka, T., Skoultchi, A., Matthews, J., Scott, H.S., *et al.* (2007) Modifiers of epigenetic reprogramming show paternal effects in the mouse. *Nat Genet* **39**, 614-622. [In eng]

Conine, C.C., Sun, F., Song, L., Rivera-Perez, J.A., and Rando, O.J. (2018) Small RNAs Gained during Epididymal Transit of Sperm Are Essential for Embryonic Development in Mice. *Dev Cell*.

Cooper, T.G. (1996) Epididymis and sperm function. *Andrologia* **28 Suppl 1**, 57-59.

Dacheux, J.L., and Dacheux, F. (2014) New insights into epididymal function in relation to sperm maturation. *Reproduction* **147**, R27-42.

De Iuliis, G.N., Thomson, L.K., Mitchell, L.A., Finnie, J.M., Koppers, A.J., Hedges, A., Nixon, B., and Aitken, R.J. (2009) DNA damage in human spermatozoa is highly correlated with the efficiency of chromatin remodeling and the formation of 8-hydroxy-2'-deoxyguanosine, a marker of oxidative stress. *Biol Reprod* **81**, 517-524.

- Fan, Y., Liu, Y., Xue, K., Gu, G., Fan, W., Xu, Y., and Ding, Z. (2015) Diet-induced obesity in male C57BL/6 mice decreases fertility as a consequence of disrupted blood-testis barrier. *PLoS One* **10**, e0120775.
- Fejes, I., Koloszar, S., Szollosi, J., Zavaczki, Z., and Pal, A. (2005) Is semen quality affected by male body fat distribution? *Andrologia* **37**, 155-159. [In eng]
- Fraga, C.G., Motchnik, P.A., Shigenaga, M.K., Helbock, H.J., Jacob, R.A., and Ames, B.N. (1991) Ascorbic acid protects against endogenous oxidative DNA damage in human sperm. *Proc Natl Acad Sci U S A* **88**, 11003-11006.
- Fullston, T., Palmer, N.O., Owens, J.A., Mitchell, M., Bakos, H.W., and Lane, M. (2012) Diet-induced paternal obesity in the absence of diabetes diminishes the reproductive health of two subsequent generations of mice. *Hum Reprod* **27**, 1391-1400.
- Garcia, O.P., Long, K.Z., and Rosado, J.L. (2009) Impact of micronutrient deficiencies on obesity. *Nutr Rev* **67**, 559-572.
- Geva, E., Bartoov, B., Zabludovsky, N., Lessing, J.B., Lerner-Geva, L., and Amit, A. (1996) The effect of antioxidant treatment on human spermatozoa and fertilization rate in an in vitro fertilization program. *Fertil Steril* **66**, 430-434.
- Ghanayem, B.I., Bai, R., Kissling, G.E., Travlos, G., and Hoffler, U. (2010) Diet-induced obesity in male mice is associated with reduced fertility and potentiation of acrylamide-induced reproductive toxicity. *Biol Reprod* **82**, 96-104.
- Haffner, S.M., Valdez, R.A., Stern, M.P., and Katz, M.S. (1993) Obesity, body fat distribution and sex hormones in men. *Int J Obes Relat Metab Disord* **17**, 643-649.
- Hammoud, A.O., Meikle, A.W., Reis, L.O., Gibson, M., Peterson, C.M., and Carrell, D.T. (2012) Obesity and male infertility: a practical approach. *Semin Reprod Med* **30**, 486-495.
- Hammoud, A.O., Wilde, N., Gibson, M., Parks, A., Carrell, D.T., and Meikle, A.W. (2008) Male obesity and alteration in sperm parameters. *Fertil Steril* **90**, 2222-2225.
- Handelsman, D.J., and Swerdloff, R.S. (1985) Male gonadal dysfunction. *Clin Endocrinol Metab* **14**, 89-124.
- Hardy, G., and Hardy, I. (2004) Selenium: the Se-XY nutraceutical. *Nutrition* **20**, 590-593.
- Hedger, M.P. (2011) Immunophysiology and pathology of inflammation in the testis and epididymis. *J Androl* **32**, 625-640.
- Horak, S., Polanska, J., and Widlak, P. (2003) Bulky DNA adducts in human sperm: relationship with fertility, semen quality, smoking, and environmental factors. *Mutat Res* **537**, 53-65.

Ibrahim, H.A., Zhu, Y., Wu, C., Lu, C., Ezekwe, M.O., Liao, S.F., and Huang, K. (2012) Selenium-enriched probiotics improves murine male fertility compromised by high fat diet. *Biol Trace Elem Res* **147**, 251-260.

Jensen, T.K., Andersson, A.M., Jorgensen, N., Andersen, A.G., Carlsen, E., Petersen, J.H., and Skakkebaek, N.E. (2004) Body mass index in relation to semen quality and reproductive hormones among 1,558 Danish men. *Fertil Steril* **82**, 863-870.

Ji, B.T., Shu, X.O., Linet, M.S., Zheng, W., Wacholder, S., Gao, Y.T., Ying, D.M., and Jin, F. (1997) Paternal cigarette smoking and the risk of childhood cancer among offspring of nonsmoking mothers. *J Natl Cancer Inst* **89**, 238-244.

Johnson, R.C., and Schoeni, R.F. (2011) Early-life origins of adult disease: national longitudinal population-based study of the United States. *Am J Public Health* **101**, 2317-2324.

Kessopoulou, E., Powers, H.J., Sharma, K.K., Pearson, M.J., Russell, J.M., Cooke, I.D., and Barratt, C.L. (1995) A double-blind randomized placebo cross-over controlled trial using the antioxidant vitamin E to treat reactive oxygen species associated male infertility. *Fertil Steril* **64**, 825-831.

Kort, H.I., Massey, J.B., Elsner, C.W., Mitchell-Leef, D., Shapiro, D.B., Witt, M.A., and Roudebush, W.E. (2006) Impact of body mass index values on sperm quantity and quality. *J Androl* **27**, 450-452. [In eng]

Lane, M., McPherson, N.O., Fullston, T., Spillane, M., Sandeman, L., Kang, W.X., and Zander-Fox, D.L. (2014) Oxidative stress in mouse sperm impairs embryo development, fetal growth and alters adiposity and glucose regulation in female offspring. *PLoS One* **9**, e100832.

Lord, T., and Aitken, R.J. (2015) Fertilization stimulates 8-hydroxy-2'-deoxyguanosine repair and antioxidant activity to prevent mutagenesis in the embryo. *Dev Biol* **406**, 1-13.

Magnusdottir, E.V., Thorsteinsson, T., Thorsteinsdottir, S., Heimisdottir, M., and Olafsdottir, K. (2005) Persistent organochlorines, sedentary occupation, obesity and human male subfertility. *Hum Reprod* **20**, 208-215.

McLachlan, R.I., O'Donnell, L., Meachem, S.J., Stanton, P.G., de Kretser, D.M., Pratis, K., and Robertson, D.M. (2002) Identification of specific sites of hormonal regulation in spermatogenesis in rats, monkeys, and man. *Recent Prog Horm Res* **57**, 149-179.

McPherson, N.O., Bakos, H.W., Owens, J.A., Setchell, B.P., and Lane, M. (2013) Improving metabolic health in obese male mice via diet and exercise restores embryo development and fetal growth. *PLoS One* **8**, e71459.

- McPherson, N.O., Fullston, T., Kang, W.X., Sandeman, L.Y., Corbett, M.A., Owens, J.A., and Lane, M. (2016) Paternal under-nutrition programs metabolic syndrome in offspring which can be reversed by antioxidant/vitamin food fortification in fathers. *Sci Rep* **6**, 27010.
- Mitchell, M., Bakos, H.W., and Lane, M. (2011) Paternal diet-induced obesity impairs embryo development and implantation in the mouse. *Fertil Steril* **95**, 1349-1353.
- Mortazavi, M., Salehi, I., Alizadeh, Z., Vahabian, M., and Roushandeh, A.M. (2014) Protective Effects of Antioxidants on Sperm Parameters and Seminiferous Tubules Epithelium in High Fat-fed Rats. *J Reprod Infertil* **15**, 22-28.
- Mosbah, R., Yousef, M.I., and Mantovani, A. (2015) Nicotine-induced reproductive toxicity, oxidative damage, histological changes and haematotoxicity in male rats: the protective effects of green tea extract. *Exp Toxicol Pathol* **67**, 253-259.
- Nonogaki, T., Noda, Y., Narimoto, K., Shiotani, M., Mori, T., Matsuda, T., and Yoshida, O. (1992) Localization of CuZn-superoxide dismutase in the human male genital organs. *Hum Reprod* **7**, 81-85.
- Oakberg, E.F. (1957) Duration of spermatogenesis in the mouse. *Nature* **180**, 1137-1138.
- Olson, G.E., NagDas, S.K., and Winfrey, V.P. (2002) Structural Differentiation of Spermatozoa During Post-Testicular Maturation. In 'The Epididymis: From Molecules to Clinical Practice.' (Eds. B Robaire and BT Hinton) pp. 371-387. (Springer US)
- Olson, G.E., Winfrey, V.P., Hill, K.E., and Burk, R.F. (2004) Sequential development of flagellar defects in spermatids and epididymal spermatozoa of selenium-deficient rats. *Reproduction* **127**, 335-342.
- Omu, A.E., Al-Azemi, M.K., Kehinde, E.O., Anim, J.T., Oriowo, M.A., and Mathew, T.C. (2008) Indications of the mechanisms involved in improved sperm parameters by zinc therapy. *Med Princ Pract* **17**, 108-116.
- Palmer, N.O., Bakos, H.W., Fullston, T., and Lane, M. (2012a) Impact of obesity on male fertility, sperm function and molecular composition. *Spermatogenesis* **2**, 253-263.
- Palmer, N.O., Bakos, H.W., Owens, J.A., Setchell, B.P., and Lane, M. (2012b) Diet and exercise in an obese mouse fed a high-fat diet improve metabolic health and reverse perturbed sperm function. *Am J Physiol Endocrinol Metab* **302**, E768-780.
- Pauli, E.M., Legro, R.S., Demers, L.M., Kunselman, A.R., Dodson, W.C., and Lee, P.A. (2008) Diminished paternity and gonadal function with increasing obesity in men. *Fertil Steril* **90**, 346-351. [In eng]

- Perry, A.C., Jones, R., and Hall, L. (1993) Isolation and characterization of a rat cDNA clone encoding a secreted superoxide dismutase reveals the epididymis to be a major site of its expression. *Biochem J* **293 ( Pt 1)**, 21-25.
- Reichenberg, A., Gross, R., Weiser, M., Bresnahan, M., Silverman, J., Harlap, S., Rabinowitz, J., Shulman, C., Malaspina, D., Lubin, G., *et al.* (2006) Advancing paternal age and autism. *Arch Gen Psychiatry* **63**, 1026-1032.
- Reis, L.O., and Dias, F.G. (2012) Male fertility, obesity, and bariatric surgery. *Reprod Sci* **19**, 778-785.
- Rejraji, H., Vernet, P., and Drevet, J.R. (2002) GPX5 is present in the mouse caput and cauda epididymidis lumen at three different locations. *Mol Reprod Dev* **63**, 96-103.
- Robaire, B., Hinton, B.T., and Orgebin-Crist, M. (2006) The Epididymis. In 'Knobil and Neill's Physiology of Reproduction.' (Ed. JD Neill). (Elsevier)
- Rybar, R., Kopecka, V., Prinosilova, P., Markova, P., and Rubes, J. (2011) Male obesity and age in relationship to semen parameters and sperm chromatin integrity. *Andrologia* **43**, 286-291.
- Saleh, R.A., and Agarwal, A. (2002) Oxidative stress and male infertility: from research bench to clinical practice. *J Androl* **23**, 737-752.
- Sallmen, M., Sandler, D.P., Hoppin, J.A., Blair, A., and Baird, D.D. (2006) Reduced fertility among overweight and obese men. *Epidemiology* **17**, 520-523. [In eng]
- Sanchez-Gutierrez, M., Garcia-Montalvo, E.A., Izquierdo-Vega, J.A., and Del Razo, L.M. (2008) Effect of dietary selenium deficiency on the in vitro fertilizing ability of mice spermatozoa. *Cell Biol Toxicol* **24**, 321-329.
- Santiso, R., Tamayo, M., Gosalvez, J., Meseguer, M., Garrido, N., and Fernandez, J.L. (2010) Simultaneous determination in situ of DNA fragmentation and 8-oxoguanine in human sperm. *Fertil Steril* **93**, 314-318.
- Shaman, J.A., Yamauchi, Y., and Ward, W.S. (2007) The sperm nuclear matrix is required for paternal DNA replication. *J Cell Biochem* **102**, 680-688.
- Sharma, U., Sun, F., Conine, C.C., Reichholz, B., Kukreja, S., Herzog, V.A., Ameres, S.L., and Rando, O.J. (2018) Small RNAs Are Trafficked from the Epididymis to Developing Mammalian Sperm. *Dev Cell*.
- Showell, M.G., Brown, J., Yazdani, A., Stankiewicz, M.T., and Hart, R.J. (2011) Antioxidants for male subfertility. *Cochrane Database Syst Rev*, CD007411. [In eng]
- Sipos, A., Rasmussen, F., Harrison, G., Tynelius, P., Lewis, G., Leon, D.A., and Gunnell, D. (2004) Paternal age and schizophrenia: a population based cohort study. *BMJ* **329**, 1070.

- Suleiman, S.A., Ali, M.E., Zaki, Z.M., el-Malik, E.M., and Nasr, M.A. (1996) Lipid peroxidation and human sperm motility: protective role of vitamin E. *J Androl* **17**, 530-537.
- Tufek, N.H., Altunkaynak, M.E., Altunkaynak, B.Z., and Kaplan, S. (2015) Effects of thymoquinone on testicular structure and sperm production in male obese rats. *Syst Biol Reprod Med* **61**, 194-204.
- Walker, W.H. (2011) Testosterone signaling and the regulation of spermatogenesis. *Spermatogenesis* **1**, 116-120.
- Ward, W.S. (2010) Function of sperm chromatin structural elements in fertilization and development. *Mol Hum Reprod* **16**, 30-36.
- Whitfield, M., Pollet-Villard, X., Levy, R., Drevet, J.R., and Saez, F. (2015) Posttesticular sperm maturation, infertility, and hypercholesterolemia. *Asian J Androl* **17**, 742-748.
- WHO (2015) Obesity and Overweight, Fact Sheet no. 311, Body Mass Index Classifications. World Health Organization.
- Wong, W.Y., Merkus, H.M., Thomas, C.M., Menkveld, R., Zielhuis, G.A., and Steegers-Theunissen, R.P. (2002) Effects of folic acid and zinc sulfate on male factor subfertility: a double-blind, randomized, placebo-controlled trial. *Fertil Steril* **77**, 491-498.
- Zareba, P., Colaci, D.S., Afeiche, M., Gaskins, A.J., Jorgensen, N., Mendiola, J., Swan, S.H., and Chavarro, J.E. (2013) Semen quality in relation to antioxidant intake in a healthy male population. *Fertil Steril* **100**, 1572-1579.
- Zhang, E., Xu, F., Liang, H., Yan, J., Xu, H., Li, Z., Wen, X., and Weng, J. (2015) GLP-1 Receptor Agonist Exenatide Attenuates the Detrimental Effects of Obesity on Inflammatory Profile in Testis and Sperm Quality in Mice. *Am J Reprod Immunol*.
- Zini, A., San Gabriel, M., and Baazeem, A. (2009) Antioxidants and sperm DNA damage: a clinical perspective. *J Assist Reprod Genet* **26**, 427-432.
- Zribi, N., Chakroun, N.F., Elleuch, H., Abdallah, F.B., Ben Hamida, A.S., Gargouri, J., Fakhfakh, F., and Keskes, L.A. (2011) Sperm DNA fragmentation and oxidation are independent of malondialdehyde. *Reprod Biol Endocrinol* **9**, 47.

Chapter 5 (conventional format)

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**The Relationship Between Sperm Oxidative Stress and ART Outcomes  
and the Effectiveness of Dietary Micronutrient Supplementation in  
Reducing Obesity-Induced Sperm Oxidative Stress (Pilot Study)**

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## 5.1. Chapter Link

As shown in the previous chapters, dietary micronutrient supplement intake in a mouse model of male obesity improved sperm oxidative stress, sperm function and pregnancy outcome. Micronutrient supplement intake in obese male mice reduced sperm intracellular ROS and improved sperm morphology and fertilization rates in addition to improved fetal outcomes, which may be explained by the observed reduction in oxidative DNA lesions in both mature sperm and the paternal pronucleus of the subsequent embryo. Given these findings in a mouse model, a clinical prospective study was conducted to establish the relationship between oxidative DNA lesions in human sperm and fertility treatment outcomes including pregnancy rates. Further, given that the diet-induced obese mouse models from previous chapters showed reductions in sperm oxidative stress with micronutrient supplementation of a high fat diet, the impact of dietary micronutrient supplement intake on sperm intracellular ROS and oxidative DNA modifications were assessed in men with an increased BMI.

## 5.2. Abstract

The prevalence of male obesity is increasing worldwide and happens to occur with a concurrent reduction in male fertility and fecundity. Men with an increased BMI have been characterized with increased sperm oxidative stress with further reports demonstrating that sperm oxidative stress is a main contributor to male infertility. Also, sperm oxidative stress and subsequent oxidative DNA damage in the male germ line is linked to perturbed offspring health. The effectiveness of micronutrient and antioxidant supplements to improve sperm oxidative stress in the growing population of obese men is unknown. This prospective study examined the impact of dietary micronutrient supplementation on sperm oxidative stress markers and assisted reproductive technology (ART) fertility treatment outcomes in 131 non-smoking couples with a normal weight, overweight or obese male partner and a non-obese female partner. The male and female partners were aged <45 and <38 years old, respectively. Couples were stratified by the male partner's BMI and whether the male partner was consuming micronutrient supplements. Sperm oxidative stress markers in live sperm cells were measured using the 2',7'-dichlorodihydrofluorescein diacetate (H<sub>2</sub>DCFDA) and the MitoSOX Red (MSR) probe. Further, oxidative DNA lesions were assessed by detecting presence of 8-hydroxy-2'-deoxyguanosine (8-OHdG). Conventional sperm parameters were also examined including sperm motility, concentration and morphology in addition to fertility treatment outcomes including fertilization rates, embryo development, blastocyst quality, embryo utilization, pregnancy rates and live birth rates. The results demonstrated that there was a positive association between sperm intracellular ROS and sperm 8-OHdG lesions and a negative association between sperm 8-OHdG lesions and fertilization rates. Also, men with increased sperm 8-OHdG lesions had a reduced chance of achieving a pregnancy.

Also, this study showed that micronutrient supplementation in obese men reduced sperm intracellular reactive oxygen species (ROS) and sperm oxidative DNA 8-OHdG lesions compared to obese men not consuming micronutrient supplements. Overall, these findings illustrate the effectiveness of dietary micronutrient supplementation in reducing obesity-induced sperm oxidative stress and sperm 8-OHdG lesions. However, further research determining the dietary and lifestyle habits of these couples and which micronutrient(s) are responsible for the improvements in fertility treatment outcomes are warranted.

### 5.3. Introduction

Parallel to the global increase in the prevalence of male obesity is a decrease in male fertility and fecundity (Swan *et al.* 2000). One of the main proposed mechanisms responsible for the compromised reproductive health and subfertility in obese males is sperm oxidative stress (McPherson and Lane 2015). Oxidative stress is an imbalance between reactive oxygen species and antioxidants whereby the levels of reactive oxygen species exceed that of antioxidants (Henkel 2011). Reactive oxygen species (ROS) include superoxide anions ( $O_2^-$ ), hydroxyl radicals (OH) and hydrogen peroxide ( $H_2O_2$ ). Many studies have reported an increase in ROS activity in sperm in several types of male infertility (Aitken *et al.* 1991; D'Agata *et al.* 1990; de Lamirande *et al.* 1995; Iwasaki and Gagnon 1992; Kodama *et al.* 1997; Sharma and Agarwal 1996; Shen *et al.* 1999; Zini *et al.* 1993) however, only in more recent years have researchers shown an increase of ROS activity in sperm and semen from obese men (Tunc *et al.* 2011; Yang *et al.* 2016).

In sperm, ROS react with cellular components including lipids, proteins and DNA resulting in lipid peroxidation and DNA damage (Aitken *et al.* 1989; Alvarez *et al.* 1987; Hughes *et al.* 1996). Most DNA damage in sperm is oxidatively induced by ROS resulting in DNA lesions, mainly 8-hydroxy-2'-deoxyguanosine (8-OHdG) (De Iuliis *et al.* 2009a; Noblanc *et al.* 2013), an oxidized guanine residue within sperm DNA (Noblanc *et al.* 2013). Epidemiological studies suggest that oxidative stress in the male germ line, including oxidative DNA lesions (8-OHdG), is a mediator for perturbed offspring health from various paternal environmental and lifestyle factors such as cigarette smoking and drug exposure (Fraga *et al.* 1996; Ji *et al.* 1997; Trasler 2009).

Healthy dietary patterns, characterized by elevated intake of vegetables, fruits and legumes; have been shown to improve sperm quality, particularly sperm motility and concentration, in men from couples undergoing assisted reproductive technology (ART) treatment (Oostingh *et al.* 2017) and in men from the general population (Cutillas-Tolin *et al.* 2015; Liu *et al.* 2015). Although dietary micronutrient supplements are no substitute for a healthy diet (Troppmann *et al.* 2002), couples opt for dietary supplements to ensure adequate nutritional intake (NHMRC 2006). In infertile men, studies investigating the effects of antioxidant and micronutrient intake have demonstrated improved sperm quality (Zini *et al.* 2009) and increased pregnancy rates (Gharagozloo and Aitken 2011). One example of a sub-cellular effect of dietary supplementation on sperm include a reduction in sperm 8-OHdG lesions, which has been demonstrated with vitamin C supplementation as when the dose of supplementation was reduced, sperm 8-OHdG modifications increased (Fraga *et al.* 1991).

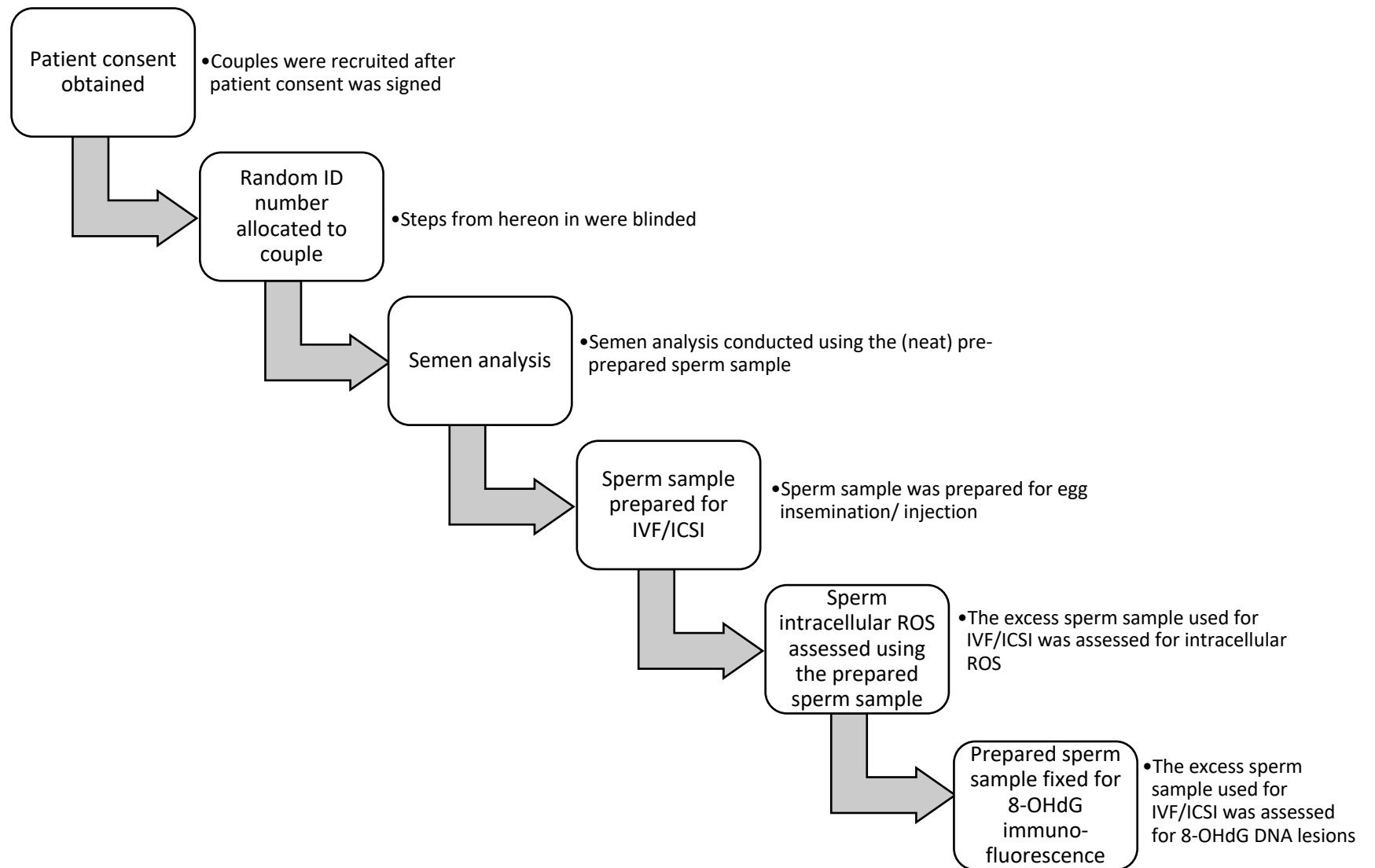
Overall, investigative studies of sperm quality and function suggest a protective role of micronutrients in sperm oxidative stress and DNA integrity (Zini *et al.* 2009). However, the effectiveness of dietary micronutrient supplements, such as vitamin E, zinc, vitamin C, folic acid or lycopene, in improving sperm quality, sperm function and reproductive outcomes in the growing population of obese men remains unknown. Therefore, this prospective study examined the relationships between sperm intracellular ROS, sperm 8-OHDG lesions, fertilization rates and pregnancy outcomes. Further investigations assessed the impact of micronutrient supplementation on sperm intracellular ROS and sperm oxidative DNA lesions in normal weight, overweight and obese men in addition to the effects of supplements on ART treatment outcomes stratified by the male partner's BMI. The results demonstrate that micronutrient supplementation is most effective in

obese men for the reduction of sperm intracellular ROS and 8-OHdG lesions. Although the effect of supplements on pregnancy rates stratified by the male partner's BMI could not be concluded as the data was statistically underpowered, there was a negative association between oxidative DNA lesions and achieving a pregnancy when analyzing couples as a whole population (i.e. not stratified by male BMI).

#### **5.4. Materials and methods**

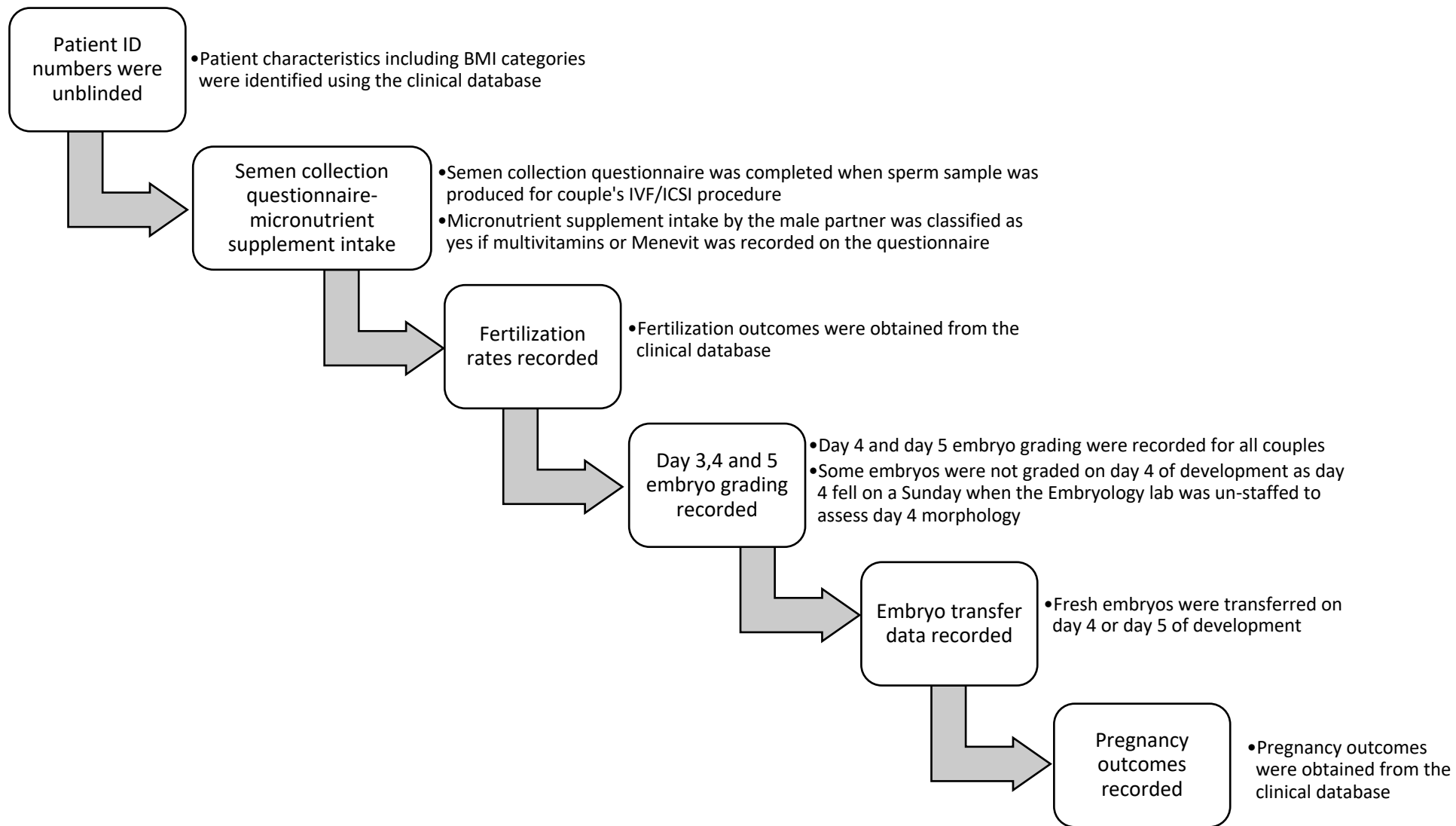
Please refer to Chapter 2, section 2.2 for the materials and methods.

The experimental design is detailed in Figures 5.1 and 5.2. The distribution of participants at different stages of analysis are outlined in Figures 5.3 and 5.4.

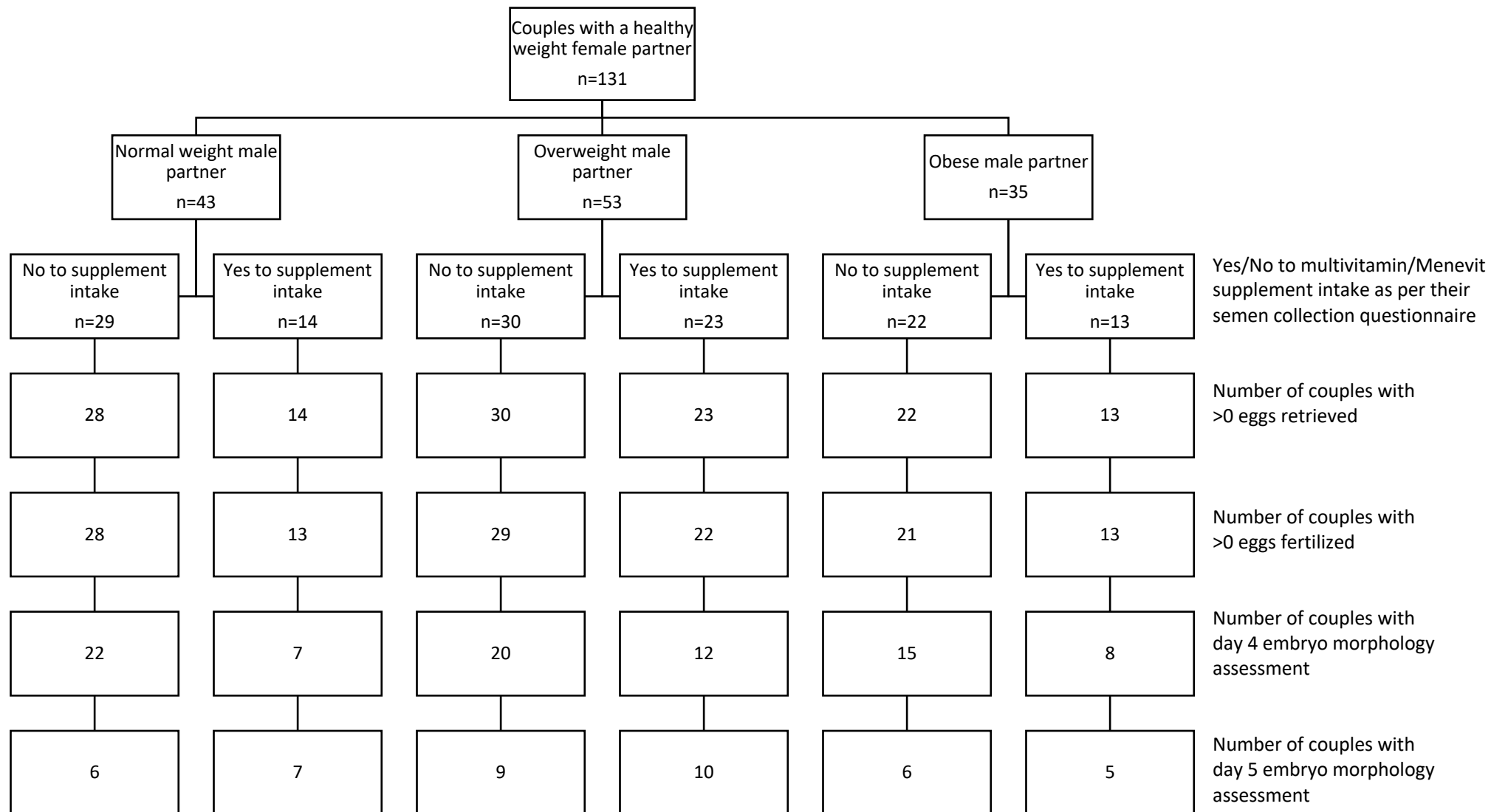




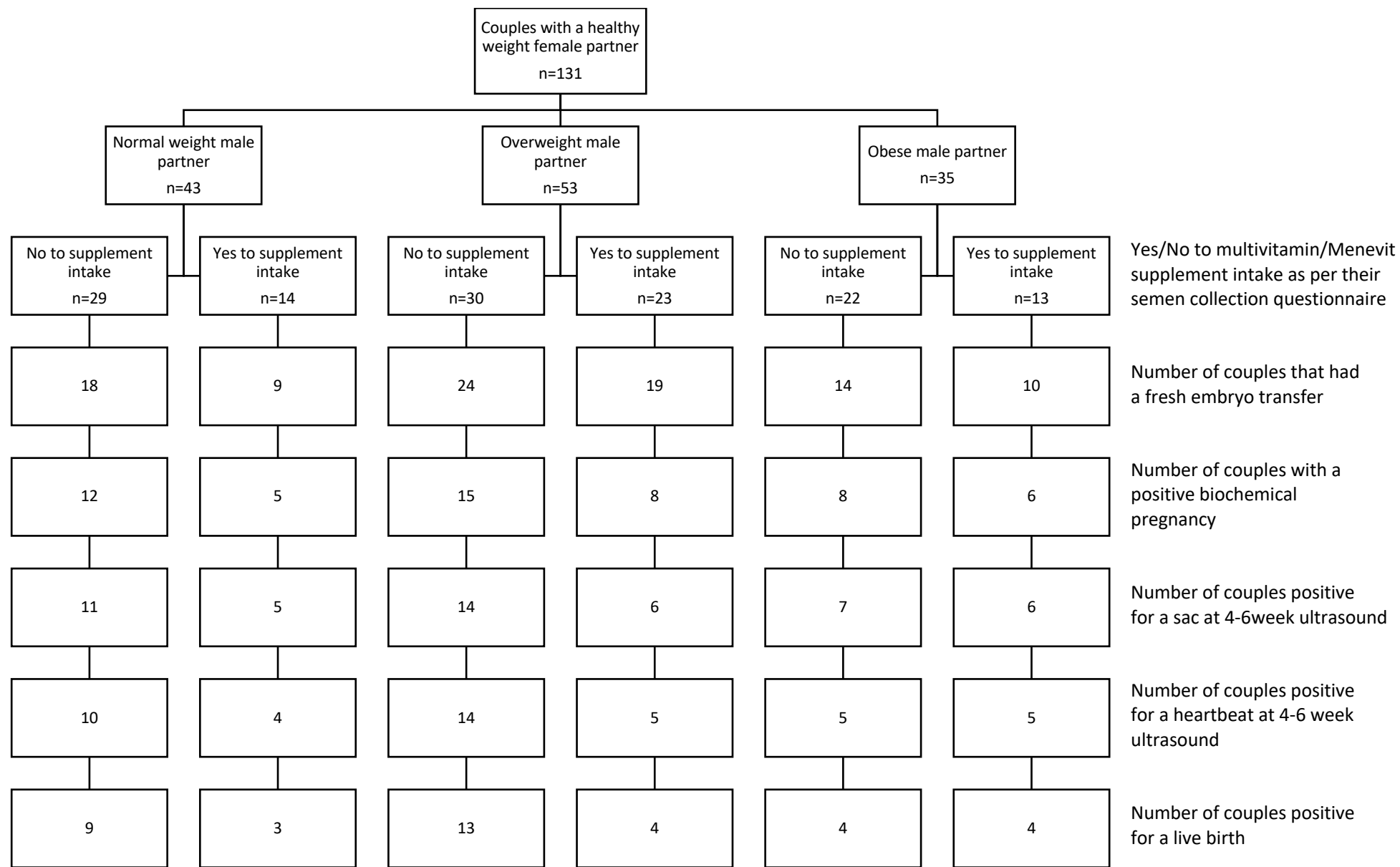
**Figure 5.1** A flow chart outlining the experimental plan. After patient consent, all sperm assessments were conducted blinded and a semen analysis was performed using the (neat) pre-prepared sperm sample. Sperm intracellular ROS markers and 8-OHdG immunofluorescence were measured using the excess prepared sperm sample that was used for the couple's IVF/ICSI procedure.



**Figure 5.2** A flow chart outlining the experimental plan after sperm assessments were conducted. After patient ID numbers were un-blinded, the clinical database was used to identify patient characteristics including the male partner's BMI and whether they were positive for multivitamin/ Menevit (micronutrient) supplement intake. Patient fertility treatment cycle outcomes were also obtained from their records including fertilization, embryo development and pregnancy outcomes.



**Figure 5.3** The distribution of patients based on the male partner's BMI and micronutrient supplementation stratification. The number of patients are outlined for each step of their fertility treatment cycle outcomes including >0 eggs collected at oocyte retrieval, >0 eggs fertilized, day 4 and day 5 embryo morphology assessments. Some embryos were day 4 on a Sunday and were not morphologically assessed. Day 5 embryos that were assessed on day 4 and had embryos that were frozen or transferred on day 4 were not included in day 5 analysis.



**Figure 5.4** The distribution of patients and pregnancy outcomes based on the male partner's BMI and micronutrient supplementation stratification. Some couples did not undergo a fresh embryo transfer due to ovarian hyperstimulation syndrome (OHSS) or pre-implantation genetic screening for aneuploidy (PGT-A) without a fresh embryo transfer.

## **5.5. Results**

### **5.5.1. Patient population characteristics**

The total number of couples included in the final analysis was 131 couples stratified by male BMI (i) normal weight men (n=43), (ii) overweight men (n=53), (iii) obese men (n=35), and each BMI category was divided by whether they consumed dietary supplementation intake in a 3 x 2 experimental design (Figure 5.3). Also, the distributions of participants at different levels of data analysis are illustrated in Figures 5.3 and 5.4.

Patient population characteristics were not different between groups including paternal age, maternal BMI, maternal age, the number of previous ART treatment cycles the couple had undergone, and the female partner's AMH and baseline FSH levels (Table 5.1). Patient cycle characteristics were also investigated and there was no difference in the double embryo transfer rate, the number of patients that underwent preimplantation genetic screening for aneuploidy (PGT-A), men with a medical condition taking regular medication and the couple's infertility diagnosis including unexplained conditions, polycystic ovary syndrome (PCOS), endometriosis, tubal issues, semen defect, anovulation/oligomenorrhea, diminished ovarian reserve and other conditions such as genetic and immunological conditions (Table 5.2).



**Table 5.1** Patient population characteristics.

	Normal weight		Overweight		Obese	
	No supplements	With supplements	No supplements	With supplements	No supplements	With supplements
Number of patients	29	14	30	23	22	13
Paternal BMI	23.4 ± 0.3 <sup>a</sup>	23.5 ± 0.3 <sup>a</sup>	26.9 ± 0.2 <sup>b</sup>	27.6 ± 0.3 <sup>b</sup>	34.1 ± 0.7 <sup>c</sup>	34.0 ± 0.9 <sup>c</sup>
Paternal age	32.8 ± 0.6	35.6 ± 0.8	33.5 ± 0.9	34.3 ± 0.9	35.1 ± 0.9	33.2 ± 1.1
Maternal BMI	24.6 ± 0.7	24.7 ± 1.3	24.6 ± 0.5	24.6 ± 0.9	25.6 ± 0.8	28.1 ± 1.4
Maternal age	31.4 ± 0.6	33.6 ± 0.8	32.0 ± 0.7	31.8 ± 0.7	32.2 ± 0.7	31.7 ± 1.0
Number of previous cycles	0.5 ± 0.1	1.1 ± 0.6	0.8 ± 0.2	0.5 ± 0.2	0.3 ± 0.1	0.6 ± 0.3
AMH (pmol/L)	31.4 ± 5.6	26.4 ± 4.9	27.3 ± 3.8	29.3 ± 4.8	29.8 ± 3.7	30.2 ± 6.2
Baseline FSH (mIU/mL)	6.9 ± 0.5	6.7 ± 0.5	6.9 ± 0.6	7.6 ± 0.6	7.9 ± 0.9	7.1 ± 0.6

Data are expressed as mean ± SEM and analyzed by a 3x2 general linear model. Different letters denote significance at P<0.05 and an absence of superscript letters denotes no statistical difference.

**Table 5.2** Patient population and ART cycle characteristics.

	Normal weight		Overweight		Obese	
	No supplements	With supplements	No supplements	With supplements	No supplements	With supplements
Double embryo transfer	1 (3.4%)	1 (7.1%)	0	1 (4.3%)	1 (4.5%)	0
Preimplantation genetic screening (PGT-A) <sup>1</sup>	5 (17.2%)	3 (21.4%)	2 (6.7%)	2 (8.7%)	7 (31.8%)	3 (23.1%)
Male medical condition/medication	3 (10.3%)	1 (7.1%)	4 (13.3%)	1 (4.3%)	2 (9.1%)	0
Infertility factor - Female	19 (65.5%)	11 (78.6%)	21 (70%)	11 (47.8%)	14 (63.6%)	9 (69.2%)
Infertility factor - Male	5 (17.2%)	1 (7.1%)	5 (16.7%)	7 (30.4%)	5 (22.7%)	2 (15.4%)
Infertility factor- Female & Male	5 (17.2%)	2 (14.3%)	4 (13.3%)	5 (21.7%)	3 (13.6%)	2 (15.4%)
<b>Infertility diagnosis</b>						
Unexplained	8 (27.6%)	3 (21.4%)	8 (26.7%)	7 (30.4%)	5 (22.7%)	4 (30.8%)
Polycystic ovary syndrome (PCOS)	4 (13.8%)	1 (7.1%)	2 (6.7%)	2 (8.7%)	1 (4.5%)	3 (23.1%)
Endometriosis	3 (10.3%)	2 (14.3%)	3 (10.0%)	1 (4.3%)	2 (9.1%)	0
Tubal	1 (3.4%)	0	3 (10%)	0	3 (13.6%)	0

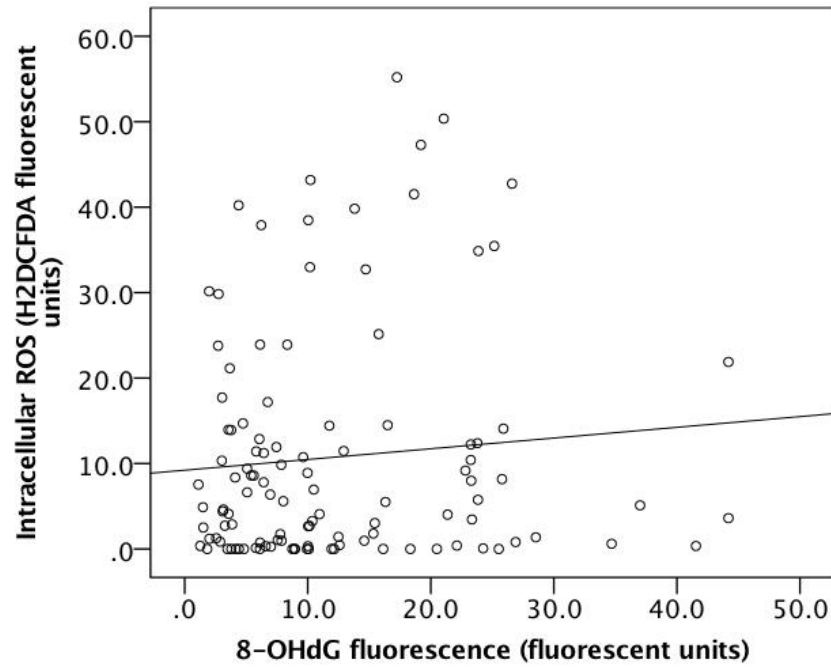
Semen defect	9 (31%)	3 (21.4%)	7 (23.3%)	11 (47.8%)	8 (36.4%)	4 (30.8%)
Anovulation/oligomenorrhea	3 (10.3%)	2 (14.3%)	5 (16.7%)	2 (8.7%)	0	1 (7.7%)
Diminished ovarian reserve	3 (10.3%)	3 (21.4%)	3 (10%)	3 (13%)	2 (9.1%)	2 (15.4%)
Other (e.g. genetic, immunological, premature ovarian dysfunction, recurrent miscarriage, other)	3 (10.3%)	2 (14.3%)	2 (6.7%)	2 (8.7%)	3 (13.6%)	1 (7.7%)

<sup>1</sup> Preimplantation genetic screening for aneuploidy (PGT-A) patients did not undergo an embryo transfer.

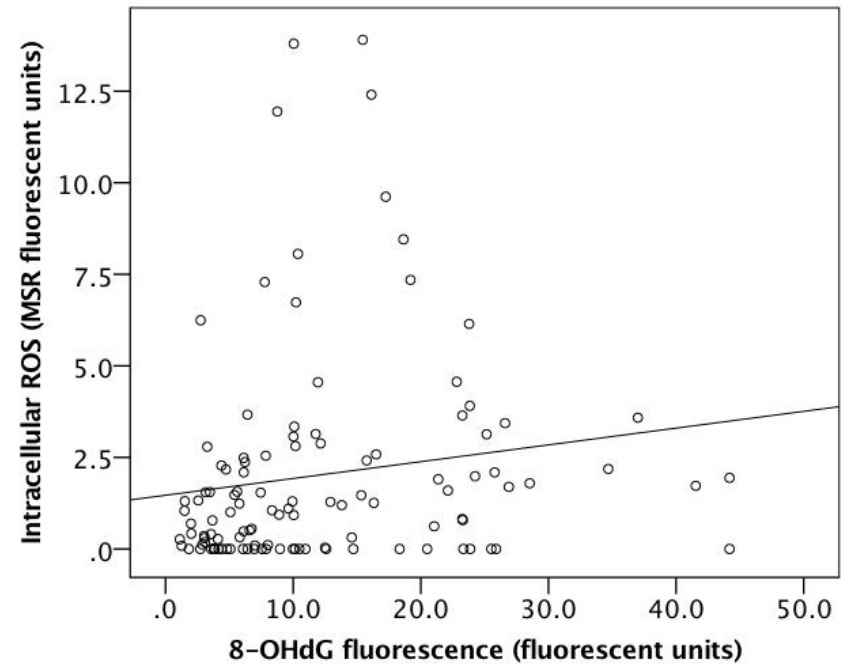
Data are expressed as n values (%: proportion of patients) and analyzed by a 3x2 general linear model. No statistical differences were detected.

### **5.5.2. Relationship between sperm oxidative DNA lesions and sperm intracellular ROS**

Sperm intracellular ROS fluorescence, measured by both H<sub>2</sub>DCFDA and MSR probes, and sperm 8-OHdG fluorescence from the washed sperm sample that was used for the couple's ART procedure, were quantified. There was a positive association between sperm 8-OHdG fluorescence and H<sub>2</sub>DCFDA fluorescence, reflecting mainly sperm intracellular hydroxyl radicals and peroxynitrite ( $r=0.09$ , Figure 5.5). Also, there was a positive association between sperm 8-OHdG fluorescence and MSR fluorescence, representing primarily intracellular mitochondrial superoxide ( $r=0.15$ , Figure 5.5).



(a)

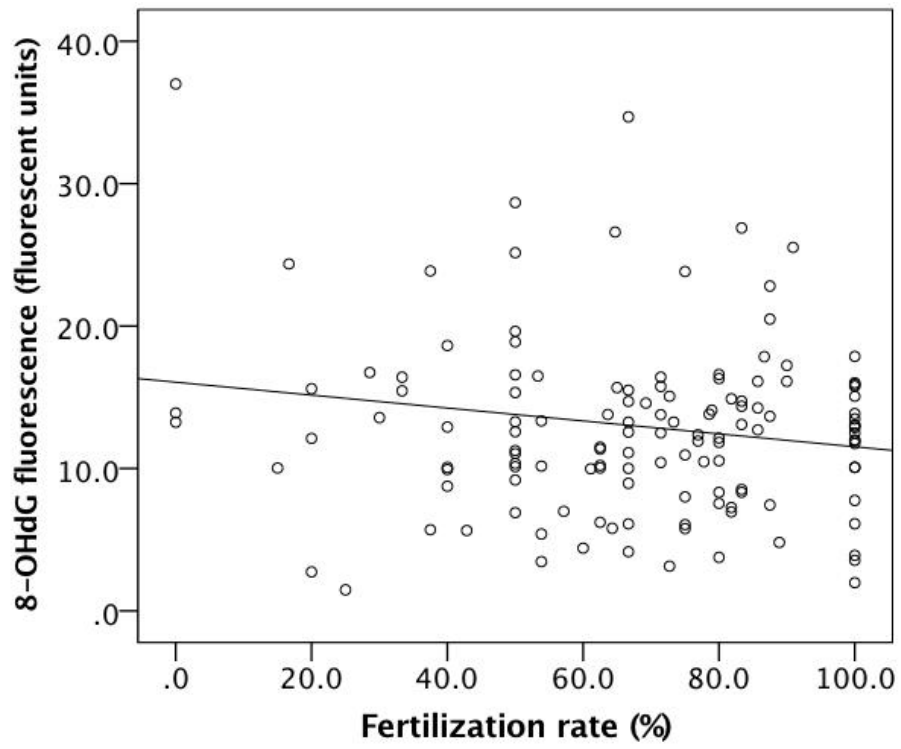


(b)

**Figure 5.5** The association between sperm 8-OHdG fluorescence and intracellular ROS. Sperm intracellular ROS was measured by (a) H<sub>2</sub>DCFDA ( $r=0.09$ ,  $p<0.05$ ) and (b) MSR fluorescence ( $r=0.15$ ,  $p<0.01$ ). Data analyzed by a Spearman correlation.

### **5.5.3. Relationship between sperm oxidative DNA lesions and fertilization rates**

Given that 8-OHdG DNA lesions are a byproduct of sperm intracellular ROS and the positive association observed above between sperm 8-OHdG fluorescence and sperm intracellular ROS fluorescence (results section 5.5.2), the relationship between sperm 8-OHdG DNA lesions and fertilization rates was assessed. There was a negative association between sperm 8-OHdG fluorescence and fertilization rates ( $r=-0.17$ , Figure 5.6).



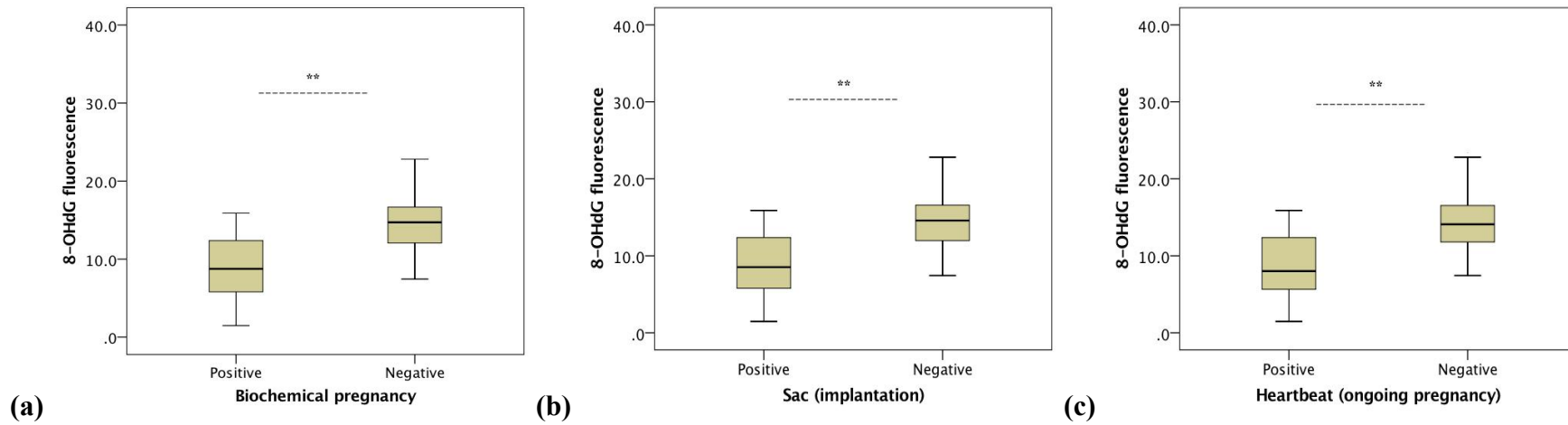
**Figure 5.6** The association between sperm 8-OHdG fluorescence and fertilization rates. Data analyzed by a Spearman correlation ( $r=-0.17$ ,  $p<0.05$ ).

#### **5.5.4. Relationship between oxidative DNA lesions and pregnancy outcome**

There was a negative relationship between sperm 8-OHdG fluorescence and achieving a biochemical pregnancy (positive serum  $\beta$ -hCG test), implantation (presence of a fetal sac on ultrasound examination) and an ongoing pregnancy (presence of a fetal heart on ultrasound examination) (Figure 5.7).

Given the negative relationship between sperm oxidative DNA lesions and achieving a pregnancy, sperm 8-OHdG fluorescence values were quartiled to assess the odds ratio for achieving a pregnancy. The odds of achieving a biochemical pregnancy, implantation (sac) and an ongoing (heartbeat) pregnancy were reduced as sperm 8-OHdG fluorescence increased (Table 5.3). Men with sperm from 8-OHdG Q3 and Q4 had reduced odds of achieving a biochemical pregnancy by 39% and 19% respectively, less than men with sperm from 8-OHdG Q1 (Table 5.3). Men with sperm from 8-OHdG Q2, Q3 and Q4 had reduced odds of achieving implantation by 16%, 49% and 35% respectively, less than men with sperm from 8-OHdG Q1 (Table 5.3). Also, men with sperm from 8-OHdG Q2, Q3 and Q4 had reduced odds of achieving an ongoing pregnancy by 29%, 22% and 38% respectively, less than men with sperm from 8-OHdG Q1 (Table 5.3).





**Figure 5.7** The association between sperm 8-OHdG fluorescence and pregnancy outcomes including (a) biochemical pregnancy (b) implantation (positive for a sac at ultrasound) and (c) ongoing pregnancy (positive for a heartbeat at ultrasound) \*\*p<0.01.

**Table 5.3** The odds of achieving a positive biochemical pregnancy, implantation and an ongoing pregnancy with increasing sperm 8-hydroxyguanosine (8-OHdG) fluorescence. All 8-OHdG fluorescence quartiles were compared to 8-OHdG quartile 1 (Q1); i.e. the lowest fluorescent values.

	Odds ratio	95% Confidence Interval		P value
		Lower	Upper	
<b>Biochemical Pregnancy</b>				
8-OHdG Q2	0.895	0.725	1.097	0.214
8-OHdG Q3	0.608	0.358	0.902	0.000
8-OHdG Q4	0.814	0.625	1.037	0.013
<b>Implantation (sac)</b>				
8-OHdG Q2	0.844	0.661	1.059	0.037
8-OHdG Q3	0.508	0.207	0.865	0.000
8-OHdG Q4	0.646	0.413	0.921	0.000
<b>Ongoing Pregnancy (heartbeat)</b>				
8-OHdG Q2	0.708	0.495	0.959	0.000
8-OHdG Q3	0.783	0.533	1.096	0.000
8-OHdG Q4	0.622	0.375	0.914	0.000

Data expressed as odds ratio with 95% lower and upper confidence intervals.

### **5.5.5. Effect of BMI and micronutrient supplementation on conventional sperm parameters**

Semen volume was recorded and sperm motility, sperm concentration and sperm morphology were assessed before the sample was prepared for the couple's intracytoplasmic sperm injection (ICSI) or standard insemination procedure. Sperm motility and concentration were not different between BMI groups and were not affected by micronutrient supplementation (Table 5.4). The percentage of sperm with normal morphology however was increased with micronutrient supplementation in obese men compared to obese men not taking supplements (Table 5.4). Sperm morphology was not affected by micronutrient supplementation in normal weight and overweight men (Table 5.4).

**Table 5.4** The effects of male BMI and dietary micronutrient supplementation on semen analysis measures including semen volume, sperm motility, sperm concentration and sperm morphology assessed before the sample was prepared for the couple's ICSI or standard insemination procedure; i.e. the neat sperm sample.

	Normal weight		Overweight		Obese	
	No supplements	With supplements	No supplements	With supplements	No supplements	With supplements
Number of patients	29	14	30	23	22	13
Semen volume (ml)	3.5 ± 0.3	3.5 ± 0.4	3.7 ± 0.3	3.0 ± 0.3	2.8 ± 0.3	3.4 ± 0.4
Motility (%)	50.2 ± 3.4	50.0 ± 4.9	49.9 ± 3.4	39.8 ± 3.8	45.2 ± 4.0	54.0 ± 5.2
Concentration (10 <sup>6</sup> /ml)	72.2 ± 10.4	77.8 ± 15.0	75.4 ± 10.5	57.6 ± 12.0	54.5 ± 12.3	52.8 ± 15.3
Morphology (%)	5.0 ± 0.4 <sup>ab</sup>	6.1 ± 0.6 <sup>b</sup>	4.6 ± 0.5 <sup>a</sup>	4.2 ± 0.5 <sup>a</sup>	4.5 ± 0.5 <sup>a</sup>	6.4 ± 0.7 <sup>b</sup>

Data are expressed as mean ± SEM and analyzed by a 3x2 general linear model. Different letters denote significance at P<0.05 and an absence of superscript letters denotes no statistical difference.

### **5.5.6. Effect of BMI and micronutrient supplementation on sperm oxidative stress**

Sperm oxidative stress measures including intracellular ROS and oxidative DNA lesions were assessed using the prepared sperm sample used for standard insemination or ICSI. Sperm oxidative stress measures were assessed from the prepared sperm sample as it was the sample used to inseminate or inject the couple's oocytes and limited the influence of seminal variables which may affect sperm intracellularly.

#### ***5.5.6.1 Sperm total intracellular reactive oxygen species***

Quantified H<sub>2</sub>DCFDA fluorescence reflecting total intracellular ROS was increased in sperm from obese men compared to normal and overweight men (Table 5.5). Total sperm intracellular ROS was reduced with micronutrient supplementation in sperm from obese men to a level similar to that from normal and overweight men (Table 5.5). Micronutrient supplementation did not have an effect on sperm intracellular ROS from normal and overweight men (Table 5.5).

#### ***5.5.6.2 Sperm mitochondrial reactive oxygen species***

Quantified MSR fluorescence in sperm increased as BMI increased with significantly higher levels in overweight compared to normal weight men and a further increase in obese men compared to overweight men (Table 5.6). MSR fluorescence was reduced with micronutrient supplementation in sperm from overweight and obese men compared to overweight and obese men not taking supplements (Table 5.6). There was no effect of micronutrient supplementation on sperm from normal weight men (Table 5.6).

### ***5.5.6.3 Sperm DNA 8-hydroxyguanosine (8-OHdG)***

Quantified DNA 8-OHdG fluorescence in sperm increased as BMI increased with elevated levels in sperm from overweight men compared to normal weight men and a further increase in obese men compared to overweight and normal weight men (Table 5.7). Sperm 8-OHdG fluorescence was reduced with micronutrient supplementation in all BMI categories (Table 5.7).

**Table 5.5** The effects of male BMI and dietary micronutrient supplementation on sperm intracellular total reactive oxygen species (ROS) fluorescence using the 2',7'-dichlorodihydrofluorescein diacetate (H<sub>2</sub>DCFDA) probe assessed using the prepared sperm sample used for standard insemination or ICSI; i.e. the washed sperm sample.

	Normal weight		Overweight		Obese	
	No	With	No	With	No	With
	supplements	supplements	supplements	supplements	supplements	supplements
Number of patients	29	14	30	23	22	13
H <sub>2</sub> DCFDA fluorescence (fluorescent units)	5.8 ± 0.4 <sup>a</sup>	7.5 ± 0.6 <sup>a</sup>	7.1 ± 0.4 <sup>a</sup>	6.6 ± 0.6 <sup>a</sup>	23.2 ± 0.8 <sup>b</sup>	5.8 ± 0.6 <sup>a</sup>

Data are expressed as mean ± SEM. Data are analyzed by a mixed effects (3x2) model adjusting for clustering in each male (100 sperm per male). Different letters denote significance at P<0.05.

**Table 5.6** The effects of male BMI and dietary micronutrient supplementation on sperm intracellular reactive oxygen species (ROS) fluorescence using the MitoSOX Red (MSR) probe assessed using the prepared sperm sample used for standard insemination or ICSI; i.e. the washed sperm sample.

	Normal weight		Overweight		Obese	
	No	With	No	With	No	With
	supplements	supplements	supplements	supplements	supplements	supplements
Number of patients	29	14	30	23	22	13
MSR fluorescence (fluorescent units)	1.2 ± 0.1 <sup>a</sup>	1.1 ± 0.1 <sup>a</sup>	1.7 ± 0.1 <sup>b</sup>	1.2 ± 0.1 <sup>a</sup>	4.6 ± 0.2 <sup>c</sup>	1.9 ± 0.2 <sup>b</sup>

Data are expressed as mean ± SEM. Data are analyzed by a mixed effects (3x2) model adjusting for clustering in each male (100 sperm per male). Different letters denote significance at P<0.05.



**Table 5.7** The effects of male BMI and dietary micronutrient supplementation on sperm DNA 8-hydroxyguanosine (8-OHdG) fluorescence using the prepared sperm sample used for standard insemination or ICSI; i.e. the washed sperm sample.

	Normal weight		Overweight		Obese	
	No supplements	With supplements	No supplements	With supplements	No supplements	With supplements
Number of patients	29	14	30	23	22	13
8-OHdG fluorescence (fluorescent units)	10.6 ± 0.3 <sup>a</sup>	6.7 ± 0.4 <sup>b</sup>	16.0 ± 0.3 <sup>c</sup>	9.8 ± 0.3 <sup>a</sup>	19.4 ± 0.3 <sup>d</sup>	7.3 ± 0.4 <sup>b</sup>

Data are expressed as mean ± SEM. Data are analyzed by a mixed effects (3x2) model adjusting for clustering in each male (100 sperm per male). Different letters denote significantly distinct groups at P<0.05.

### **5.5.7. Fertilization rates**

Although both ICSI and standard insemination fertilization rates were not different between BMI groups and were not affected by micronutrient supplementation, the percentage of embryos with failed fertilization was reduced with supplementation in normal weight men compared to normal weight men not taking supplements (Table 5.8). There was no effect of BMI or micronutrient supplementation on fertilization rates when ICSI and standard insemination fertilization rates were analyzed separately (Table 5.8).

**Table 5.8** The effects of male BMI and dietary micronutrient supplementation on fertilization rates (per patient) for intracytoplasmic sperm injection (ICSI), standard insemination (IVF) and both ICSI and IVF ART procedures.

	Normal weight		Overweight		Obese	
	No supplements	With supplements	No supplements	With supplements	No supplements	With supplements
<b>ICSI fertilization rates</b>						
Number of patients	24	14	27	23	21	11
Mean number of oocytes	8.0 ± 1.1	7.3 ± 2.1	6.6 ± 0.8	7.3 ± 0.8	8.1 ± 1.3	8.6 ± 1.4
Fertilization rate <sup>1</sup> (%)	70.5	84.4	63.2	62.7	69.2	69.6
Oocytes that failed to fertilize <sup>2</sup> (%)	21.9	10.2	14.6	22.5	17.1	15.9
Abnormal fertilization <sup>3</sup> (%)	3.1	3.8	8.8	9.1	9.7	6.6
Non-viable <sup>4</sup> (%)	5.6	5.3	11.9	7.2	5.3	7.9
<b>IVF fertilization rates</b>						
Number of patients	6	1	11	3	4	2
Mean number of oocytes	8.5 ± 2.2	-	5.6 ± 0.8	8.0 ± 1.0	6.5 ± 1.2	9.5 ± 0.5
Fertilization rate <sup>1</sup> (%)	61.3	0.0	72.0	61.0	70.0	47.0

Oocytes that failed to fertilize <sup>2</sup> (%)	25.8	100.0	18.3	16.0	22.3	45.1
Abnormal fertilization <sup>3</sup> (%)	10.1	0.0	9.3	12.3	0	7.9
Non-viable <sup>4</sup> (%)	0.8	0.0	4.2	8.3	6.7	0
<b>ICSI and IVF fertilization rates</b>						
Number of patients	28	14	30	23	22	13
Mean number of oocytes	8.0 ± 1.1	8.4 ± 2.4	7.9 ± 0.9	8.3 ± 0.9	8.9 ± 1.2	10.3 ± 1.7
Fertilization rate <sup>1</sup> (%)	66.9	80.8	65.8	61.3	68.5	66.2
Oocytes that failed to fertilize <sup>2</sup> (%)	23.8 <sup>a</sup>	10.2 <sup>b</sup>	15.7 <sup>ab</sup>	22.5 <sup>ab</sup>	17.3 <sup>ab</sup>	20.4 <sup>ab</sup>
Abnormal fertilization <sup>3</sup> (%)	4.7	3.8	8.0	9.1	9.2	6.8
Non-viable <sup>4</sup> (%)	4.8	5.3	10.7	7.2	5.0	6.7

<sup>1</sup> The presence of two pronuclei (2PN) and two polar bodies (2PB).

<sup>2</sup> Oocytes that lacked the presence of pronuclei (2PN) and two polar bodies (2PB).

<sup>3</sup> Abnormal fertilization rate reflecting parthenogenetic oocytes or the presence of three or more pronuclei.

<sup>4</sup> Non-viable rate reflecting the proportion of lysed eggs/embryos.

Data are expressed as mean ± SEM and as proportion of patients (%), and analyzed by a binary logistic regression. Different letters denote significance at P<0.05 and an absence of superscript letters denotes no statistical difference.

### **5.5.8. Embryo development, blastocyst quality and embryo utilization**

On days 4 and 5 of culture, embryo morphology was morphologically assessed, as these time points are post-embryonic genome activation and therefore can be influenced by the quality of the male (paternal) genome. Day 4 embryo morphology was not affected by BMI however the proportion of grade 2A embryos (partially compacted morulae or early blastocysts) was increased and the proportion of grade 3 embryos (embryos with  $\geq 8$  cells and no compaction) was reduced with micronutrient supplementation in normal weight men (Table 5.9). On day 5 of embryo culture, blastocyst development (Table 5.10) was assessed in addition to blastocyst inner cell mass (ICM) and trophectoderm (TE) quality (Table 5.11), however conclusive results were not attained due to underpowered statistical analysis. The statistical analyses on day 5 were underpowered due to reduced n values as many embryos were frozen or transferred (utilized) on day 4 of embryo culture. The percentage of transferred and/or frozen embryos was assessed to measure embryo utilization and was not different between BMI groups and not affected by micronutrient supplementation (Table 5.12).

**Table 5.9** The effects of male BMI and dietary micronutrient supplementation on day 4 embryo morphology.

	Normal weight		Overweight		Obese	
	No supplements	With supplements	No supplements	With supplements	No supplements	With supplements
Number of patients	22	7	20	12	15	8
Mean number of embryos	5.1 ± 0.8	4.4 ± 1.9	6.8 ± 0.9	6.5 ± 1.0	6.2 ± 0.9	9.0 ± 1.4
Grade 1 <sup>1</sup> (%)	18.8	19.4	17.0	15.4	23.7	27.8
Grade 2A <sup>2</sup> (%)	12.5 <sup>a</sup>	38.7 <sup>b</sup>	23.7 <sup>ab</sup>	12.8 <sup>ab</sup>	29.0 <sup>b</sup>	23.6 <sup>ab</sup>
Grade 2B <sup>3</sup> (%)	19.6	22.6	18.5	34.6	11.8	11.1
Grade 3 <sup>4</sup> (%)	31.3 <sup>a</sup>	9.7 <sup>b</sup>	29.6 <sup>a</sup>	26.9 <sup>a</sup>	14.0 <sup>b</sup>	23.6 <sup>ab</sup>
Grade 4 <sup>5</sup> (%)	17.9	9.7	11.1	10.3	21.5	13.9

<sup>1</sup> Early blastocysts or compacted morulae

<sup>2</sup> Partially compacted morulae (>70% compaction) with no anomalies or early blastocysts and compacted morulae with minimal anomalies

<sup>3</sup> Partially compacted morulae (<70% compaction) with no or minimal anomalies

<sup>4</sup> Embryos with ≥8 cells and no compaction with no or minimal anomalies or early blastocysts and compacted morulae with above minimal anomalies

<sup>5</sup> Embryos with <8 cells or embryos with ≥8 cells, no compaction and above minimal anomalies

Data are expressed as mean ± SEM and as a proportion of fertilized embryos (%) and analyzed by a binary logistic regression weighted to the total number of fertilized embryos per couple. Different letters denote significantly distinct groups at P<0.05 and an absence of superscript letters denotes no statistical difference.

**Table 5.10** The effects of male BMI and dietary micronutrient supplementation on day 5 embryo morphology.

	Normal weight		Overweight		Obese	
	No supplements	With supplements	No supplements	With supplements	No supplements	With supplements
Number of patients	6	7	9	10	6	5
Mean number of embryos	7.2 ± 1.7	8.0 ± 3.6	3.6 ± 0.8	4.5 ± 0.9	7.8 ± 2.1	2.6 ± 0.7
<16 cells (%)	16.3	17.9	15.6	20.2	14.8	15.3
Morula (%)	13.8	12.3	15.6	11.2	16.5	13.7
Early blastocyst (%)	34.9	27.1	21.9	25.8	22.8	30.1
Full blastocyst (%)	14.0	14.1	25.1	23.2	24.8	17.7
Expanded blastocyst (%)	21.0	28.6	22.0	20.0	21.3	23.0

Data are expressed as a mean ± SEM and as a proportion of fertilized embryos (%) and analyzed by a binary logistic regression weighted to the total number of fertilized embryos per couple.

Note: the analysis in the table above is not statistically powered.



**Table 5.11** The effects of male BMI and dietary micronutrient supplementation on blastocyst inner cell mass and trophectoderm morphology.

	Normal weight		Overweight		Obese	
	No supplements	With supplements	No supplements	With supplements	No supplements	With supplements
Number of patients	4	3	8	8	4	3
Mean number of blastocysts	2.5 ± 0.4	4.6 ± 1.6	1.4 ± 0.3	1.6 ± 0.3	2.4 ± 0.3	1.1 ± 0.2
<b>Inner cell mass</b>						
Grade A <sup>1</sup> (%)	35.4	63.5	75.0	29.2	54.2	50.0
Grade B <sup>2</sup> (%)	45.8	25.7	25.0	70.8	39.6	50.0
Grade C <sup>3</sup> (%)	18.8	10.8	0.0	0.0	6.3	0.0
<b>Trophectoderm</b>						
Grade A <sup>4</sup> (%)	47.9	55.0	43.8	41.7	50.0	33.4
Grade B <sup>5</sup> (%)	45.8	27.9	56.3	41.7	40.6	66.7
Grade C <sup>6</sup> (%)	6.3	17.1	0.0	16.7	9.4	0.0

<sup>1</sup> Many tightly packed cells

<sup>2</sup> Several (loose) cells

<sup>3</sup> Very few cells

<sup>4</sup> Many cells forming a cohesive layer

<sup>5</sup> Few cells forming a loose epithelium

<sup>6</sup> Very few cells

Data are expressed as a mean  $\pm$  SEM and as a proportion of blastocyst-staged embryos (%) and analyzed by a binary logistic regression weighted to the total number of blastocyst-staged embryos per couple. Different letters denote significantly distinct groups at  $P < 0.05$  and an absence of superscript letters denotes no statistical difference.

Note: the analysis in the table above is not statistically powered.

**Table 5.12** The effects of male BMI and dietary micronutrient supplementation on embryo utilization.

	Normal weight		Overweight		Obese	
	No supplements	With supplements	No supplements	With supplements	No supplements	With supplements
Number of patients	28	13	29	22	21	13
Mean number of embryos	5.5 ± 0.7	6.2 ± 2.1	5.6 ± 0.7	5.4 ± 0.7	6.4 ± 0.9	6.5 ± 1.2
Transferred and/or frozen embryos (%)	57.4	72.9	66.6	54.4	65.8	61.1

Data are expressed as mean ± SEM and as a proportion of fertilized embryos (%) and analyzed by a binary logistic regression weighted to the total number of fertilized embryos per couple. No statistical differences were detected.

### **5.5.9. Pregnancy outcome**

Biochemical pregnancy rates (positive serum  $\beta$ -hCG test), implantation rates (presence of a fetal sac on ultrasound examination), ongoing pregnancy rates (presence of a fetal heart on ultrasound examination) and live birth rates were not different BMI groups and were not affected by micronutrient supplementation (Table 5.13). However, it must be acknowledged that the pregnancy outcomes analysis was statistically underpowered.

**Table 5.13** The effects of male BMI and dietary micronutrient supplementation on pregnancy outcomes.

	Normal weight		Overweight		Obese	
	No supplements	With supplements	No supplements	With supplements	No supplements	With supplements
<b>Per embryo transfer</b>						
Number of patients	18	9	24	19	14	10
Biochemical pregnancy (%)	66.7	55.6	62.5	42.1	57.1	60.0
Implantation (%)	61.1	55.6	58.4	31.6	50.0	60.0
Ongoing pregnancy (%)	55.6	44.4	58.4	26.3	35.7	50.0
Live birth (%)	50.0	33.4	54.2	21.1	28.6	40.0
<b>Per oocyte pick-up</b>						
Number of patients	28	14	29	22	21	13
Biochemical pregnancy (%)	42.9	35.7	51.7	36.4	38.1	46.2
Implantation (%)	39.3	35.7	48.3	27.3	33.3	46.2
Ongoing pregnancy (%)	35.7	28.6	48.3	22.7	23.8	38.5
Live birth (%)	32.1	21.4	44.8	18.2	19.0	30.8

Data are expressed as a proportion of embryo transfers and oocyte pick-ups (%) and analyzed by a binary logistic regression. No statistical differences were detected.

Note: the analysis in the table above is not statistically powered.

## 5.6. Discussion

This study demonstrated that there is a negative association between sperm intracellular ROS and sperm oxidative DNA lesions in addition to negative associations between sperm oxidative DNA modifications and fertilization and pregnancy rates. Dietary micronutrient supplementation reduced sperm oxidative stress in these men primarily observed by reduced oxidative DNA lesions in normal weight, overweight and obese men. Also, dietary micronutrient supplementation was most effective in sperm oxidative stress in obese men whereby sperm intracellular ROS and oxidative DNA modifications were reduced with supplement intake compared to obese men not consuming supplements. These findings suggest that micronutrient supplementation is effective in the amelioration of sperm oxidative stress-induced subfertility.

The oxidative DNA adduct, 8-OHdG, is formed as a result from ROS attack and therefore, the sperm intracellular ROS data in addition to the 8-OHdG fluorescence data was combined regardless of male BMI and micronutrient supplementation to identify the association between sperm intracellular ROS and 8-OHdG lesions. This correlation analysis found that both H<sub>2</sub>DCFDA and MSR fluorescence had a positive association with 8-OHdG fluorescence. Previous studies have suggested that hydroxyl radicals are the main type of ROS contributing to the formation of 8-OHdG DNA adducts (Kasai *et al.* 1986; Takeuchi *et al.* 1997; Valavanidis *et al.* 2009). Specifically, the hydroxyl radical is most likely formed from mitochondrial-generated superoxide and the subsequent Fenton reaction resulting from normal respiration (Takeuchi *et al.* 1997) and 8-OHdG is induced by the hydroxyl radical at the C-8 position of deoxyguanosine in DNA (Kasai *et al.* 1986). Based on previous work in different cell types, it is plausible to suggest that

the extremely reactive hydroxyl radical most likely comes in reactive proximity to the nucleus from the midpiece, where the mitochondria are mainly located in the sperm cell, resulting in 8-OHdG formation (Bowen 2011).

Given the observed positive associations between sperm intracellular ROS and oxidative DNA lesions (8-OHdG) and that 8-OHdG adducts are a byproduct of ROS attack on sperm, the association between sperm 8-OHdG lesions and sperm function measures such as fertilization rates was assessed. When correlating sperm 8-OHdG lesion fluorescence with fertilization rates from all couples regardless of the male partner's BMI and supplementation status, there was a negative association between sperm 8-OHdG fluorescence and fertilization rates. Although previous studies, including a meta-analysis, have shown a negative correlation between sperm oxidative stress and successful fertilization following IVF/ICSI treatment (Agarwal *et al.* 2005), these studies measured ROS in semen as opposed to this study that investigated 8-OHdG lesions in sperm.

Interestingly, the non-stratified data (regardless of male BMI and micronutrient supplement intake) indicates that sperm oxidative DNA modifications had an inverse relationship with pregnancy and implantation rates. As sperm 8-OHdG fluorescence increased, the likelihood of achieving a biochemical pregnancy, implantation and an ongoing pregnancy was reduced, which is consistent with previous reports of a reduced odds ratio of achieving a biochemical pregnancy with increased sperm oxidative DNA lesions (Loft *et al.* 2003). The negative association between sperm oxidative stress and pregnancy outcome implicates compromised DNA integrity as a potential contributor of pregnancy loss (Carrell *et al.* 2003). This may be due to oxidative DNA lesions/mutations and molecular alterations in the male germ line such as altered gene expression and



epigenetic DNA methylation which may persist into the preimplantation embryo (Cirio *et al.* 2008; Trasler 2009). Also, the reduced odds ratio of achieving a positive pregnancy with increased sperm oxidative DNA damage may serve as a potential predictor for pregnancy outcomes in patients undergoing ART treatment.

After assessing the relationships between sperm 8-OHdG lesions and fertility treatment outcomes including fertilization and pregnancy rates, the impact of micronutrient supplementation on ART outcomes were evaluated in these couples. Micronutrient supplementation did not have an effect on sperm motility or concentration however, there was an increase in normal sperm morphology with micronutrient supplement intake within the obese men category. Previous studies have shown no effect of supplement intake (zinc, vitamin E, vitamin C, selenium or carnitines) on sperm morphology, however these studies have been conducted in men diagnosed with either athenozoospermia (Omu *et al.* 2008), male factor infertility (Keskes-Ammar *et al.* 2003; Kodama *et al.* 1997; Vicari and Calogero 2001; Vicari *et al.* 2002), or high percentage of sperm DNA fragmentation (Greco *et al.* 2005a; Greco *et al.* 2005b). Further, most of these studies used different combinations of between 1 and 3 micronutrients including zinc (Omu *et al.* 2008), vitamins C and E (Greco *et al.* 2005a; Greco *et al.* 2005b; Kodama *et al.* 1997; Omu *et al.* 2008), vitamin E and selenium (Keskes-Ammar *et al.* 2003), and L-carnitine and acetyl-L-carnitine (Vicari and Calogero 2001) except for one study that used a combination of micronutrient supplements with a total of 60 men (Tremellen *et al.* 2007) as opposed to the 131 men recruited in this study. Therefore, the reasons contributing to this discrepancy in improved sperm morphology with micronutrient supplementation include patient cohort characteristics, different combinations of micronutrient supplements or a single micronutrient and different sample sizes.

In addition to conventional sperm parameters, this study examined intracellular ROS in live sperm from normal weight, overweight and obese men from the sperm sample used for the couple's IVF/ICSI procedure as this limits the influence of variables from the seminal fluid that may affect sperm intracellularly. Sperm intracellular ROS was measured using two different probes reflecting mainly peroxynitrite and hydroxyl radicals concentrations (H<sub>2</sub>DCFDA) in addition to mitochondrial ROS primarily superoxide (MSR) concentrations (Purdey *et al.* 2015). Both probes showed increased sperm intracellular ROS in obese men compared to both overweight and normal weight men. These results illustrate that ROS production does not only occur in the seminal fluid from obese men (Tunc *et al.* 2011; Yang *et al.* 2016) but also develops in sperm intracellularly. This increase in oxidative stress in both sperm and seminal fluid may be due to the presence of leukocytes (Aitken *et al.* 1994; Aitken *et al.* 1995) and/or defective and immature sperm (Gomez *et al.* 1996; Sakkas *et al.* 2003). Obesity has been defined as a chronic state of inflammation (Johnson *et al.* 2012) and this immune activation may extend to the male reproductive tract increasing leukocyte activity, mainly neutrophils and macrophages, and seminal oxidative stress (Tunc *et al.* 2011). It is postulated that sperm intracellular ROS are generated by defective and immature sperm characterized with (i) large cytoplasmic residues containing NADPH (Aziz *et al.* 2004) and/or (ii) increased mitochondrial metabolism (Koppers *et al.* 2008). Interestingly, this study showed that micronutrient supplement intake in obese men reduces sperm intracellular ROS ultimately alleviating sperm oxidative stress. In semen from infertile men, there are lower levels of antioxidants and micronutrients compared to fertile men (Alkan *et al.* 1997; Garrido *et al.* 2004; Lewis *et al.* 1995) suggesting that dietary micronutrient supplementation may lessen obesity-induced sperm oxidative stress by balancing the levels anti-oxidative micronutrients and reducing ROS generation.

An additional oxidative stress marker, oxidative DNA lesions (8-OHdG), was also assessed from the sperm sample used for the couple's IVF/ICSI procedure. 8-OHdG, the most common oxidized base modification (De Iuliis *et al.* 2009b), is an oxidized guanine base adduct within sperm DNA resulting from ROS attack including hydroxyl radicals and superoxide (Noblanc *et al.* 2013) and is associated with subfertility and reduced pregnancy rates (Thomson *et al.* 2011), which is consistent with the findings in this study. When 8-OHdG fluorescence was quantified, a positive relationship was identified between BMI and sperm 8-OHdG lesions whereby 8-OHdG fluorescence increased as BMI increased. Interestingly, micronutrient supplementation showed to be an effective treatment option in all men despite BMI as sperm 8-OHdG fluorescence was reduced in normal weight, overweight and obese men. This reduction in sperm 8-OHdG lesions with micronutrient supplementation in men from all BMI categories may be due to factors beyond BMI whereby individuals from the normal weight and overweight BMI categories have sperm with oxidative DNA damage possibly due to low micronutrient and antioxidant food intake, perturbed metabolic profiles (Amiri *et al.* 2011), infection (Aitken and Roman 2008) or exposure to environmental pollutants (Xu *et al.* 2003).

Dietary vitamin C supplementation has been shown to reduce 8-OHdG lesions in sperm from smokers (Fraga *et al.* 1991). Further, a combination of vitamin C, vitamin E and glutathione supplement intake has also shown to reduce 8-OHdG adducts in sperm from infertile men (Kodama *et al.* 1997) suggesting that these micronutrients may contribute to the reduction in sperm oxidative DNA damage. Overall, this is the first demonstration of that sperm 8-OHdG modifications is increased with an increased BMI and that dietary micronutrient supplementation can reduce these lesions.

Sperm fertilization capacity and fertilization rates were not affected by BMI or micronutrient supplement intake however a difference may be detected in a larger cohort of participants. On day 4 of embryo development, the proportion of partially compacted morulae or early blastocysts was increased with micronutrient supplementation in normal weight men. There are some studies that report no effect of micronutrient supplement therapy in men undergoing fertility treatment on embryo morphology (Greco *et al.* 2005b; Tremellen *et al.* 2007) whereas others report an improvement in embryo quality with anti-oxidative micronutrient therapy (Kacem 2014). However, these studies differed significantly in terms of micronutrient supplement composition; a combination of micronutrients (Kacem 2014; Tremellen *et al.* 2007) versus vitamins C and E only (Greco *et al.* 2005b), and the study methodology; same male before and after supplement therapy (Greco *et al.* 2005b; Kacem 2014) versus a treatment and placebo group (Tremellen *et al.* 2007). Further, these studies did not report the BMI of male participants and additional differences in other BMI categories from this study may be identified in a larger sample size of participants. Although pregnancy and live birth rates from the BMI and micronutrient supplementation stratified dataset could not be concluded due to a statistically underpowered analysis, the impact of micronutrient supplement intake on pregnancy outcomes has been reviewed and found that supplement intake increases pregnancy and live birth rates in subfertile couples undergoing ART treatment (Gharagozloo and Aitken 2011; Showell *et al.* 2011).

Overall, these novel findings show that oxidative DNA damage in sperm is inversely associated with successful fertilization and pregnancy. Further, this study demonstrates a potential mechanism for dietary micronutrient supplementation in improving fertility treatment outcomes by reducing sperm oxidative stress-induced subfertility. However, it

is not possible to determine if dietary and lifestyle habits had an impact and which specific micronutrients contribute to the observed improvements in fertility treatment outcomes, which are limitations of this study. Future research with increased sample sizes to ensure adequate statistical power is required to conclude the impact of micronutrient supplementation on pregnancy outcomes.

## 5.7. References

- Agarwal, A., Allamaneni, S.S., Nallella, K.P., George, A.T., and Mascha, E. (2005) Correlation of reactive oxygen species levels with the fertilization rate after in vitro fertilization: a qualified meta-analysis. *Fertil Steril* **84**, 228-231.
- Aitken, J., Krausz, C., and Buckingham, D. (1994) Relationships between Biochemical Markers for Residual Sperm Cytoplasm, Reactive Oxygen Species Generation, and the Presence of Leukocytes and Precursor Germ-Cells in Human Sperm Suspensions. *Molecular Reproduction and Development* **39**, 268-279. [In English]
- Aitken, R.J., Buckingham, D.W., Brindle, J., Gomez, E., Baker, H.W.G., and Irvine, D.S. (1995) Analysis of Sperm Movement in Relation to the Oxidative Stress Created by Leukocytes in Washed Sperm Preparations and Seminal Plasma. *Human Reproduction* **10**, 2061-2071. [In English]
- Aitken, R.J., Clarkson, J.S., and Fishel, S. (1989) Generation of reactive oxygen species, lipid peroxidation, and human sperm function. *Biol Reprod* **41**, 183-197.
- Aitken, R.J., Irvine, D.S., and Wu, F.C. (1991) Prospective analysis of sperm-oocyte fusion and reactive oxygen species generation as criteria for the diagnosis of infertility. *Am J Obstet Gynecol* **164**, 542-551.
- Aitken, R.J., and Roman, S.D. (2008) Antioxidant systems and oxidative stress in the testes. *Adv Exp Med Biol* **636**, 154-171.
- Alkan, I., Simsek, F., Haklar, G., Kervancioglu, E., Ozveri, H., Yalcin, S., and Akdas, A. (1997) Reactive oxygen species production by the spermatozoa of patients with idiopathic infertility: relationship to seminal plasma antioxidants. *J Urol* **157**, 140-143.
- Alvarez, J.G., Touchstone, J.C., Blasco, L., and Storey, B.T. (1987) Spontaneous Lipid-Peroxidation and Production of Hydrogen-Peroxide and Superoxide in Human-Spermatozoa Superoxide-Dismutase as Major Enzyme Protectant against Oxygen-Toxicity. *Journal of Andrology* **8**, 338-348. [In English]
- Amiri, I., Karimi, J., Piri, H., Goodarzi, M.T., Tavilani, H., Khodadadi, I., and Ghorbani, M. (2011) Association between nitric oxide and 8-hydroxydeoxyguanosine levels in semen of diabetic men. *Syst Biol Reprod Med* **57**, 292-295.
- Aziz, N., Saleh, R.A., Sharma, R.K., Lewis-Jones, I., Esfandiari, N., Thomas, A.J., Jr., and Agarwal, A. (2004) Novel association between sperm reactive oxygen species production, sperm morphological defects, and the sperm deformity index. *Fertil Steril* **81**, 349-354.
- Bowen, P.E. (2011) Hydroxylated Nucleotides. In 'Biomarkers for Antioxidant Defense and Oxidative Damage.' (Ed. RM Russell). (John Wiley & Sons)

Carrell, D.T., Liu, L., Peterson, C.M., Jones, K.P., Hatasaka, H.H., Erickson, L., and Campbell, B. (2003) Sperm DNA fragmentation is increased in couples with unexplained recurrent pregnancy loss. *Arch Androl* **49**, 49-55. [In eng]

Cirio, M.C., Martel, J., Mann, M., Toppings, M., Bartolomei, M., Trasler, J., and Chaillet, J.R. (2008) DNA methyltransferase 1o functions during preimplantation development to preclude a profound level of epigenetic variation. *Dev Biol* **324**, 139-150.

Cutillas-Tolin, A., Minguez-Alarcon, L., Mendiola, J., Lopez-Espin, J.J., Jorgensen, N., Navarrete-Munoz, E.M., Torres-Cantero, A.M., and Chavarro, J.E. (2015) Mediterranean and western dietary patterns are related to markers of testicular function among healthy men. *Hum Reprod* **30**, 2945-2955.

D'Agata, R., Vicari, E., Moncada, M.L., Sidoti, G., Calogero, A.E., Fornito, M.C., Minacapilli, G., Mongioi, A., and Polosa, P. (1990) Generation of reactive oxygen species in subgroups of infertile men. *Int J Androl* **13**, 344-351.

De Iuliis, G.N., Thomson, L.K., Mitchell, L.A., Finnie, J.M., Koppers, A.J., Hedges, A., Nixon, B., and Aitken, R.J. (2009a) DNA Damage in Human Spermatozoa Is Highly Correlated with the Efficiency of Chromatin Remodeling and the Formation of 8-Hydroxy-2'-Deoxyguanosine, a Marker of Oxidative Stress. *Biology of Reproduction* **81**, 517-524. [In English]

De Iuliis, G.N., Thomson, L.K., Mitchell, L.A., Finnie, J.M., Koppers, A.J., Hedges, A., Nixon, B., and Aitken, R.J. (2009b) DNA damage in human spermatozoa is highly correlated with the efficiency of chromatin remodeling and the formation of 8-hydroxy-2'-deoxyguanosine, a marker of oxidative stress. *Biol Reprod* **81**, 517-524.

de Lamirande, E., Leduc, B.E., Iwasaki, A., Hassouna, M., and Gagnon, C. (1995) Increased reactive oxygen species formation in semen of patients with spinal cord injury. *Fertil Steril* **63**, 637-642.

Fraga, C.G., Motchnik, P.A., Shigenaga, M.K., Helbock, H.J., Jacob, R.A., and Ames, B.N. (1991) Ascorbic acid protects against endogenous oxidative DNA damage in human sperm. *Proc Natl Acad Sci U S A* **88**, 11003-11006.

Fraga, C.G., Motchnik, P.A., Wyrobek, A.J., Rempel, D.M., and Ames, B.N. (1996) Smoking and low antioxidant levels increase oxidative damage to sperm DNA. *Mutat Res* **351**, 199-203.

Garrido, N., Meseguer, M., Simon, C., Pellicer, A., and Remohi, J. (2004) Pro-oxidative and anti-oxidative imbalance in human semen and its relation with male fertility. *Asian J Androl* **6**, 59-65.

Gharagozloo, P., and Aitken, R.J. (2011) The role of sperm oxidative stress in male infertility and the significance of oral antioxidant therapy. *Hum Reprod* **26**, 1628-1640.

Gomez, E., Buckingham, D.W., Brindle, J., Lanzafame, F., Irvine, D.S., and Aitken, R.J. (1996) Development of an image analysis system to monitor the retention of

residual cytoplasm by human spermatozoa: Correlation with biochemical markers of the cytoplasmic space, oxidative stress, and sperm function. *Journal of Andrology* **17**, 276-287. [In English]

Greco, E., Iacobelli, M., Rienzi, L., Ubaldi, F., Ferrero, S., and Tesarik, J. (2005a) Reduction of the incidence of sperm DNA fragmentation by oral antioxidant treatment. *J Androl* **26**, 349-353.

Greco, E., Romano, S., Iacobelli, M., Ferrero, S., Baroni, E., Minasi, M.G., Ubaldi, F., Rienzi, L., and Tesarik, J. (2005b) ICSI in cases of sperm DNA damage: beneficial effect of oral antioxidant treatment. *Hum Reprod* **20**, 2590-2594.

Henkel, R.R. (2011) Leukocytes and oxidative stress: dilemma for sperm function and male fertility. *Asian J Androl* **13**, 43-52.

Hughes, C.M., Lewis, S.E., McKelvey-Martin, V.J., and Thompson, W. (1996) A comparison of baseline and induced DNA damage in human spermatozoa from fertile and infertile men, using a modified comet assay. *Mol Hum Reprod* **2**, 613-619.

Iwasaki, A., and Gagnon, C. (1992) Formation of reactive oxygen species in spermatozoa of infertile patients. *Fertil Steril* **57**, 409-416.

Ji, B.T., Shu, X.O., Linet, M.S., Zheng, W., Wacholder, S., Gao, Y.T., Ying, D.M., and Jin, F. (1997) Paternal cigarette smoking and the risk of childhood cancer among offspring of nonsmoking mothers. *J Natl Cancer Inst* **89**, 238-244.

Johnson, A.R., Milner, J.J., and Makowski, L. (2012) The inflammation highway: metabolism accelerates inflammatory traffic in obesity. *Immunol Rev* **249**, 218-238.

Kacem, O.H., M; Zedini, C.; Meddeb, S.; Saidi, H.; Chaib, A.; Ali, H.; Bibi, M.; Saad, A.; Khairi, H.; Aijna, M. (2014) Beneficial Effect of an Oral Antioxidant Supplement (Fertimax2) on IVF-ICSI Outcomes: A Preliminary Clinical Study. *Advances in Reproductive Sciences*, 47-56.

Kasai, H., Crain, P.F., Kuchino, Y., Nishimura, S., Ootsuyama, A., and Tanooka, H. (1986) Formation of 8-hydroxyguanine moiety in cellular DNA by agents producing oxygen radicals and evidence for its repair. *Carcinogenesis* **7**, 1849-1851.

Keskes-Ammar, L., Feki-Chakroun, N., Rebai, T., Sahnoun, Z., Ghozzi, H., Hammami, S., Zghal, K., Fki, H., Damak, J., and Bahloul, A. (2003) Sperm oxidative stress and the effect of an oral vitamin E and selenium supplement on semen quality in infertile men. *Arch Androl* **49**, 83-94.

Kodama, H., Yamaguchi, R., Fukuda, J., Kasai, H., and Tanaka, T. (1997) Increased oxidative deoxyribonucleic acid damage in the spermatozoa of infertile male patients. *Fertil Steril* **68**, 519-524.

Koppers, A.J., De Iuliis, G.N., Finnie, J.M., McLaughlin, E.A., and Aitken, R.J. (2008) Significance of mitochondrial reactive oxygen species in the generation of oxidative stress in spermatozoa. *Journal of Clinical Endocrinology & Metabolism* **93**, 3199-3207.



- Lewis, S.E., Boyle, P.M., McKinney, K.A., Young, I.S., and Thompson, W. (1995) Total antioxidant capacity of seminal plasma is different in fertile and infertile men. *Fertil Steril* **64**, 868-870.
- Liu, C.Y., Chou, Y.C., Chao, J.C., Hsu, C.Y., Cha, T.L., and Tsao, C.W. (2015) The Association between Dietary Patterns and Semen Quality in a General Asian Population of 7282 Males. *PLoS One* **10**, e0134224.
- Loft, S., Kold-Jensen, T., Hjollund, N.H., Giwercman, A., Gylleborg, J., Ernst, E., Olsen, J., Scheike, T., Poulsen, H.E., and Bonde, J.P. (2003) Oxidative DNA damage in human sperm influences time to pregnancy. *Hum Reprod* **18**, 1265-1272.
- McPherson, N.O., and Lane, M. (2015) Male obesity and subfertility, is it really about increased adiposity? *Asian J Androl* **17**, 450-458.
- NHMRC (2006) Nutrient Reference Values for Australia and New Zealand including Recommended Dietary Intakes. *National Health and Medical Research Council* **N37**.
- Nobranc, A., Damon-Soubeyrand, C., Karrich, B., Henry-Berger, J., Cadet, R., Saez, F., Guiton, R., Janny, L., Pons-Rejraji, H., Alvarez, J.G., *et al.* (2013) DNA oxidative damage in mammalian spermatozoa: where and why is the male nucleus affected? *Free Radic Biol Med* **65**, 719-723.
- Omu, A.E., Al-Azemi, M.K., Kehinde, E.O., Anim, J.T., Oriowo, M.A., and Mathew, T.C. (2008) Indications of the mechanisms involved in improved sperm parameters by zinc therapy. *Med Princ Pract* **17**, 108-116.
- Oostingh, E.C., Steegers-Theunissen, R.P., de Vries, J.H., Laven, J.S., and Koster, M.P. (2017) Strong adherence to a healthy dietary pattern is associated with better semen quality, especially in men with poor semen quality. *Fertil Steril* **107**, 916-923 e912.
- Purdey, M.S., Connaughton, H.S., Whiting, S., Schartner, E.P., Monro, T.M., Thompson, J.G., Aitken, R.J., and Abell, A.D. (2015) Boronate probes for the detection of hydrogen peroxide release from human spermatozoa. *Free Radic Biol Med* **81**, 69-76.
- Sakkas, D., Seli, E., Bizzaro, D., Tarozzi, N., and Manicardi, G.C. (2003) Abnormal spermatozoa in the ejaculate: abortive apoptosis and faulty nuclear remodelling during spermatogenesis. *Reprod Biomed Online* **7**, 428-432.
- Sharma, R.K., and Agarwal, A. (1996) Role of reactive oxygen species in male infertility. *Urology* **48**, 835-850.
- Shen, H.M., Chia, S.E., and Ong, C.N. (1999) Evaluation of oxidative DNA damage in human sperm and its association with male infertility. *J Androl* **20**, 718-723.
- Showell, M.G., Brown, J., Yazdani, A., Stankiewicz, M.T., and Hart, R.J. (2011) Antioxidants for male subfertility. *Cochrane Database Syst Rev*, CD007411. [In eng]

Swan, S.H., Elkin, E.P., and Fenster, L. (2000) The question of declining sperm density revisited: an analysis of 101 studies published 1934-1996. *Environ Health Perspect* **108**, 961-966.

Takeuchi, T., Matsugo, S., and Morimoto, K. (1997) Mutagenicity of oxidative DNA damage in Chinese hamster V79 cells. *Carcinogenesis* **18**, 2051-2055.

Thomson, L.K., Zieschang, J.A., and Clark, A.M. (2011) Oxidative deoxyribonucleic acid damage in sperm has a negative impact on clinical pregnancy rate in intrauterine insemination but not intracytoplasmic sperm injection cycles. *Fertil Steril* **96**, 843-847.

Trasler, J.M. (2009) Epigenetics in spermatogenesis. *Mol Cell Endocrinol* **306**, 33-36.

Tremellen, K., Miari, G., Froiland, D., and Thompson, J. (2007) A randomised control trial examining the effect of an antioxidant (Menevit) on pregnancy outcome during IVF-ICSI treatment. *Aust N Z J Obstet Gynaecol* **47**, 216-221.

Troppmann, L., Gray-Donald, K., and Johns, T. (2002) Supplement use: is there any nutritional benefit? *J Am Diet Assoc* **102**, 818-825.

Tunc, O., Bakos, H.W., and Tremellen, K. (2011) Impact of body mass index on seminal oxidative stress. *Andrologia* **43**, 121-128.

Valavanidis, A., Vlachogianni, T., and Fiotakis, C. (2009) 8-hydroxy-2' - deoxyguanosine (8-OHdG): A critical biomarker of oxidative stress and carcinogenesis. *J Environ Sci Health C Environ Carcinol Ecotoxicol Rev* **27**, 120-139.

Vicari, E., and Calogero, A.E. (2001) Effects of treatment with carnitines in infertile patients with prostatic-vesiculo-epididymitis. *Hum Reprod* **16**, 2338-2342.

Vicari, E., La Vignera, S., and Calogero, A.E. (2002) Antioxidant treatment with carnitines is effective in infertile patients with prostatovesiculoepididymitis and elevated seminal leukocyte concentrations after treatment with nonsteroidal anti-inflammatory compounds. *Fertil Steril* **78**, 1203-1208.

Xu, D.X., Shen, H.M., Zhu, Q.X., Chua, L., Wang, Q.N., Chia, S.E., and Ong, C.N. (2003) The associations among semen quality, oxidative DNA damage in human spermatozoa and concentrations of cadmium, lead and selenium in seminal plasma. *Mutat Res* **534**, 155-163.

Yang, Q., Zhao, F., Hu, L., Bai, R., Zhang, N., Yao, G., and Sun, Y. (2016) Effect of paternal overweight or obesity on IVF treatment outcomes and the possible mechanisms involved. *Sci Rep* **6**, 29787.

Zini, A., de Lamirande, E., and Gagnon, C. (1993) Reactive oxygen species in semen of infertile patients: levels of superoxide dismutase- and catalase-like activities in seminal plasma and spermatozoa. *Int J Androl* **16**, 183-188.

Zini, A., San Gabriel, M., and Baazeem, A. (2009) Antioxidants and sperm DNA damage: a clinical perspective. *J Assist Reprod Genet* **26**, 427-432.

Chapter 6 (conventional format)

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**Live Birth Rates from ART are Increased with Dietary Micronutrient  
Supplementation in Men from all BMI Categories**

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## 6.1 Chapter link

In Chapter 5, prospective data demonstrated that there is negative association between sperm oxidative DNA lesions and a successful pregnancy whereby the odds of achieving a pregnancy was reduced as sperm oxidative 8-OHdG lesion fluorescence increased. Further, the clinical data from the previous chapter showed that micronutrient supplement intake reduced sperm oxidative stress measures including sperm intracellular ROS and sperm oxidative DNA 8-OHdG lesions. However, the effectiveness of micronutrient supplement intake on pregnancy outcomes could not be concluded in the previous chapter due to small sample sizes and subsequent inadequate statistical power. Ideally, the golden standard of a multicentred double-blinded randomized controlled trial (RCT) could be considered to evaluate the effectiveness of micronutrient supplementation on ART outcomes and sperm oxidative stress measures in men undergoing fertility treatment however and unfortunately, a RCT is beyond the magnitude of a PhD project. Therefore, a retrospective study was conducted to address the inadequate statistical power from the previous chapter to establish the effectiveness of dietary micronutrient supplement intake on ART outcomes and ultimately live birth rates.

## 6.2 Abstract

Male obesity is strongly associated with subfertility and compromised assisted reproduction outcomes that include reduced live birth rates. It is apparent that obesity is increasingly associated with micronutrient deficiencies due to consumption of energy dense but nutrient deficient foods, creating the paradigm that sub-fertility in obese men could be in part related to micronutrient deficiencies. Micronutrient deficiencies can lead to impaired sperm quality but with micronutrient supplement intake, fertility treatment outcomes are improved in normal weight men. However, the effectiveness of this treatment in the growing population of obese men has not been established. This retrospective study investigated the effects of dietary micronutrient supplement intake on fertility treatment outcomes in non-smoking couples with a normal weight, overweight or obese male partner (aged <45 years) and a non-obese female partner (aged <38 years). Couples were stratified by the male partner's BMI and whether they were consuming dietary micronutrient supplements. Fertility treatment outcomes were assessed and included semen volume, sperm motility, sperm concentration, fertilization rates, days 4 and 5 embryo morphology, blastocyst inner cell mass (ICM) and trophectoderm (TE) quality, embryo utilization, biochemical and ongoing pregnancy rates, implantation rates, pregnancy loss, live birth rates and live birth outcomes including gestational length and gender of the infant and their weight at birth. The results demonstrated that micronutrient supplementation in obese men increased standard insemination fertilization rates and improved embryo morphology compared to obese men not consuming micronutrient supplements. Also, micronutrient supplement intake increased implantation, ongoing pregnancy and live birth rates in normal weight, overweight and obese men. Overall, these results demonstrate the effectiveness of dietary micronutrient supplementation on

assisted reproduction outcomes in men with varying BMIs, primarily alleviating the growing prevalence of obesity-induced male subfertility and achieving an ongoing pregnancy and live birth.

### 6.3 Introduction

Obesity is a global epidemic with 37% of men worldwide having an elevated body mass index (BMI) and are thus categorized as overweight or obese (Ng *et al.* 2014). According to the World Health Organization (WHO 2015), being overweight or obese increases the risk of developing cardiovascular diseases, diabetes, musculoskeletal disorders, some cancers (including endometrial, prostate, liver and kidney) ultimately resulting in more deaths than being underweight (WHO 2015). Recently, there is increasing awareness of an obesity and malnutrition paradox as the excessive dietary calorie intake commonly associated with obesity is linked to high rates of micronutrient deficiencies (Kaidar-Person *et al.* 2008). Parallel to the increase in the prevalence of obese men and their micronutrient deficiencies is a global decrease in male fertility and fecundity (Swan *et al.* 2000). Men with an increased BMI are more likely to be subfertile than normal weight men with reduced sperm motility and concentration (Hammoud *et al.* 2008; Hofny *et al.* 2010; Jensen *et al.* 2004), increased sperm DNA damage (Chavarro *et al.* 2010; Kort *et al.* 2006), reduced fertilization rates (Bakos *et al.* 2011; Merhi *et al.* 2013) and reduced pregnancy rates (Bakos *et al.* 2011; Colaci *et al.* 2012; Keltz *et al.* 2010; Merhi *et al.* 2013) leading to an overall reduced reproductive potential.

There are several proposed mechanisms responsible for the effects of obesity on male subfertility including reproductive hormone imbalances and subsequent dysregulation of the hypothalamic pituitary gonadal (HPG) axis (Jarow *et al.* 1993; Roth *et al.* 2008), elevated scrotal temperature (Hjollund *et al.* 2000; Jung and Schuppe 2007), and increased reactive oxygen species (ROS) and oxidative stress (McPherson and Lane 2015). The generation of ROS are a result of inflammation and/or oxidative stress, which

occurs when ROS concentrations exceed antioxidants levels (Agarwal *et al.* 2006a). Studies have shown a correlation between increased seminal ROS and an increased BMI (Tunc *et al.* 2011), which is associated with male infertility (Agarwal *et al.* 2006b).

Lifestyle changes to achieve a healthy balance of energy expenditure and ultimately weight loss are recommended for the clinical management of infertility in overweight and obese men however, maintaining a nutrient-rich diet and exercise plan can be difficult for individuals with demanding lifestyles. Therefore, another treatment strategy includes the dietary intake of micronutrient supplements to reduce sperm oxidative stress and subsequently, improve sperm parameters and reproductive potential (Gharagozloo and Aitken 2011; Tremellen *et al.* 2007). To date, the efficacy of dietary micronutrient supplementation on pregnancy outcomes has only been established in men with severe male factor infertility and shown to increase pregnancy rates in couples (Tremellen *et al.* 2007). Currently, the effectiveness of micronutrient supplementation has not been investigated in men who are overweight or obese.

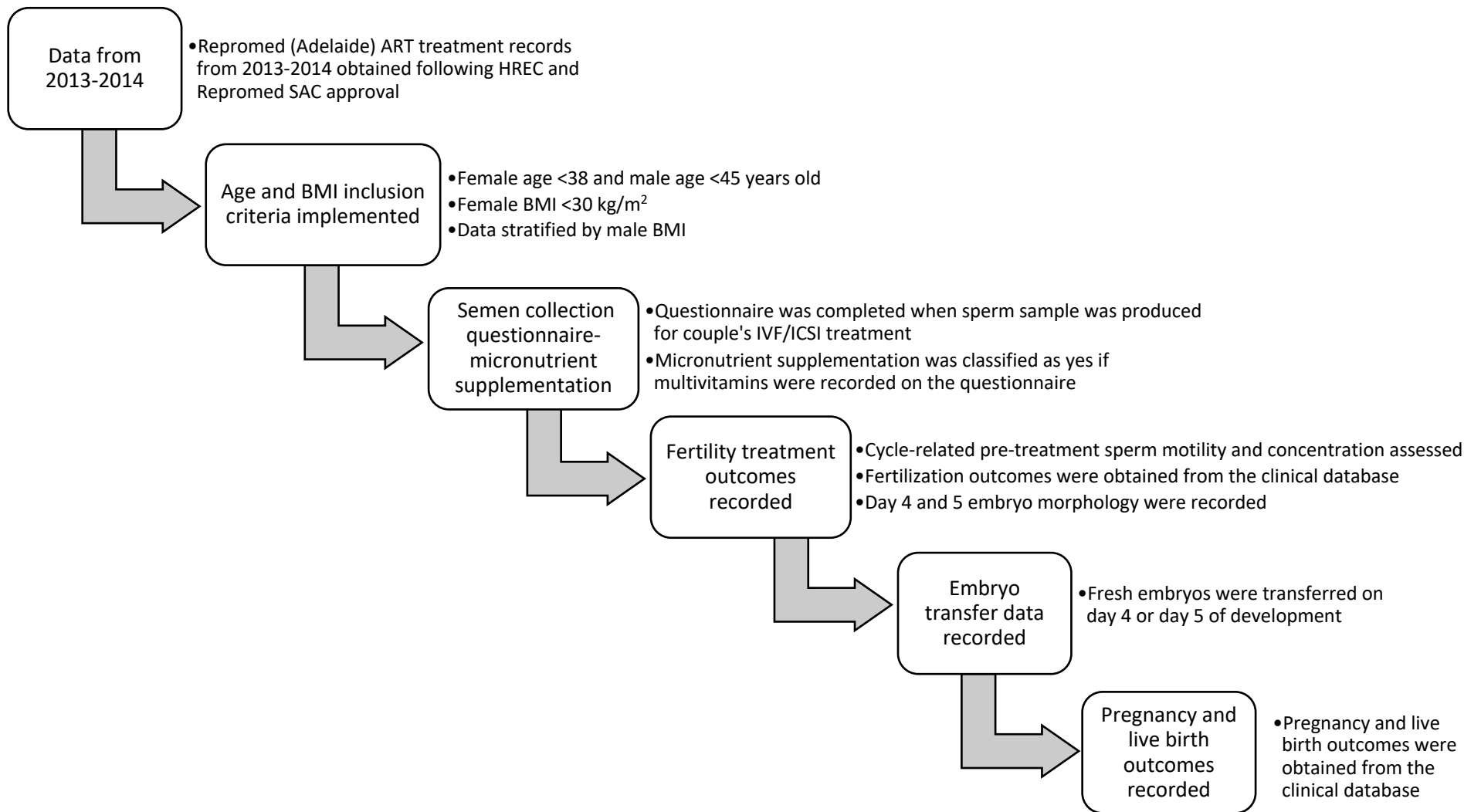
This retrospective study investigates the effectiveness of dietary micronutrient supplement intake by normal weight, overweight or obese male men on sperm motility, sperm concentration, and outcomes of their ART cycle including fertilization rates, embryo development, pregnancy outcomes and live birth rates.



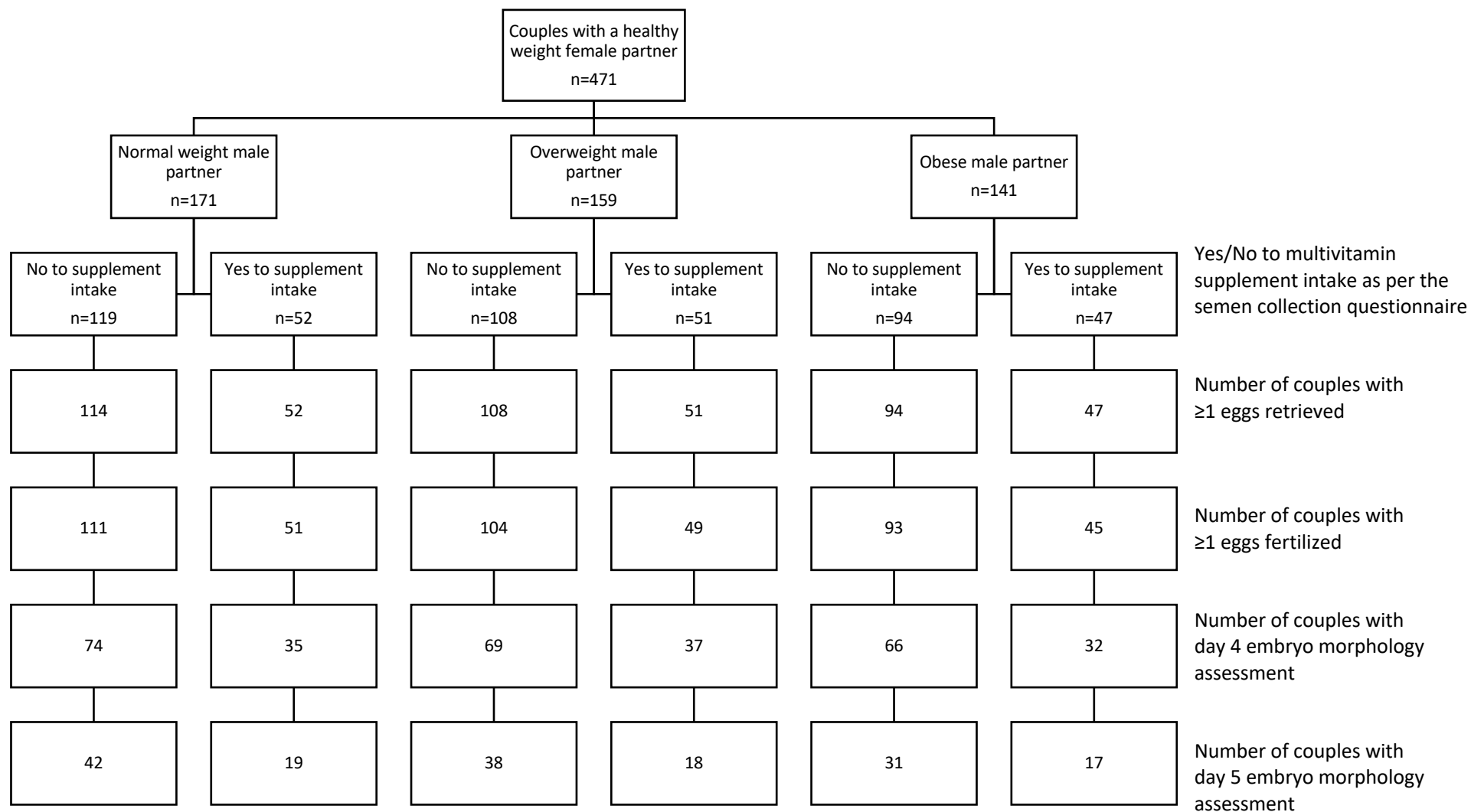
## **6.4 Materials and methods**

Please refer to Chapter 2, section 2.2 for materials and methods.

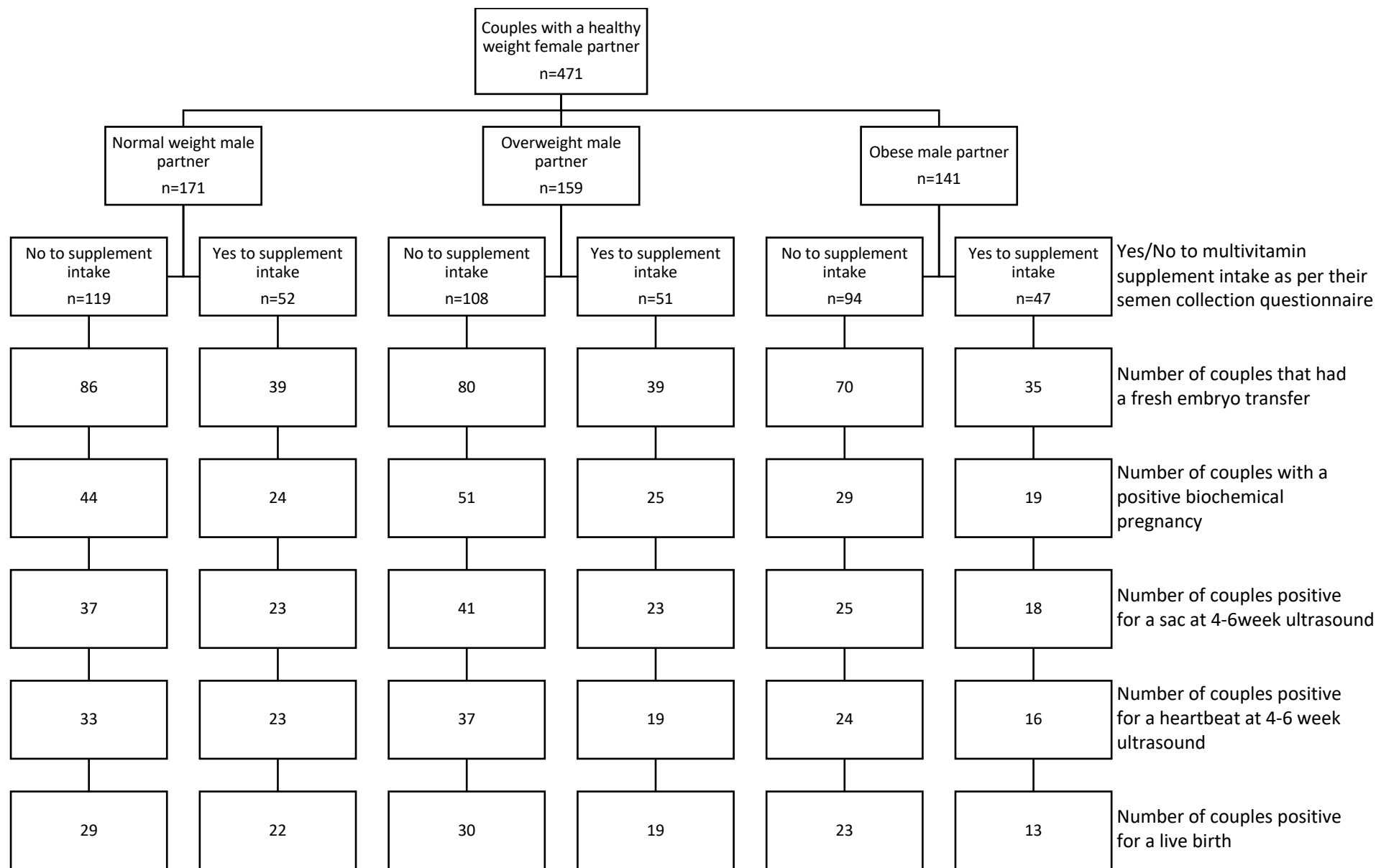
The experimental design is illustrated in Figure 6.1 and distribution of the number of participants at different stages of analysis are outlined in Figures 6.2 and 6.3.



**Figure 6.1** A flow chart outlining the experimental plan. The clinical database was used to implement the inclusion criteria, and to identify patient characteristics including the male partner's BMI and whether they were positive for multivitamin (micronutrient) supplement intake. Fertility treatment cycle outcomes were also obtained from their records including fertilization rates, embryo development, pregnancy and live birth outcomes.



**Figure 6.2** The distribution of patients based on the male partner's BMI and micronutrient supplementation stratification. The number of patients are outlined for each step of their fertility treatment cycle outcomes including  $\geq 1$  eggs collected at oocyte retrieval,  $\geq 1$  eggs fertilized, day 4 and day 5 embryo morphology assessments. Some embryos were day 4 on a Sunday and were not morphologically assessed. Day 5 embryos that were assessed on day 4 and had embryos that were frozen or transferred on day 4 were not included in day 5 analysis to eliminate skewing of data.



**Figure 6.3** The distribution of patients and pregnancy outcomes based on the male partner's BMI and micronutrient supplementation stratification. Some couples did not undergo a fresh embryo transfer due to ovarian hyperstimulation syndrome (OHSS) or pre-implantation genetic screening for aneuploidy (PGT-A) without a fresh embryo transfer.

## **6.5 Results**

### **6.5.1 Patient population**

A total of 471 couples were included in the final analysis stratified by male BMI (i) normal weight men (n=171), (ii) overweight men (n=159), (iii) obese men (n=141), and each BMI category was divided by whether they consumed dietary supplementation intake in a 3 x 2 experimental design as outlined in Figure 6.2 and Table 6.1. Also, the distributions of participants at different levels of data analysis are illustrated in Figures 6.2 and 6.3.

Patient population characteristics were analyzed and no differences in paternal age, maternal age, maternal BMI, number of previous ART cycles the couple had undergone, female AMH and baseline FSH levels were detected (Table 6.2). Further, there was no difference in most infertility etiologies (polycystic ovary syndrome (PCOS), endometriosis, tubal issues, semen defect, anovulation/oligomenorrhea and other conditions including genetic, immunological, premature ovarian dysfunction and recurrent miscarriage; Table 6.2) or the mean number of embryos transferred, proportion of double embryo transfers or the use of preimplantation genetic screening. However, the number and proportion of couples with unexplained and diminished ovarian reserve infertility diagnoses, respectively were different between the overweight men categories (with and without micronutrient supplements). Further, couples in the category of obese men with supplement intake had a reduced prevalence of diminished ovarian reserve as their infertility diagnosis compared to couples with an obese male partner.



**Table 6.1** Distribution of patients by male BMI and dietary micronutrient supplementation.

	<b>Normal weight</b>	<b>Normal weight + supplement intake</b>	<b>Overweight</b>	<b>Overweight + supplement intake</b>	<b>Obese</b>	<b>Obese + supplement intake</b>
Number of patients	119	52	108	51	94	47
Paternal BMI	23.3 ± 0.1	23.3 ± 0.2	27.1 ± 0.1	27.2 ± 0.2	32.7 ± 0.3	33.1 ± 0.4
Morbid obese (BMI > 40)					2 (2.1%)	1 (2.1%)

Data are expressed as mean ± SEM and as n values (%: proportion of patients) for morbid obese men.

**Table 6.2** Patient population characteristics.

	Normal weight		Overweight		Obese	
	No supplements	With supplements	No supplements	With supplements	No supplements	With supplements
Paternal age	33.7 ± 0.4	34.2 ± 0.6	34.7 ± 0.4	34.2 ± 0.6	34.9 ± 0.4	34.2 ± 0.6
Maternal age	33.6 ± 0.4	32.6 ± 0.5	32.5 ± 0.3	32.5 ± 0.4	32.5 ± 0.4	32.1 ± 0.6
Maternal BMI	23.2 ± 0.3	22.2 ± 0.4	23.8 ± 0.3	24.4 ± 0.4	23.8 ± 0.3	24.5 ± 0.5
Number of previous cycles	1.1 ± 0.1	1.1 ± 0.2	1.2 ± 0.2	0.9 ± 0.2	0.8 ± 0.1	0.6 ± 0.1
AMH (pmol/L)	33.5 ± 2.8	38.7 ± 4.0	29.6 ± 2.1	26.3 ± 3.6	35.3 ± 2.9	34.9 ± 4.4
Baseline FSH (mIU/mL)	7.7 ± 0.4	7.0 ± 0.3	7.0 ± 0.2	7.2 ± 0.5	7.1 ± 0.4	7.8 ± 0.4
Number of embryos transferred	1.1 ± 0.03	1.1 ± 0.04	1.1 ± 0.03	1.1 ± 0.05	1.1 ± 0.04	1.1 ± 0.05
Double embryo transfer	5 (4.2%)	2 (3.8%)	8 (7.4%)	4 (7.8%)	8 (8.5%)	3 (6.4%)
Preimplantation genetic screening (PGT-A) <sup>1</sup>	11 (9.2%)	10 (19.2%)	19 (17.6%)	6 (11.8%)	18 (19.1%)	6 (12.8%)
Female smoker	3 (2.5%)	0	0	1 (2.0%)	0	1 (2.1%)

Male medical condition/medication	13 (10.9%)	6 (11.5%)	15 (13.9%)	4 (7.8%)	6 (6.4%)	2 (4.3%)
Infertility factor- Female	63 (52.9%)	20 (38.5%)	63 (58.3%)	19 (37.3%)	54 (57.5%)	22 (46.8%)
Infertility factor- Male	22 (18.5%)	18 (34.6%)	24 (22.2%)	20 (39.2%)	13 (13.8%)	13 (27.7%)
Infertility factor- Female and Male	34 (28.6%)	14 (26.9%)	21 (19.5%)	12 (23.5%)	27 (28.7%)	12 (25.5%)
<b>Infertility Diagnosis</b>						
Unexplained	18 (15.1%) <sup>ab</sup>	13 (25.0%) <sup>ab</sup>	33 (30.6%) <sup>a</sup>	7 (13.7%) <sup>b</sup>	21(22.3%) <sup>ab</sup>	10(21.3%) <sup>ab</sup>
Polycystic ovary syndrome (PCOS)	11 (9.2%)	7 (13.5%)	8 (7.4%)	2 (3.9%)	9 (9.6%)	3 (6.4%)
Endometriosis	19 (16.0%)	3 (5.8%)	11 (10.2%)	3 (5.9%)	15 (16.0%)	10 (21.3%)
Tubal	12 (10.0%)	5 (9.6%)	10 (9.3%)	6 (11.8%)	4 (4.3%)	0
Semen defect	51 (42.9%)	30 (57.7%)	37 (34.3%)	31 (60.8%)	34 (36.2%)	24 (51.1%)
Anovulation/oligomenorrhea	15 (12.6%)	2 (3.8%)	6 (5.6%)	1 (2.0%)	10 (10.6%)	2 (4.3%)
Diminished ovarian reserve	8 (6.7%) <sup>ab</sup>	4 (7.7%) <sup>ab</sup>	10 (9.3%) <sup>a</sup>	10 (19.6%) <sup>b</sup>	14 (14.9%) <sup>b</sup>	3 (6.4%) <sup>a</sup>

Other (e.g. genetic, immunological, premature ovarian dysfunction, recurrent miscarriage, other)	20 (16.8%)	3 (5.8%)	14 (13.0%)	4 (7.8%)	17 (18.1%)	8 (17.0%)
<b>Type of micronutrient supplementation</b>						
Menevit	-	37 (71%)	-	36 (70%)	-	31 (66%)
Multivitamins	-	13 (25%)	-	11 (22%)	-	10 (21%)
Menevit and multivitamins	-	2 (4%)	-	4 (8%)	-	6 (13%)

<sup>1</sup> Preimplantation genetic screening for aneuploidy (PGT-A) patients did not undergo an embryo transfer.

Data are expressed as mean  $\pm$  SEM and as n values (%: proportion of patients) and analyzed by a 3x2 general linear model. Different letters denote significance at  $P < 0.05$  and an absence of superscript letters denotes no statistical difference.

### **6.5.2 Pre-treatment sperm assessment**

Semen volume was not different between BMI groups and was not affected by micronutrient supplement intake (Table 6.3). Sperm concentration and sperm motility from the neat sperm sample (before sperm preparation) were also not affected by either BMI or micronutrient supplement intake (Table 6.3).

**Table 6.3** The effects of male BMI and dietary micronutrient supplementation on cycle-related pre-treatment semen assessment.

	Normal weight		Overweight		Obese	
	No supplements	With supplements	No supplements	With supplements	No supplements	With supplements
Semen volume (ml)	3.3 ± 0.1	3.0 ± 0.2	3.4 ± 0.1	3.3 ± 0.2	3.3 ± 0.1	2.9 ± 0.2
Sperm concentration (10 <sup>6</sup> /ml)	58.4 ± 3.7	56.8 ± 6.2	65.9 ± 5.6	53.8 ± 5.9	44.9 ± 3.8	60.4 ± 7.2
Motility (%)	53.7 ± 1.5	52.6 ± 2.1	53.7 ± 1.4	49.8 ± 2.7	48.2 ± 1.9	54.9 ± 3.0

Data are expressed as mean ± SEM and analyzed by a 3x2 general linear model. No statistical differences were detected.

### **6.5.3 Fertilization rates**

Fertilization rates after intracytoplasmic sperm injection (ICSI) were not different with male BMI and were not affected by micronutrient supplement intake (Table 6.4). However, fertilization rates resulting from the standard insemination procedure (IVF) were reduced in obese men compared to that from overweight men (Table 6.4) and were increased with micronutrient supplementation in obese men compared to obese men not consuming micronutrient supplements (Table 6.4). Fertilization rates from both the combined ICSI and IVF methods were not different between BMI groups and were not affected by supplement intake (Table 6.4).

**Table 6.4** The effects of male BMI and dietary micronutrient supplementation on fertilization rates for intracytoplasmic sperm injection (ICSI), standard insemination (IVF) and both ICSI and IVF ART procedures.

	Normal weight		Overweight		Obese	
	No supplements	With supplements	No supplements	With supplements	No supplements	With supplements
<b>ICSI fertilization rates</b>						
Number of patients	103	51	90	49	86	44
Mean number of oocytes	7.2 ± 0.5	8.5 ± 0.8	7.9 ± 0.4	7.7 ± 1.4	7.8 ± 0.5	7.6 ± 0.6
Fertilization rate <sup>1</sup> (%)	73.7	74.0	72.1	69.2	72.1	70.8
Non-viable <sup>2</sup> (%)	6.9	6.7	7.5	6.7	9.8	6.9
Abnormal fertilization <sup>3</sup> (%)	4.9	5.8	6.0	6.3	4.6	3.9
Oocytes that failed to fertilize <sup>4</sup> (%)	14.7	13.6	14.4	17.8	13.5	18.5
<b>IVF fertilization rates</b>						
Number of patients	24	6	27	7	19	6



Mean number of oocytes	6.4 ± 0.7	8.7 ± 0.7	7.3 ± 0.5	8.1 ± 0.9	9.1 ± 0.6	7.8 ± 1.0
Fertilization rate <sup>1</sup> (%)	61.1 <sup>ab</sup>	52.0 <sup>a</sup>	67.4 <sup>b</sup>	59.6 <sup>ab</sup>	52.8 <sup>a</sup>	72.1 <sup>b</sup>
Non-viable <sup>2</sup> (%)	1.3	0.0	1.5	0.0	1.7	4.3
Abnormal fertilization <sup>3</sup> (%)	13.0	5.8	9.2	12.3	9.2	12.6
Oocytes that failed to fertilize <sup>4</sup> (%)	24.7 <sup>ab</sup>	42.4 <sup>a</sup>	21.9 <sup>ab</sup>	28.3 <sup>ab</sup>	36.2 <sup>a</sup>	10.5 <sup>b</sup>

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**ICSI and IVF fertilization rates**

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Number of patients	114	52	108	51	94	47
Mean number of oocytes	8.0 ± 0.6	9.3 ± 0.8	8.4 ± 0.5	8.5 ± 1.0	9.0 ± 0.5	8.1 ± 0.7
Fertilization rate <sup>1</sup> (%)	70.7	71.5	71.0	66.9	68.1	70.9
Non-viable <sup>2</sup> (%)	7.0	6.0	6.5	6.5	8.2	6.5
Abnormal fertilization <sup>3</sup> (%)	6.5	5.8	6.8	6.9	5.4	5.2
Oocytes that failed to fertilize <sup>4</sup> (%)	15.8	16.7	15.7	19.6	18.3	17.3

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<sup>1</sup> The presence of two pronuclei (2PN) and two polar bodies (2PB).

<sup>2</sup> Non-viable rate reflecting the proportion of lysed or degenerate eggs/embryos.

<sup>3</sup> Abnormal fertilization rate reflecting parthenogenetic oocytes or the presence of three or more pronuclei.

<sup>4</sup> Oocytes that lacked the presence of two pronuclei (2PN) and two polar bodies (2PB).

Data are expressed as n values, mean  $\pm$  SEM or a proportion of patients (%) and analyzed by a 3x2 general linear model weighted to the total number of oocytes per couple. Different letters denote significance at  $P < 0.05$  and an absence of superscript letters denotes no statistical difference.

#### **6.5.4 Embryo development, blastocyst quality and embryo utilization**

Embryo morphology was assessed on day 4 and day 5 of embryo development as these time points (post-embryonic genome activation) in embryo development may be affected by the quality of the male (paternal) genome. There was no effect of BMI on day 4 of embryo development however, micronutrient supplementation improved embryo morphological score in obese men (Table 6.5) with the percentage of grade 1 embryos (best quality) on day 4 (early blastocysts and compacted morulae) being increased with supplementation in obese men compared with obese men without micronutrient supplementation. The converse is observed with fewer poor grade embryos produced when obese men were consuming micronutrients compared to men who did not (Table 6.5). On day 5 of culture, BMI and micronutrient supplementation did not have an effect on blastocyst development rates or blastocyst ICM and TE quality (Tables 6.6 and 6.7). The percentage of transferred and/or frozen embryos (embryo utilization), was not affected by BMI but was increased with micronutrient supplementation in normal weight men compared to normal weight men not taking supplements (Table 6.8).

**Table 6.5** The effects of male BMI and dietary micronutrient supplementation on day 4 embryo morphology.

	Normal weight		Overweight		Obese	
	No supplements	With supplements	No supplements	With supplements	No supplements	With supplements
Number of patients	74	35	69	37	66	32
Mean number of embryos	5.3 ± 0.5	6.5 ± 0.8	6.1 ± 0.5	5.4 ± 0.5	6.4 ± 0.4	6.4 ± 0.8
Grade 1 <sup>1</sup> (%)	8.7 <sup>ab</sup>	9.8 <sup>ab</sup>	11.7 <sup>ab</sup>	6.9 <sup>a</sup>	6.4 <sup>a</sup>	14.4 <sup>b</sup>
Grade 2A <sup>2</sup> (%)	20.7	15.2	19.2	19.7	18.2	16.7
Grade 2B <sup>3</sup> (%)	18.5	17.4	18.1	16.6	13.8	18.0
Grade 3 <sup>4</sup> (%)	33.0 <sup>ab</sup>	28.1 <sup>a</sup>	23.7 <sup>ac</sup>	28.1 <sup>a</sup>	39.2 <sup>b</sup>	20.8 <sup>c</sup>
Grade 4 <sup>5</sup> (%)	18.9	29.3	26.8	29.3	22.6	29.9

<sup>1</sup> Early blastocysts or compacted morulae

<sup>2</sup> Partially compacted morulae (>70% compaction) with no anomalies or early blastocysts and compacted morulae with minimal anomalies

<sup>3</sup> Partially compacted morulae (<70% compaction) with no or minimal anomalies

<sup>4</sup> Embryos with ≥8 cells and no compaction with no or minimal anomalies

<sup>5</sup> Embryos with <8 cells or embryos with ≥8 cells, no compaction and above minimal anomalies

Data are expressed as n values, mean ± SEM or a proportion of fertilized embryos (%) and analyzed by a 3x2 general linear model weighted to the total number of fertilized embryos per couple. Different letters denote significance at P<0.05 and an absence of superscript letters denotes no statistical difference.

**Table 6.6** The effects of male BMI and dietary micronutrient supplementation on day 5 embryo morphology.

	Normal weight		Overweight		Obese	
	No supplements	With supplements	No supplements	With supplements	No supplements	With supplements
Number of patients	42	19	38	18	31	17
Mean number of embryos	6.4 ± 0.4	7.5 ± 0.5	6.4 ± 0.3	7.4 ± 0.7	5.1 ± 0.3	5.1 ± 0.5
<16c (%)	30.0	33.0	30.0	32.0	31.0	30.0
Morula (%)	16.0	12.0	20.0	16.0	19.0	12.0
Early blastocyst (%)	22.0	26.0	22.0	26.0	24.0	27.0
Full blastocyst (%)	13.0	14.0	16.0	15.0	15.0	16.0
Expanded blastocyst (%)	19.0	15.0	12.0	11.0	11.0	15.0
Full blastocyst (% total blastocysts)	33.0	34.0	33.0	25.0	32.0	23.0
Expanded blastocyst (% total blastocysts)	33.0	27.0	23.0	24.0	28.0	29.0

Data are expressed as n values, mean  $\pm$  SEM or a proportion of fertilized embryos/total blastocysts (%) and analyzed by a 3x2 general linear model weighted to the total number of fertilized embryos per couple. No statistical differences were detected.

**Table 6.7** The effects of male BMI and dietary micronutrient supplementation on blastocyst inner cell mass and trophectoderm grading.

	Normal weight		Overweight		Obese	
	No supplements	With supplements	No supplements	With supplements	No supplements	With supplements
Number of patients	27	13	25	7	16	10
Mean number of blastocysts	3.0 ± 0.4	3.7 ± 0.7	2.6 ± 0.3	3.3 ± 0.7	2.6 ± 0.4	1.5 ± 0.2
<b>Inner cell mass</b>						
Grade A <sup>1</sup> (%)	47.5	37.5	54.7	34.8	57.1	40.0
Grade B <sup>2</sup> (%)	45.0	60.4	37.5	58.2	33.4	46.7
Grade C <sup>3</sup> (%)	7.5	2.1	7.8	7.0	9.5	13.3
<b>Trophectoderm</b>						
Grade A <sup>4</sup> (%)	47.5	41.7	43.8	56.5	52.4	46.7
Grade B <sup>5</sup> (%)	42.5	52.1	46.9	43.5	38.1	33.3
Grade C <sup>6</sup> (%)	10.0	6.3	9.4	0.0	9.5	20.0

<sup>1</sup> Many tightly packed cells



<sup>2</sup> Several (loose) cells

<sup>3</sup> Very few cells

<sup>4</sup> Many cells forming a cohesive layer

<sup>5</sup> Few cells forming a loose epithelium

<sup>6</sup> Very few cells

Data are expressed as n values, mean  $\pm$  SEM or a proportion of blastocyst-staged embryos (%) and analyzed by a 3x2 general linear model weighted to the total number of blastocyst-staged embryos per couple. No statistical differences were detected.

**Table 6.8** The effects of male BMI and dietary micronutrient supplementation on embryo utilization.

	Normal weight		Overweight		Obese	
	No supplements	With supplements	No supplements	With supplements	No supplements	With supplements
Number of patients	111	51	104	49	93	45
Mean number of embryos	5.8 ± 0.4	6.3 ± 0.6	6.0 ± 0.4	5.2 ± 0.7	5.9 ± 0.4	5.3 ± 0.6
Transferred and/or frozen embryos (%)	51.6 <sup>a</sup>	62.2 <sup>b</sup>	52.2 <sup>a</sup>	57.6 <sup>ab</sup>	55.8 <sup>ab</sup>	56.1 <sup>ab</sup>

Data are expressed as n values, mean ± SEM or a proportion of fertilized embryos (%) and analyzed by a 3x2 general linear model weighted to the total number of fertilized embryos per couple. Different letters denote significance at P<0.05.

### **6.5.5 Pregnancy and live birth outcomes**

Pregnancy outcomes were analyzed as a proportion of couples that underwent an embryo transfer in addition to as a proportion of patients that underwent an oocyte retrieval (Table 6.9). In both analyses (per embryo transfer and per oocyte retrieval), implantation rates, measured by a positive sac upon ultrasound examination, were not affected by BMI but increased with micronutrient supplementation in all men regardless of their BMI category (Table 6.9). Ongoing pregnancy rates, measured by the presence of a fetal heart on ultrasound examination, were also increased with micronutrient supplementation but in normal weight and obese men compared to normal weight and obese men not taking supplements in both analyses (per embryo transfer and per oocyte retrieval, Table 6.9). Although not affected by BMI, live birth rates as a proportion of patients that underwent an embryo transfer were increased with micronutrient supplement intake in all BMI categories (Table 6.9). However, live birth rates as a proportion of patients that underwent an oocyte retrieval was only increased in normal weight men with micronutrient supplement intake compared to normal weight men not taking supplements (Table 6.9). Live birth outcomes were also assessed and there was no effect of BMI or micronutrient supplementation on gestational length, sex ratios and infant weight (Table 6.10).

**Table 6.9** The effects of male BMI and dietary micronutrient supplementation on pregnancy outcomes.

	Normal weight		Overweight		Obese	
	No supplements	With supplements	No supplements	With supplements	No supplements	With supplements
<b>Per embryo transfer</b>						
Number of patients	86	39	80	39	70	35
Biochemical pregnancy (%)	51.2	61.5	63.8	64.1	41.4	54.3
Implantation (%)	43.0 <sup>a</sup>	59.0 <sup>b</sup>	51.2 <sup>a</sup>	59.0 <sup>b</sup>	35.7 <sup>a</sup>	51.4 <sup>b</sup>
Ongoing pregnancy (%)	38.4 <sup>a</sup>	59.0 <sup>b</sup>	46.3 <sup>abc</sup>	48.7 <sup>abc</sup>	34.3 <sup>a</sup>	45.7 <sup>c</sup>
Pregnancy loss <sup>1</sup> (%)	15.9 <sup>a</sup>	4.2 <sup>b</sup>	19.6 <sup>a</sup>	8.0 <sup>b</sup>	17.2 <sup>a</sup>	5.3 <sup>b</sup>
Live birth (%)	33.7 <sup>a</sup>	56.4 <sup>b</sup>	37.5 <sup>c</sup>	48.7 <sup>b</sup>	32.9 <sup>a</sup>	37.1 <sup>c</sup>
<b>Per oocyte retrieval<sup>2</sup></b>						
Number of patients	102	41	89	44	76	40
Biochemical pregnancy (%)	43.1	58.5	56.2	59.1	38.2	47.5
Implantation (%)	37.3 <sup>ab</sup>	56.1 <sup>c</sup>	46.1 <sup>b</sup>	54.5 <sup>c</sup>	32.9 <sup>a</sup>	45.0 <sup>c</sup>

Ongoing pregnancy (%)	32.4 <sup>a</sup>	53.7 <sup>b</sup>	41.6 <sup>c</sup>	45.5 <sup>c</sup>	32.9 <sup>a</sup>	40.0 <sup>c</sup>
Pregnancy loss <sup>1</sup> (%)	34.1 <sup>a</sup>	8.3 <sup>b</sup>	34.0 <sup>a</sup>	26.9 <sup>a</sup>	20.7 <sup>a</sup>	31.6 <sup>a</sup>
Live birth (%)	28.4 <sup>a</sup>	53.7 <sup>b</sup>	37.1 <sup>ab</sup>	43.2 <sup>ab</sup>	30.3 <sup>a</sup>	32.5 <sup>ab</sup>

<sup>1</sup> Pregnancy loss was defined as a loss that occurred between a positive  $\beta$ -hCG test and live birth.

<sup>2</sup> Per oocyte retrieval rates referred to couples with the intention to treat and excluded couples with non-paternal factors that could contribute to a loss of pregnancy such as couples that did not undergo an embryo transfer due to ovarian hyperstimulation syndrome (OHSS) or PGT-A.

Data are expressed as n values and a proportion of embryo transfers and oocyte retrieval (%) and analyzed by a binary logistic regression. Different letters denote significance at  $P < 0.05$  and an absence of superscript letters denotes no statistical difference.

**Table 6.10** The effects of male BMI and dietary micronutrient supplementation on live birth outcomes.

	Normal weight		Overweight		Obese	
	No supplements	With supplements	No supplements	With supplements	No supplements	With supplements
Number of patients	29	22	30	19	23	13
Gestational length (weeks)	39.2 ± 0.6	38.0 ± 0.7	38.6 ± 0.5	38.3 ± 0.5	39.5 ± 0.6	38.4 ± 0.6
Male infant (%)	31.0	50.0	30.0	42.1	26.1	53.8
Female infant (%)	69.0	50.0	70.0	57.9	73.9	46.2
Male weight at birth (kg)	3.13 ± 0.21	3.05 ± 0.47	3.45 ± 0.30	3.22 ± 0.17	3.21 ± 0.15	3.53 ± 0.23
Female weight at birth (kg)	3.08 ± 0.15	3.22 ± 0.14	3.13 ± 0.31	3.00 ± 0.17	3.16 ± 0.30	3.10 ± 0.29

Data are expressed as n values, mean ± SEM or a proportion of live birth (%) and analyzed by a 3x2 general linear model for quantitative data and by a binary logistic regression for binomial data. No statistical differences were detected.

## 6.6 Discussion

This study illustrates that dietary micronutrient supplement intake was effective in improving fertility treatment outcomes in normal weight, overweight and obese men albeit to differing degrees. In obese men, micronutrient supplementation improved fertilization rates and embryo morphology, in addition to subsequent increases in implantation rates, and ongoing pregnancy and live birth rates.

Although previous investigations in men with unspecified BMIs have shown improvements in sperm motility with the intake of vitamin C (Dawson *et al.* 1990), vitamin E (Suleiman *et al.* 1996), zinc (Omu *et al.* 1998), a combination of selenium and vitamins A, C and E (Keskes-Ammar *et al.* 2003; Nozha *et al.* 2001; Scott *et al.* 1998), L-carnitine (Balercia *et al.* 2005; Biagiotti *et al.* 2003; Lenzi *et al.* 2004; Lombardo *et al.* 2002), and coenzyme Q10 (Balercia *et al.* 2009; Safarinejad 2009), the data from this study showed no effect of micronutrient supplementation on sperm motility and concentration. This is likely due to differences in study designs whereby the majority of previous studies were prospective randomised controlled trials whereas this study was retrospective in design. Also, the micronutrients and antioxidants used in previous studies were different as this study included supplements such as multivitamins.

Standard insemination fertilization rates were increased with micronutrient supplementation in obese men. The distinction of fertilization rates based on *standard insemination* method suggests that obesity has an impact on the sperm's capacity to undergo acrosomal reaction and sperm-oocyte binding which are improved by micronutrient supplementation. In contrast, ICSI fertilization rates were not altered

whereby these events are by-passed. This increase in standard insemination fertilization rates with micronutrient supplementation may be due to a reduction of ROS generation in sperm cells (Agarwal *et al.* 2014), which in turn reduces lipid peroxidation of the sperm plasma membrane and maintains membrane fluidity and integrity, which is crucial for successful sperm-oocyte binding, fusion and ultimately fertilization (Aitken *et al.* 1989; Geva *et al.* 1996).

On days 4 and 5 of culture, embryos were graded based on morphological characteristics. Micronutrient supplementation in obese men appeared to increase the rate of development post-compaction with more embryos reaching the morulae/blastocyst stage on day 4 of embryo culture, a time point post-embryonic genome activation (Braude *et al.* 1988). This may be explained by potential reductions in sperm oxidative stress and oxidative DNA damage with micronutrient supplementation, which reductions may persist to the subsequent embryo through the paternal pronucleus and result in timely embryo developmental competence (Conine *et al.* 2018). Although the effects of paternal BMI on day 4 embryo development have not been previously investigated (Campbell *et al.* 2015), blastocyst development is known to be reduced by an increasing BMI (Bakos *et al.* 2011). The improvements in embryo morphology with micronutrient supplement intake did not persist to day 5 of culture and did not have an effect on blastocyst development or quality, determined by the ICM size and shape and TE cell number. Embryo utilization, reflected by the percentage of transferred and frozen embryos, was increased with micronutrient supplement intake in normal weight men which has not been previously investigated. This may be due to reduced oxidative DNA lesions in sperm and improved DNA integrity in the male germline in the subsequent embryos from normal weight men (Takahashi 2012), which may increase the overall quality and utilisation of



embryos produced by normal weight men consuming micronutrient and anti-oxidative supplements.

Implantation rates, indicated by the presence of a fetal sac; ongoing pregnancy rates, indicated by the presence of a fetal heartbeat; and live birth rates were increased with micronutrient supplement intake in all men regardless of BMI. Previous investigations demonstrate that pregnancy rates are increased with the intake of vitamin E (Suleiman *et al.* 1996), L-carnitine and acetyl L-carnitine (Cavallini *et al.* 2004; Li *et al.* 2005) and Menevit, a combination of anti-oxidative micronutrients including vitamin C, zinc, vitamin E, folic acid, lycopene, selenium and garlic oil (Tremellen *et al.* 2007); which are all micronutrients and anti-oxidative agents used by men in this study. Further, some studies have reported increased live birth rates in men of unspecified BMI with the intake of vitamin E (Kessopoulou *et al.* 1995; Suleiman *et al.* 1996) and zinc (Omu *et al.* 1998). The range of micronutrient supplements taken among the men from this study were consistent and contain similar components and doses (Appendix, Table A9), which is in line with the recommendations of the couple's clinicians. Unfortunately, due to the nature of this retrospective study, it is impossible to determine the dietary and lifestyle habits of men from each BMI category and which micronutrient(s) specifically are associated with the observed increases in pregnancy and live birth rates. These factors are the main limitations of this study as for example, some men may have implemented dietary changes due to clinical advice to include more micronutrient intake from increased fruit and vegetable intake instead of consuming micronutrient supplements. Subsequently, these men would confound their allocated BMI category without supplementation group.

Micronutrient supplement intake has been reported as a supportive therapy for the treatment of infertility in men based on the protective effects of anti-oxidative micronutrients including, and not limited to, vitamins A, C and E and carnitine, and micronutrients such as zinc and selenium (Gharagozloo and Aitken 2011; Walczak-Jedrzejowska *et al.* 2013). Although the exact mechanisms involved in the protection of fertility treatment outcomes by anti-oxidative micronutrients are unknown, it is becoming apparent that sperm oxidative stress may be a critical and underlying factor for paternal DNA damage and defective male reproductive potential (Aitken *et al.* 2003; Trasler 2009). Sperm DNA integrity is impaired with oxidative stress and increased levels of intracellular ROS as oxidized residues within sperm DNA are formed, including 8-hydroxy-2'-deoxyguanosine (8-OHdG) (Noblanc *et al.* 2013). These oxidative DNA residues and adducts have destabilizing effects on the DNA backbone leading to DNA strand breaks (Mitchell *et al.* 2011). Also, oxidative DNA lesions cannot be repaired completely as sperm cells do not possess all the enzymes required for DNA base excision and repair (Bruner *et al.* 2000; Smith *et al.* 2013). Due to this limited DNA repair capacity, sperm DNA damage may be transmitted to the oocyte, if fertilization occurs, and may contribute to impaired offspring health (Frans *et al.* 2008; Ji *et al.* 1997; Lee *et al.* 2009; Sipos *et al.* 2004). Previous studies have investigated the effects of micronutrient supplement intake on sperm DNA fragmentation and found a combination of both vitamins C and E (Greco *et al.* 2005) and zinc, (without or in combination with vitamins C and E) (Omu *et al.* 2008) supplementation reduced sperm DNA fragmentation. Also, a study investigating the effects of increasing and reducing the dose of vitamin C supplement intake found a negative association between vitamin C intake and oxidative DNA damage in sperm (Fraga *et al.* 1991). Therefore, a potential mechanism for the observed improvement in fertilization rates, embryo developmental

competence and live birth rates with micronutrient supplementation may be reduced sperm oxidative stress and DNA damage. This reduced damage from the male germ line may persist into the subsequent embryo and ultimately lessen mutational load in the embryo's genome to produce a healthy fetus (Takahashi 2012; Trasler 2009).

Interestingly, this study demonstrates that micronutrient supplement intake in all men benefits the couple's fertility treatment outcomes and ultimately pregnancy and live birth rates. Overall, these novel findings elucidate the effectiveness of micronutrient supplementation on fertility treatment outcomes in men with an increased BMI, which is vital given the concurrent obesity epidemic and prevalence of infertility among men with an increased BMI. Future research into the effects of micronutrient supplement intake and its anti-oxidative impact on the subcellular components of sperm (intracellular ROS and oxidative DNA damage) and their association with pregnancy outcomes are warranted. These future findings could clarify potential mechanisms for the mode of action of micronutrient supplement intake on sperm function, fertilization and pregnancy outcomes.

## 6.7 References

Agarwal, A., Prabakaran, S., and Allamaneni, S. (2006a) What an andrologist/urologist should know about free radicals and why. *Urology* **67**, 2-8.

Agarwal, A., Sharma, R.K., Nallella, K.P., Thomas, A.J., Jr., Alvarez, J.G., and Sikka, S.C. (2006b) Reactive oxygen species as an independent marker of male factor infertility. *Fertil Steril* **86**, 878-885.

Agarwal, A., Virk, G., Ong, C., and du Plessis, S.S. (2014) Effect of oxidative stress on male reproduction. *World J Mens Health* **32**, 1-17.

Aitken, R.J., Baker, M.A., and Sawyer, D. (2003) Oxidative stress in the male germ line and its role in the aetiology of male infertility and genetic disease. *Reprod Biomed Online* **7**, 65-70.

Aitken, R.J., Clarkson, J.S., and Fishel, S. (1989) Generation of reactive oxygen species, lipid peroxidation, and human sperm function. *Biol Reprod* **41**, 183-197.

Bakos, H.W., Henshaw, R.C., Mitchell, M., and Lane, M. (2011) Paternal body mass index is associated with decreased blastocyst development and reduced live birth rates following assisted reproductive technology. *Fertil Steril* **95**, 1700-1704.

Balercia, G., Buldreghini, E., Vignini, A., Tiano, L., Paggi, F., Amoroso, S., Ricciardo-Lamonica, G., Boscaro, M., Lenzi, A., and Littarru, G. (2009) Coenzyme Q10 treatment in infertile men with idiopathic asthenozoospermia: a placebo-controlled, double-blind randomized trial. *Fertil Steril* **91**, 1785-1792.

Balercia, G., Regoli, F., Armeni, T., Koverech, A., Mantero, F., and Boscaro, M. (2005) Placebo-controlled double-blind randomized trial on the use of L-carnitine, L-acetylcarnitine, or combined L-carnitine and L-acetylcarnitine in men with idiopathic asthenozoospermia. *Fertil Steril* **84**, 662-671.

Biagiotti, G., Cavallini, G., Modenini, F., Vitali, G., Magli, C., and Ferraretti, A. (2003) Prostaglandins pulsed down-regulation enhances carnitine therapy performance in severe idiopathic oligoasthenospermia. *Human Reproduction* **18**, 202-202. [In English]

Braude, P., Bolton, V., and Moore, S. (1988) Human gene expression first occurs between the four- and eight-cell stages of preimplantation development. *Nature* **332**, 459-461.

Bruner, S.D., Norman, D.P., and Verdine, G.L. (2000) Structural basis for recognition and repair of the endogenous mutagen 8-oxoguanine in DNA. *Nature* **403**, 859-866.

Campbell, J.M., Lane, M., Owens, J.A., and Bakos, H.W. (2015) Paternal obesity negatively affects male fertility and assisted reproduction outcomes: a systematic review and meta-analysis. *Reproductive BioMedicine Online* **31**, 593-604.

Cavallini, G., Ferraretti, A.P., Gianaroli, L., Biagiotti, G., and Vitali, G. (2004) Cinnoxicam and L-carnitine/acetyl-L-carnitine treatment for idiopathic and varicocele-associated oligoasthenospermia. *J Androl* **25**, 761-770; discussion 771-762.

Chavarro, J.E., Toth, T.L., Wright, D.L., Meeker, J.D., and Hauser, R. (2010) Body mass index in relation to semen quality, sperm DNA integrity, and serum reproductive hormone levels among men attending an infertility clinic. *Fertil Steril* **93**, 2222-2231.

Colaci, D.S., Afeiche, M., Gaskins, A.J., Wright, D.L., Toth, T.L., Tanrikut, C., Hauser, R., and Chavarro, J.E. (2012) Men's body mass index in relation to embryo quality and clinical outcomes in couples undergoing in vitro fertilization. *Fertil Steril* **98**, 1193-1199 e1191.

Conine, C.C., Sun, F., Song, L., Rivera-Perez, J.A., and Rando, O.J. (2018) Small RNAs Gained during Epididymal Transit of Sperm Are Essential for Embryonic Development in Mice. *Dev Cell*.

Dawson, E.B., Harris, W.A., and Powell, L.C. (1990) Relationship between ascorbic acid and male fertility. *World Rev Nutr Diet* **62**, 1-26.

Fraga, C.G., Motchnik, P.A., Shigenaga, M.K., Helbock, H.J., Jacob, R.A., and Ames, B.N. (1991) Ascorbic acid protects against endogenous oxidative DNA damage in human sperm. *Proc Natl Acad Sci U S A* **88**, 11003-11006.

Frans, E.M., Sandin, S., Reichenberg, A., Lichtenstein, P., Langstrom, N., and Hultman, C.M. (2008) Advancing paternal age and bipolar disorder. *Arch Gen Psychiatry* **65**, 1034-1040.

Geva, E., Bartoov, B., Zabludovsky, N., Lessing, J.B., Lerner-Geva, L., and Amit, A. (1996) The effect of antioxidant treatment on human spermatozoa and fertilization rate in an in vitro fertilization program. *Fertil Steril* **66**, 430-434.

Gharagozloo, P., and Aitken, R.J. (2011) The role of sperm oxidative stress in male infertility and the significance of oral antioxidant therapy. *Hum Reprod* **26**, 1628-1640.

Greco, E., Iacobelli, M., Rienzi, L., Ubaldi, F., Ferrero, S., and Tesarik, J. (2005) Reduction of the incidence of sperm DNA fragmentation by oral antioxidant treatment. *J Androl* **26**, 349-353.

Hammoud, A.O., Wilde, N., Gibson, M., Parks, A., Carrell, D.T., and Meikle, A.W. (2008) Male obesity and alteration in sperm parameters. *Fertil Steril* **90**, 2222-2225.

Hjollund, N.H., Bonde, J.P., Jensen, T.K., and Olsen, J. (2000) Diurnal scrotal skin temperature and semen quality. The Danish First Pregnancy Planner Study Team. *Int J Androl* **23**, 309-318.

- Hofny, E.R., Ali, M.E., Abdel-Hafez, H.Z., Kamal Eel, D., Mohamed, E.E., Abd El-Azeem, H.G., and Mostafa, T. (2010) Semen parameters and hormonal profile in obese fertile and infertile males. *Fertil Steril* **94**, 581-584.
- Jarow, J.P., Kirkland, J., Koritnik, D.R., and Cefalu, W.T. (1993) Effect of obesity and fertility status on sex steroid levels in men. *Urology* **42**, 171-174. [In eng]
- Jensen, T.K., Andersson, A.M., Jorgensen, N., Andersen, A.G., Carlsen, E., Petersen, J.H., and Skakkebaek, N.E. (2004) Body mass index in relation to semen quality and reproductive hormones among 1,558 Danish men. *Fertil Steril* **82**, 863-870.
- Ji, B.T., Shu, X.O., Linet, M.S., Zheng, W., Wacholder, S., Gao, Y.T., Ying, D.M., and Jin, F. (1997) Paternal cigarette smoking and the risk of childhood cancer among offspring of nonsmoking mothers. *J Natl Cancer Inst* **89**, 238-244.
- Jung, A., and Schuppe, H.C. (2007) Influence of genital heat stress on semen quality in humans. *Andrologia* **39**, 203-215.
- Kaidar-Person, O., Person, B., Szomstein, S., and Rosenthal, R.J. (2008) Nutritional deficiencies in morbidly obese patients: a new form of malnutrition? Part A: vitamins. *Obes Surg* **18**, 870-876.
- Keltz, J., Zapantis, A., Jindal, S.K., Lieman, H.J., Santoro, N., and Polotsky, A.J. (2010) Overweight men: clinical pregnancy after ART is decreased in IVF but not in ICSI cycles. *J Assist Reprod Genet* **27**, 539-544. [In eng]
- Keskes-Ammar, L., Feki-Chakroun, N., Rebai, T., Sahnoun, Z., Ghazzi, H., Hammami, S., Zghal, K., Fki, H., Damak, J., and Bahloul, A. (2003) Sperm oxidative stress and the effect of an oral vitamin E and selenium supplement on semen quality in infertile men. *Arch Androl* **49**, 83-94.
- Kessopoulou, E., Powers, H.J., Sharma, K.K., Pearson, M.J., Russell, J.M., Cooke, I.D., and Barratt, C.L. (1995) A double-blind randomized placebo cross-over controlled trial using the antioxidant vitamin E to treat reactive oxygen species associated male infertility. *Fertil Steril* **64**, 825-831.
- Kort, H.I., Massey, J.B., Elsner, C.W., Mitchell-Leef, D., Shapiro, D.B., Witt, M.A., and Roudebush, W.E. (2006) Impact of body mass index values on sperm quantity and quality. *J Androl* **27**, 450-452. [In eng]
- Lee, K.M., Ward, M.H., Han, S., Ahn, H.S., Kang, H.J., Choi, H.S., Shin, H.Y., Koo, H.H., Seo, J.J., Choi, J.E., *et al.* (2009) Paternal smoking, genetic polymorphisms in CYP1A1 and childhood leukemia risk. *Leuk Res* **33**, 250-258.
- Lenzi, A., Sgro, P., Salacone, P., Paoli, D., Gilio, B., Lombardo, F., Santulli, M., Agarwal, A., and Gandini, L. (2004) A placebo-controlled double-blind randomized trial of the

use of combined l-carnitine and l-acetyl-carnitine treatment in men with asthenozoospermia. *Fertil Steril* **81**, 1578-1584.

Li, Z., Chen, G.W., Shang, X.J., Bai, W.J., Han, Y.F., Chen, B., Teng, X.M., Meng, F.H., Zhang, B., Chen, D.N., *et al.* (2005) [A controlled randomized trial of the use of combined L-carnitine and acetyl-L-carnitine treatment in men with oligoasthenozoospermia]. *Zhonghua Nan Ke Xue* **11**, 761-764.

Lombardo, F., Gandini, L., Agarwal, A., Sgro, P., Dondero, F., and Lenzi, A. (2002) A prospective double blind placebo controlled cross over trial of carnitine therapy in selected cases of male infertility. *Fertility and Sterility* **78**, S68-S69. [In English]

McPherson, N.O., and Lane, M. (2015) Male obesity and subfertility, is it really about increased adiposity? *Asian J Androl* **17**, 450-458.

Merhi, Z.O., Keltz, J., Zapantis, A., Younger, J., Berger, D., Lieman, H.J., Jindal, S.K., and Polotsky, A.J. (2013) Male Adiposity Impairs Clinical Pregnancy Rate by In Vitro Fertilization Without Affecting Day 3 Embryo Quality. *Obesity* **21**, 1608-1612. [In English]

Mitchell, L.A., De Iuliis, G.N., and Aitken, R.J. (2011) The TUNEL assay consistently underestimates DNA damage in human spermatozoa and is influenced by DNA compaction and cell vitality: development of an improved methodology. *Int J Androl* **34**, 2-13.

Ng, M., Fleming, T., Robinson, M., Thomson, B., Graetz, N., Margono, C., Mullany, E.C., Biryukov, S., Abbafati, C., Abera, S.F., *et al.* (2014) Global, regional, and national prevalence of overweight and obesity in children and adults during 1980-2013: a systematic analysis for the Global Burden of Disease Study 2013. *Lancet* **384**, 766-781.

Noblanc, A., Damon-Soubeyrand, C., Karrich, B., Henry-Berger, J., Cadet, R., Saez, F., Guiton, R., Janny, L., Pons-Rejraji, H., Alvarez, J.G., *et al.* (2013) DNA oxidative damage in mammalian spermatozoa: where and why is the male nucleus affected? *Free Radic Biol Med* **65**, 719-723.

Nozha, C.F., Leila, A.K., Zouhir, S., Hanen, G., Khled, Z., and Tarek, R. (2001) Oxidative stress and male infertility: comparative study of combined vitamin E/selenium treatment versus vitamin B. *Human Reproduction* **16**, 111-112. [In English]

Omu, A.E., Al-Azemi, M.K., Kehinde, E.O., Anim, J.T., Oriowo, M.A., and Mathew, T.C. (2008) Indications of the mechanisms involved in improved sperm parameters by zinc therapy. *Med Princ Pract* **17**, 108-116.

Omu, A.E., Dashti, H., and Al-Othman, S. (1998) Treatment of asthenozoospermia with zinc sulphate: andrological, immunological and obstetric outcome. *Eur J Obstet Gynecol Reprod Biol* **79**, 179-184.

- Roth, M.Y., Amory, J.K., and Page, S.T. (2008) Treatment of male infertility secondary to morbid obesity. *Nat Clin Pract Endocrinol Metab* **4**, 415-419.
- Safarinejad, M.R. (2009) Efficacy of coenzyme Q10 on semen parameters, sperm function and reproductive hormones in infertile men. *J Urol* **182**, 237-248.
- Scott, R., MacPherson, A., Yates, R.W., Hussain, B., and Dixon, J. (1998) The effect of oral selenium supplementation on human sperm motility. *Br J Urol* **82**, 76-80.
- Sipos, A., Rasmussen, F., Harrison, G., Tynelius, P., Lewis, G., Leon, D.A., and Gunnell, D. (2004) Paternal age and schizophrenia: a population based cohort study. *BMJ* **329**, 1070.
- Smith, T.B., Dun, M.D., Smith, N.D., Curry, B.J., Connaughton, H.S., and Aitken, R.J. (2013) The presence of a truncated base excision repair pathway in human spermatozoa that is mediated by OGG1. *J Cell Sci* **126**, 1488-1497.
- Suleiman, S.A., Ali, M.E., Zaki, Z.M., el-Malik, E.M., and Nasr, M.A. (1996) Lipid peroxidation and human sperm motility: protective role of vitamin E. *J Androl* **17**, 530-537.
- Swan, S.H., Elkin, E.P., and Fenster, L. (2000) The question of declining sperm density revisited: an analysis of 101 studies published 1934-1996. *Environ Health Perspect* **108**, 961-966.
- Takahashi, M. (2012) Oxidative stress and redox regulation on in vitro development of mammalian embryos. *J Reprod Dev* **58**, 1-9.
- Trasler, J.M. (2009) Epigenetics in spermatogenesis. *Mol Cell Endocrinol* **306**, 33-36.
- Tremellen, K., Miari, G., Froiland, D., and Thompson, J. (2007) A randomised control trial examining the effect of an antioxidant (Menevit) on pregnancy outcome during IVF-ICSI treatment. *Aust N Z J Obstet Gynaecol* **47**, 216-221.
- Tunc, O., Bakos, H.W., and Tremellen, K. (2011) Impact of body mass index on seminal oxidative stress. *Andrologia* **43**, 121-128.
- Walczak-Jedrzejowska, R., Wolski, J.K., and Slowikowska-Hilczler, J. (2013) The role of oxidative stress and antioxidants in male fertility. *Cent European J Urol* **66**, 60-67.
- WHO (2015) Obesity and Overweight, Fact Sheet no. 311, Body Mass Index Classifications. World Health Organization.



## Chapter 7

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### **The Role of Sperm Oxidative Stress in Male Obesity and the Effectiveness of Dietary Micronutrient Supplementation – General Discussion**

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## 7.1. Introduction

The burgeoning health problem of obesity has continued despite several government initiatives with almost 2 in 3 Australian adults now classified as overweight or obese (AIHW 2016). In men, it is estimated that the odds of infertility increase 10% for every 9kg a man is overweight (Sallmen *et al.* 2006). Given the increasing evidence in animal models and human cohorts regarding male obesity at preconception and its association with poorer health outcomes in children, it is vital that treatments are assessed for their effectiveness in treating obesity-induced subfertility and the transmission of impaired health to the next generation.

Male obesity is associated with elevated sperm oxidative stress in both humans and in animal models of HFD-induced obesity (Palmer *et al.* 2012; Tunc *et al.* 2011). Sperm oxidative stress occurs when there is an imbalance between reactive oxygen species (ROS) production (elevated) and cellular enzymatic and non-enzymatic antioxidant activity (reduced). Specifically, male obesity is associated with increased sperm intracellular ROS and increased sperm DNA damage and fragmentation which in turn, is linked to poor fertilization rates, delayed embryo development, poor pregnancy rates, increased birth defects and impaired offspring health (Aitken and Curry 2011; Bakos *et al.* 2011; Bakos *et al.* 2010; Fullston *et al.* 2012; McPherson *et al.* 2013). Sperm DNA integrity is compromised by the oxidative attack of intracellular ROS resulting in not just DNA breaks, but importantly DNA base modifications and mutations that can be transmitted to potential offspring via the male germ line (Soubry *et al.* 2014). Therefore, the regulation of sperm ROS levels are vital not only for male fertility, but potentially for the lasting health consequences of his children.

Meta-analyses have demonstrated the efficacy of oral micronutrient and antioxidant supplement intake for the improvement of sperm quality, fertilization rates and pregnancy rates, in subfertile and infertile men with unspecified BMIs (Barratt *et al.* 2017; Gharagozloo and Aitken 2011; Ross *et al.* 2010; Showell *et al.* 2011; Zini *et al.* 2009). However, the effectiveness of micronutrient supplement intake in the growing population of obese males and the aetiology of obesity-induced subfertility and sperm oxidative stress has not been investigated until now. Therefore, it was hypothesized that dietary micronutrient supplementation in obese males improves fertilization rates, embryo development, implantation and pregnancy rates by reducing sperm oxidative stress and oxidative DNA damage.

## 7.2. Male obesity and sperm oxidative stress

One of the primary mechanisms linking male obesity to subfertility is sperm oxidative stress (Aitken *et al.* 2014). This is because sperm cells are extremely susceptible to oxidative stress due to their limited cytoplasmic capacity for antioxidants and antioxidant enzymes, as the bulk of the cytoplasm is shed during their maturation process during spermatogenesis (Rengan *et al.* 2012). Sources of endogenous ROS in semen and sperm include leukocytes (neutrophils and macrophages) (Aitken and Fisher 1994; Aitken *et al.* 1995), which have been found to be elevated in semen from obese men (Tunc *et al.* 2011). Also, excess residual cytoplasm in morphologically abnormal sperm (Gomez *et al.* 1996; Sakkas *et al.* 2003) along with mitochondrial electron leakage metabolism (Koppers *et al.* 2008; Vernet *et al.* 2001) have been shown to contribute to the excessive production of ROS.

Similar to the human phenotype, HFD-induced obese mice display increased sperm oxidative stress, characterised by elevated sperm intracellular ROS (Fullston *et al.* 2012; Palmer *et al.* 2012), which was also shown in data from Chapters 3 and 4 where sperm intracellular ROS was increased in HFD-induced obese mice compared to CD-fed mice. Furthermore, these chapters extended these findings and demonstrated that 8-hydroxy-deoxy-guanosine (8-OHdG) immunofluorescence, which detected oxidized guanine base adducts, was elevated in both mature sperm and in testicular spermatogenic sperm from HFD-fed mice. 8-OHdG is an abnormal DNA lesion that can impact transcription and interfere with methylation marks (Shen *et al.* 1999; Valavanidis *et al.* 2009). These findings suggest that at least a proportion of this damage is initiated in the testes in which developing germ cells, particularly spermatogonia and elongating spermatids, from diet-

induced obese mice had elevated 8-OHdG fluorescence (Chapter 3). One of the proposed mechanisms for these findings include disruption of the blood-testes barrier (BTB) and the infiltration of serum cytokines, leukocytes and ROS through seminal vesicles and into the testicular tissue (La Vignera *et al.* 2012). Another proposed mechanism is the increased gonadal adipose tissue accumulation, associated scrotal heat and increased serum levels of cholesterol, glucose, HDLs and triglycerides observed in HFD-fed mice (McPherson and Lane 2015), which may contribute to the atrophy of seminiferous tubules from the testes and impair the BTB permeability, as observed with diet-induced obesity (Fan *et al.* 2015). Further studies examining BTB permeability may explain which vascular/serum molecules, such as ROS and any of the other increased serum levels (cholesterol, glucose, triglycerides) observed in Chapter 3, may pass through the perturbed BTB resulting in 8-OHdG lesions of testicular spermatogenic sperm and reduced protection of germ cells from harmful products from the vascular environment (Haverfield *et al.* 2014).

The duration of a HFD intake also had an impact on the degree of sperm intracellular ROS production and 8-OHdG lesions. Male mice that consumed a HFD for 20 weeks (Chapter 3) produced sperm with more intracellular ROS than those that consumed a HFD for 15 weeks (Chapter 3) or 11 weeks (Chapter 4). Furthermore, sperm 8-OHdG lesions were greater in male mice fed a HFD for 20 or 15 weeks (Chapter 3) than those fed a HFD for 11 weeks (Chapter 4). Although all 3 HFD intake durations increased adiposity, there was also an increase in serum metabolites as the duration of a HFD intake increased. Male mice that consumed a HFD for 20 weeks (Chapter 3) had increased serum cholesterol, glucose, high density lipoproteins (HDLs) and triglycerides whereas mice fed a HFD for 15 weeks (Chapter 3) or 11 weeks (Chapter 4) displayed increased serum

cholesterol and HDLs only. The additional increases in glucose and triglycerides with the 20 week intake of a HFD may be due to a pre-diabetic state, insulin resistance and impaired triglyceride metabolism in these mice (Burgeiro *et al.* 2017). Overall, these differences in the degrees of damage to sperm intracellular ROS and sperm oxidative DNA lesions as the duration of HFD intake increased suggest that adiposity is not the only factor contributing to their increase in sperm oxidative stress and that their metabolic profile may also play an important role.

The increased sperm intracellular ROS and sperm 8-OHdG lesions observed from HFD-induced obese mice (Chapters 3 and 4) were similar to that seen in sperm from overweight and obese men (Chapter 5). Sperm oxidative stress measures were examined in men and showed that increased BMI was associated with increased sperm intracellular ROS. These findings are consistent with previous studies that demonstrated increased ROS content in semen (Tunc *et al.* 2011) and sperm (Yang *et al.* 2016) from men with an elevated BMI. In addition to elevated sperm intracellular ROS in obese men, sperm 8-OHdG fluorescence increased as male BMI increased, indicating a concomitant elevation of oxidative DNA lesions in sperm from overweight and obese men. There is prior evidence for a strong association between intracellular ROS and oxidative DNA modifications including 8-OHdG formation in sperm with chemically-induced oxidative stress (Aitken *et al.* 1998; De Iuliis *et al.* 2009; Oger *et al.* 2003) and in sperm from men undergoing ART treatment (Oger *et al.* 2003). The findings from Chapter 5 that demonstrated a positive association between sperm intracellular ROS (both H<sub>2</sub>DCFDA and MSR probes) and 8-OHdG fluorescence are consistent with previous studies confirming a biochemical link whereby sperm intracellular ROS lead to the formation of 8-OHdG DNA base adducts (De Iuliis *et al.* 2009; Noblanc *et al.* 2013). Given the

positive relationship between intracellular ROS generation and the formation of oxidative DNA lesions, as observed in Chapter 5 and consistent with previous literature, future studies could investigate the mutations produced by the modifications of DNA bases induced by ROS. These mutations may include genetic and epigenetic mutations that may be passed to offspring and potentially lead to compromised long-term health.

### 7.3. Sperm oxidative stress, embryo development and pregnancy

Sperm have a limited capacity to repair DNA adducts and lesions as sperm cells contain only one of the enzymes involved in the base excision repair (BER) pathway, 8-oxoguanine DNA glycosylase (OGG1) to repair DNA lesions by excising lesions from the DNA backbone (Bruner *et al.* 2000; Smith *et al.* 2013). The OGG1 enzyme excises base adducts such as 8-OHdG however, the nucleotide is not replaced due to the lack of the remaining BER enzymes (APE1, XRCC1) (Smith *et al.* 2013). Although 8-OHdG base mutations may compromise sperm DNA integrity and sperm cells are not able to completely repair 8-OHdG lesions, the zygote is able to excise 8-OHdG adducts and ultimately repair some of the oxidative DNA damage post-fertilization (Lord and Aitken 2015). However, the repair of this damage in the zygote is limited and cannot completely eliminate and excise all 8-OHdG mutagenic adducts, which is elevated in obese and subfertile males (Lord and Aitken 2015). Sperm 8-OHdG lesions are associated with reduced blastocyst formation rates (Meseguer *et al.* 2008) and asymmetry and fragmentation in the embryo (Meseguer *et al.* 2006). Further, sperm 8-OHdG is associated with perturbed offspring health including cancers and neurological disorders in children (Aitken *et al.* 2014; Lee *et al.* 2009). These associations may be explained by the findings observed in Chapter 4, which revealed that increased 8-OHdG immunofluorescence in mature sperm persisted to the paternal pronucleus in the subsequent embryo. This demonstration of inheritance of the 8-OHdG into the preimplantation embryo may suggest that this could be a part of the mechanism for the transmission of male obesity and compromised reproductive and metabolic health to the next generation (Fullston *et al.* 2013; Fullston *et al.* 2012). Chapter 4 ultimately highlights the transmission of compromised DNA integrity through the obese male germ



line to embryos with the potential to harm the health of the next generation. The excision and repair of paternally-derived 8-OHdG lesions in the subsequent embryo is vital to reduce the potential for mutagenesis (Bruner *et al.* 2000; Chabory *et al.* 2009; Ohno *et al.* 2014) and reduce the susceptibility of offspring to impaired health including metabolic syndrome (Lane *et al.* 2014; Meseguer *et al.* 2008). Future studies could further investigate the extent of oocyte repair capacity to reduce the risk of mutations post-fertilization in the zygote and to determine if this repair prevents mutagenesis in the embryo.

Similar to the data observed in the mouse models (Chapters 3 and 4), Chapter 5 demonstrated that fertilization rates and pregnancy rates were compromised with increased sperm 8-OHdG lesions (regardless of male BMI). Although 8-OHdG was not assessed in the paternal pronucleus of the human embryo, Chapter 5 indicated that fertilization rates were reduced with increased sperm oxidative DNA lesions. This may be due to elevated sperm oxidative stress and subsequent DNA modifications, which are beyond the oocyte's capacity for low level sperm DNA damage repair and consistent with previous findings (Lewis and Aitken 2005; Sukcharoen *et al.* 1995). Chapter 5 also indicated that the odds of achieving a pregnancy (biochemical, implantation or an ongoing pregnancy) were reduced by up to 49% with increases in sperm 8-OHdG lesions regardless of male BMI. Although the association between sperm 8-OHdG DNA modifications and live birth outcomes was not assessed due to statistical power limitations, previous studies have indicated that impaired sperm DNA integrity and subsequent mutations in sperm from paternal smoking is linked to recurrent pregnancy loss and congenital malformations, neurological disorders and cancers in children (Aitken *et al.* 2003; Aitken and Krausz 2001). Given the link between sperm oxidative

DNA damage, perturbed sperm DNA integrity and poor offspring health, future studies with larger sample sizes to address the power limitations in Chapter 5 could be conducted to evaluate the relationship and impact of sperm 8-OHdG DNA lesions on live birth outcomes and long-term offspring health.

One of the strengths of this project is that sperm intracellular ROS and 8-OHdG lesions were examined from the prepared (washed) sperm sample used to inseminate or inject the couple's oocytes (Chapter 5). By doing this, correlation analyses between treatment cycle outcomes and sperm oxidative stress assessments are restricted to the washed sperm sample used for oocyte insemination/injection limiting the influence of seminal variables which may affect sperm intracellularly. Overall, the sperm oxidative stress measurements assessed, primarily 8-OHdG, could be used as a clinical marker and laboratory sperm quality evaluation test to predict the odds of achieving a pregnancy and even a live birth whereby as sperm oxidative DNA damage levels increase, the chances of pregnancy decline (Chapter 5).

#### **7.4 Role of micronutrient supplementation in overweight and obese males**

The data outlined in this project showed that micronutrient supplement intake improved sperm oxidative stress, primarily sperm 8-OHdG lesions, in both diet-induced obese mice (Chapters 3 and 4) and overweight and obese men (Chapters 5 and 6). As obesity is associated with overnutrition, many obese individuals are actually micronutrient deficient, as a result of consumption of energy-dense and micronutrient-low diets. This led to the hypothesis investigated thereby creating a paradigm that in males with diets with micronutrient deficiencies, dietary supplementation may improve sperm oxidative stress parameters (sperm intracellular ROS and oxidative DNA lesions). In the mouse models, outcomes were differentiated based on the duration of micronutrient supplement treatment. Investigating different durations of intake throughout this project was vital to understand potential locations of damage (testes versus epididymis) and how micronutrient supplements may improve different aspects of sperm quality, sperm function, and if the damage found in sperm persisted to the paternal pronucleus of the subsequent embryo and affected pregnancy outcomes. Although the maximum investigated duration of 10 weeks of micronutrient supplementation (Chapter 3), a duration that spans two rounds of spermatogenesis, in obese male mice reduced adiposity relative to total body weight and improved normal sperm morphology, the reduction in sperm intracellular ROS was evident from as little as 10 days of supplement intake (Chapter 4).

It is clear from the data in Chapter 3 that a HFD causes damage to the sperm during the period of spermatogenesis. The increased 8-OHdG lesions observed in testicular sperm (elongating and elongated spermatids) from HFD-fed mice was reduced with 10 weeks

of micronutrient supplement intake. Interestingly, the reduction of 8-OHdG modifications with micronutrient supplementation in testicular sperm persisted to mature sperm (collected from the epididymis) and the paternal pronucleus of embryos derived from obese male mice. Ten days of micronutrient supplement intake spans the duration of sperm maturation during epididymal transit (Robaire *et al.* 2006), which suggests that supplement intake in obese males may reduce sperm oxidative stress and oxidative DNA lesions by improving the epididymal environment and antioxidant defence mechanisms during sperm maturation (Figure 7.1). Additionally, given that 10 and 5 weeks of micronutrient supplement intake in obese male mice also reduced sperm oxidative stress and oxidative DNA lesions to similar levels to that observed in Chapter 4 (10 day treatment) further indicates that micronutrient supplement intake may also affect spermatogenesis in the testis (Figure 7.1). Collectively, these findings highlight the possibility of a combination effect of micronutrient supplementation in both the epididymis and testes.

A possible explanation for the observed reductions in sperm 8-OHdG lesions with micronutrient supplement intake may be the observed reductions in sperm intracellular ROS in both testicular spermatogenic and mature epididymal sperm (Chapters 3 and 4). When comparing outcomes from treatment durations, the degree of reductions in sperm intracellular ROS and 8-OHdG lesions with micronutrient supplementation in diet-induced obese mice were relative to the duration of HFD consumption. Mice fed a HFD with micronutrient supplements for 10 days or 5 weeks had reduced sperm intracellular ROS that was at a level similar to that from CD-fed mice (almost restoring sperm intracellular ROS). However, 10 weeks of micronutrient supplementation in HFD-fed mice did not have an additive effect and almost restore sperm intracellular ROS as seen

in the other treatment durations albeit reducing sperm intracellular ROS compared to that from mice fed a HFD alone. This suggests that long-term intake (10 weeks) of micronutrient supplements can only reduce sperm intracellular ROS to certain degree and can not fully overcome the effects of the concurrent long-term intake of a HFD. Overall and regardless of the duration of micronutrient treatment, this finding is encouraging for mitigating effects in offspring sired by diet-induced obese fathers with elevated sperm oxidative stress, highlighting a potential treatment for the reduction or prevention of sperm oxidative DNA lesion transmission to the subsequent embryo and minimising offspring effects (Barker 2004; Fullston *et al.* 2013; Fullston *et al.* 2012; Ng *et al.* 2010). Therefore, future studies assessing the impact of dietary micronutrient supplementation on the health of offspring produced by diet-induced obese mice are warranted.

Micronutrients may pass the blood testis (selectively permeable) barrier and/or blood epididymal (semi-permeable) barrier through the vascular system and enhance antioxidant enzyme activity in the testes and epididymis (Chabory *et al.* 2009; Haverfield *et al.* 2014) to reduce the excess of ROS in sperm observed in diet-induced obese males. Antioxidants and antioxidant enzymes are dependent on micronutrients such as glutathione peroxidase (GPx), which is regulated by and contains the micronutrient selenium (Walczak-Jedrzejowska *et al.* 2013); and zinc is an essential component of the antioxidant enzyme superoxide dismutase (SOD) (Walczak-Jedrzejowska *et al.* 2013). Serum levels of the supplemented micronutrients could be examined in future studies in addition to real-time PCR analysis of antioxidant enzymes expressed in the testis and epididymis to further investigate this proposed mechanism. Also, the superiority of active ingredients and their concentrations in dietary micronutrient and antioxidant supplements need to be explored as the current literature differs considerably in terms of the use of a

single micronutrient or a combination/ formulation of micronutrients and their doses, study designs and selection criteria (Barratt *et al.* 2017). A combination of micronutrients including lipophilic and hydrophilic antioxidants seems more appropriate and effective given that oxidative stress is a non-localized heterogeneous occurrence (Gharagozloo and Aitken 2011). Additionally, given the inconsistency in literature regarding duration of dietary micronutrient and antioxidant supplementation, future studies could evaluate an optimal duration of intake to highlight a potential epididymal effect or if more benefits are observed with a longer duration of intake.

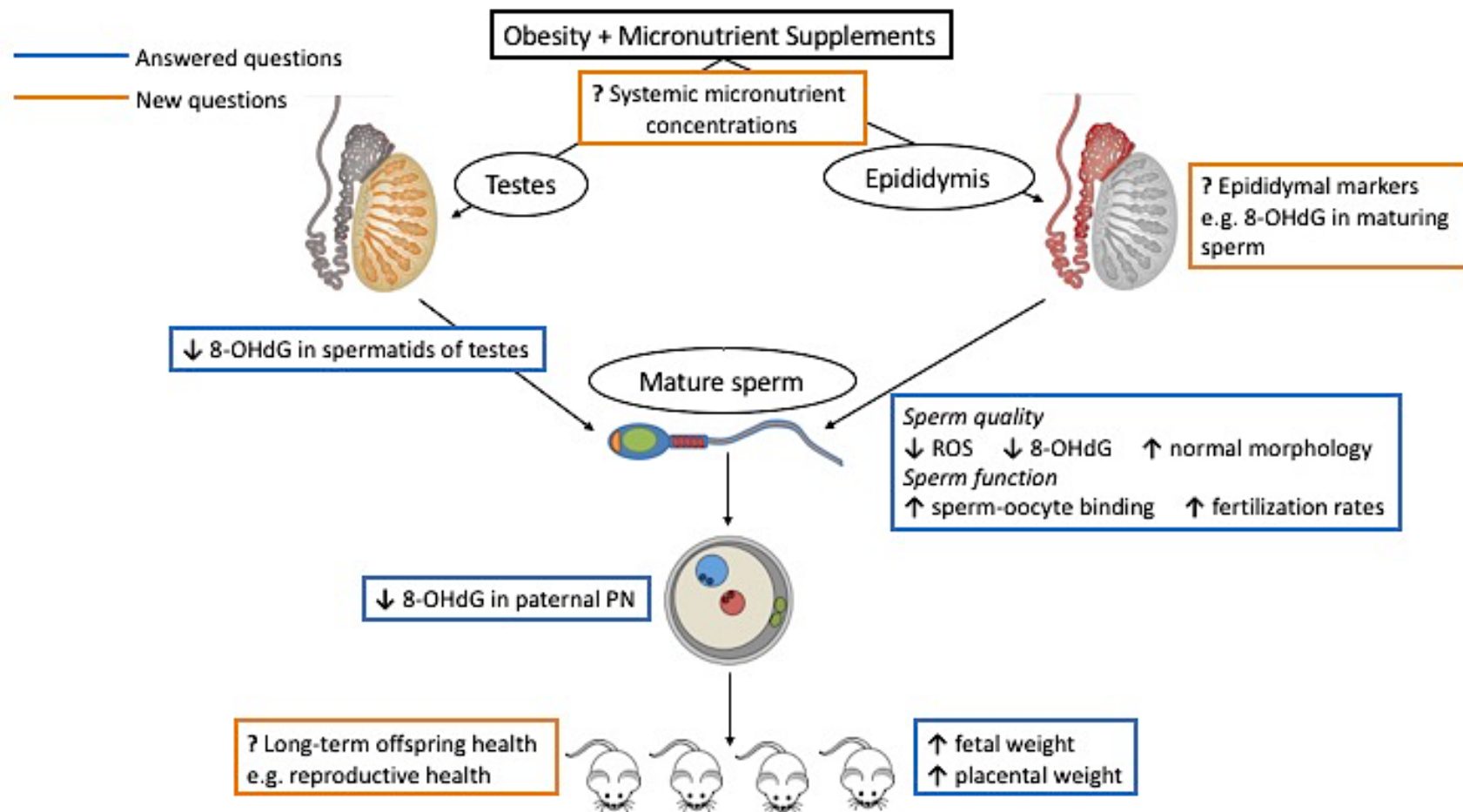
Given the reductions in sperm intracellular ROS and 8-OHdG lesions with the relatively short duration (10 days) of micronutrient supplementation observed in Chapter 4, future translational clinical studies could investigate sperm oxidative stress markers from men consuming micronutrient supplements for 10 days, which is also the duration spanning epididymal transit in humans (Robaire *et al.* 2006). Also, the design of these future investigations could include the man serving as their own control (longitudinal design) in which sperm oxidative DNA lesions (8-OHdG) could be assessed before and after micronutrient supplement intake in contrast to the data from Chapters 5 and 6 whereby men were stratified by BMI and micronutrient supplementation. Moreover, Chapter 5 suggests that 8-OHdG is a viable biomarker and predictor for a successful pregnancy as 8-OHdG was reduced and live birth rates were increased with micronutrient supplementation in men across all BMIs. However, future studies are required to confirm this potential marker with the assessment of sensitivity and specificity to confirm validity and reliability before sperm 8-OHdG immunofluorescence quantification were to be applied as a diagnostic and/or predictive test.

Data from Chapter 6 showed that micronutrient supplement intake is associated with increased standard insemination fertilization rates in obese men. This is consistent with previous studies that have shown obese men have reduced standard insemination fertilization rates (Yang *et al.* 2016) and a reduced chance of achieving a pregnancy after standard insemination but not ICSI (Keltz *et al.* 2010). This difference in fertilization rates based on the insemination method is most likely due to the ability of sperm to capacitate, undergo acrosome reaction, and enact sperm-oocyte fusion during the standard insemination method as opposed to the ICSI method in which these processes are surpassed (Anifandis *et al.* 2014). In the subsequent embryo, the embryonic genome is activated at the 4-8 cell stage (Braude *et al.* 1988; Desai *et al.* 2009), and any improvements in embryo morphology and development would be expected after this time point (day 4 of embryo development) after the effects of the activation of a damaged paternal genome would most likely be seen. This was the case in obese men whereby day 4 on-time morula and early blastocyst-staged embryo development and morphology was improved with micronutrient supplement intake. Interestingly, Chapter 6 showed for the first time that pregnancy and live birth rates can be improved with micronutrient supplementation in overweight and obese men. This may ultimately be due to the reduced sperm intracellular ROS and reduced oxidative 8-OHdG lesions observed in Chapter 5. Although these improved pregnancy outcomes were observed in couples undergoing ART treatment, similar outcomes would be expected in couples conceiving naturally in which micronutrient supplement intake in obese men may improve spermatogenesis and sperm maturation during epididymal transit (Chapters 3 and 4) optimising DNA integrity and ultimately increasing the likelihood of achieving a successful pregnancy and live birth. On a translational clinical basis, the data from Chapter 5 suggests that a feasible sperm oxidative stress test could be formulated to assess sperm intracellular ROS and 8-

OHdG, which could ultimately be used as a predictive biomarker for couples undergoing ART treatment. Furthermore, micronutrient supplementation could be suggested as a treatment option for the clinical management of men with obesity-induced subfertility and elevated sperm oxidative stress.

Unfortunately, one of the limitations from Chapters 5 and 6 is that food intake and dietary habits from these men were not recorded. Future studies could record more detailed information regarding lifestyle habits primarily dietary patterns, exercise and adherence to the consumption of micronutrient supplements, which may influence pregnancy outcomes. Also, future investigations could examine the effects of dietary micronutrient supplement intake on the health and developmental competence of children from overweight and obese men given that paternal obesity, sperm oxidative stress and impaired sperm DNA integrity is associated with perturbed offspring health (Soubry *et al.* 2013; Yeung *et al.* 2017).





**Figure 7.1:** Potential pathways, spermatogenic (testes) and/or sperm maturation (epididymis), for the effects of micronutrient supplement intake on sperm quality, sperm function, embryo quality and pregnancy outcomes in diet-induced obese males. This diagram also highlights the data identified by the project and the generation of new knowledge gaps for future research.

## **7.5 Role of micronutrient supplementation in a normal/healthy weight males**

Adherence to a healthy diet, characterised by an increased intake of vegetables and fruits, has been shown to increase sperm motility and concentration among men with unspecified BMIs in Asia, Europe, and the USA (Cutillas-Tolin *et al.* 2015; Liu *et al.* 2015; Oostingh *et al.* 2017). However, a healthy diet can be difficult to comply with due to busy work/life schedules and low compliance rates in men (Mardby *et al.* 2016; Vitale *et al.* 2016). Therefore, micronutrient supplementation is an option for individuals to achieve adequate micronutrient intake if a well balanced diet is not regularly maintained. In infertile men, micronutrient and antioxidant supplement intake is associated with improved male reproductive health outcomes including increased sperm motility, concentration, normal morphology (Ahmadi *et al.* 2016; Zareba *et al.* 2013) and increased pregnancy and live birth rates (Showell *et al.* 2011; Showell *et al.* 2014). Cochrane reviews support the efficacy of micronutrient and antioxidant supplements (Showell *et al.* 2011; Showell *et al.* 2014) however, a recent meta-analysis states that the effect is uncertain due to low-quality evidence and that further randomized placebo-controlled trials are required to draw a conclusion (Barratt *et al.* 2017).

In rodent models, whereby confounding lifestyle factors are controlled, micronutrient and antioxidant supplement intake have been shown to improve conventional sperm parameters including sperm morphology (Ceribasi *et al.* 2010; Mosbah *et al.* 2015). Further, sperm 8-OHdG lesions were reduced with the intake of a combination of micronutrient supplements (selenium, lycopene, vitamin E, vitamin C, green tea extract, zinc and folic acid) in undernourished mice (McPherson *et al.* 2016). In Chapter 3, CD fed mice, to model normal healthy weight males, were supplemented with micronutrients

for 10 weeks and had improved fertilization rates compared to mice fed the same un-supplemented CD. However, mice fed a short-term intake of micronutrient supplements for 10 days (Chapter 4) did not improve fertilization rates. The increase in fertilization rates with the longer treatment duration of 10 weeks may be due to the observed improvement in normal sperm morphology (Chapter 3), which can optimise the acrosome reaction process, sperm-oocyte fusion, and ultimately improve the odds of achieving successful fertilization (Liu and Baker 2000). Although these findings were not observed in men (Chapters 5 and 6), the percentage of day 4 embryos (post embryonic genome activation) that reached the partially compacted morula stage was improved in normal weight men that consumed micronutrient supplements compared to their normal counterparts that did not consume micronutrient supplements (Chapter 5). Also, normal weight men had improved embryo utilisation rates and increased implantation, ongoing pregnancy, and live birth rates compared to normal weight men not consuming supplements (Chapter 6). The observed improvements in on-time embryo development, embryo utilisation, implantation, pregnancy and live birth rates demonstrated in Chapter 6 may be explained by the intracellular investigations of Chapter 5, whereby normal weight men that consumed micronutrient supplements displayed reduced sperm intracellular ROS and oxidative DNA lesions. The reduction in sperm oxidative stress and 8-OHdG adducts may in turn transmit optimal sperm DNA integrity to the subsequent embryo and may contain less genomic abnormalities increasing the proper development and morphology of embryos (Meseguer *et al.* 2006; Meseguer *et al.* 2008) and increasing the chance of a live birth (Loft *et al.* 2003; Thomson *et al.* 2011).

It was unanticipated yet interesting to see that normal weight men had improvements with micronutrient supplement intake in not only sperm oxidative stress markers but also

clinical outcomes including live birth. These findings suggest that normal weight men may also be micronutrient deficient or may have other underlying pathologies that induce high levels of ROS. Secondary markers should be investigated in future studies that assess the systemic profiles of all men. A blood examination may provide better insight into micronutrient deficiencies, metabolic markers, inflammatory markers and other pathologies (beyond an increased BMI) that may impact the effectiveness of micronutrient supplementation. Given that subfertility is multifactorial (McPherson and Lane 2015), future studies should investigate adiposity and muscle mass using a body composition analysis in addition to systemic serum analyses, including lipids, glucose, insulin, leptin and inflammatory markers, in all men despite their BMI classification.

## 7.6 Clinical relevance and significance

The role of micronutrient supplement intake in male obesity, in both a mouse model of HFD-induced obesity and in overweight and obese men, has shown to be effective in improving sperm intracellular ROS, sperm oxidative DNA damage, fertilization rates, embryo development and pregnancy outcomes. Interestingly, there were also benefits observed in CD-fed mice and normal weight men whereby micronutrient supplementation reduced sperm oxidative DNA damage and improving fertilization rates, embryo development, embryo utilisation and pregnancy rates.

Male mice fed a HFD supplemented with micronutrients had reduced sperm intracellular ROS and reduced sperm 8-OHdG lesions in addition to improvements in sperm morphology, sperm binding, and fertilization rates. These improvements were observed from as little as 10 days of intake, which spanned the duration of epididymal transport, as opposed to 5 or 10 weeks of intake, durations that covered one or two rounds of spermatogenesis respectively. Moreover, male mice that were treated with 10 days of dietary micronutrient supplementation produced embryos with reduced oxidative DNA lesions in the paternal pronucleus and had increased implantation rates and increased fetal weight. In men, dietary micronutrient supplementation was associated with increased fertilization rates in obese men in addition to improved embryo morphology and pregnancy outcomes including increased implantation, ongoing pregnancy and live birth rates in all men regardless of BMI. Although additional dietary and lifestyle factors were not reported, there was an obvious increase in sperm intracellular ROS and oxidative 8-OHdG DNA lesions in obese men which was reduced with micronutrient supplement intake.

Overall, the mouse model suggests that dietary micronutrient supplementation may impact the testes (spermatogenesis) and/or the epididymis (sperm maturation) as a potential mode of action, which should be investigated further in future studies. Furthermore and to address the project's hypothesis, the combined mouse and clinical data highlight the effectiveness of dietary micronutrient supplement intake in improving subfertility in obese males, primarily improving fertilization rates, embryo development, implantation, pregnancy and live birth rates in addition to alleviating compromised sperm DNA integrity (8-OHdG).

## 7.7 References

Ahmadi, S., Bashiri, R., Ghadiri-Anari, A., and Nadjarzadeh, A. (2016) Antioxidant supplements and semen parameters: An evidence based review. *Int J Reprod Biomed (Yazd)* **14**, 729-736.

AIHW (2016) Australia's Health 2016. Australian Institute of Health and Welfare.

Aitken, J., and Fisher, H. (1994) Reactive oxygen species generation and human spermatozoa: the balance of benefit and risk. *Bioessays* **16**, 259-267.

Aitken, R.J., Baker, M.A., and Sawyer, D. (2003) Oxidative stress in the male germ line and its role in the aetiology of male infertility and genetic disease. *Reprod Biomed Online* **7**, 65-70.

Aitken, R.J., Buckingham, D.W., Brindle, J., Gomez, E., Baker, H.W.G., and Irvine, D.S. (1995) Analysis of Sperm Movement in Relation to the Oxidative Stress Created by Leukocytes in Washed Sperm Preparations and Seminal Plasma. *Human Reproduction* **10**, 2061-2071. [In English]

Aitken, R.J., and Curry, B.J. (2011) Redox Regulation of Human Sperm Function: From the Physiological Control of Sperm Capacitation to the Etiology of Infertility and DNA Damage in the Germ Line. *Antioxidants & Redox Signaling* **14**, 367-381. [In English]

Aitken, R.J., Gordon, E., Harkiss, D., Twigg, J.P., Milne, P., Jennings, Z., and Irvine, D.S. (1998) Relative impact of oxidative stress on the functional competence and genomic integrity of human spermatozoa. *Biol Reprod* **59**, 1037-1046.

Aitken, R.J., and Krausz, C. (2001) Oxidative stress, DNA damage and the Y chromosome. *Reproduction* **122**, 497-506.

Aitken, R.J., Smith, T.B., Jobling, M.S., Baker, M.A., and De Iuliis, G.N. (2014) Oxidative stress and male reproductive health. *Asian J Androl* **16**, 31-38.

Anifandis, G., Messini, C., Dafopoulos, K., Sotiriou, S., and Messinis, I. (2014) Molecular and cellular mechanisms of sperm-oocyte interactions opinions relative to in vitro fertilization (IVF). *Int J Mol Sci* **15**, 12972-12997.

Bakos, H.W., Henshaw, R.C., Mitchell, M., and Lane, M. (2011) Paternal body mass index is associated with decreased blastocyst development and reduced live birth rates following assisted reproductive technology. *Fertil Steril* **95**, 1700-1704.

Bakos, H.W., Mitchell, M., Setchell, B.P., and Lane, M. (2010) The effect of paternal diet-induced obesity on sperm function and fertilization in a mouse model. *Int J Androl* **34**, 402-410.

Barker, D.J. (2004) The developmental origins of chronic adult disease. *Acta Paediatr Suppl* **93**, 26-33.

- Barratt, C.L.R., Bjorndahl, L., De Jonge, C.J., Lamb, D.J., Osorio Martini, F., McLachlan, R., Oates, R.D., van der Poel, S., St John, B., Sigman, M., *et al.* (2017) The diagnosis of male infertility: an analysis of the evidence to support the development of global WHO guidance-challenges and future research opportunities. *Hum Reprod Update* **23**, 660-680.
- Braude, P., Bolton, V., and Moore, S. (1988) Human gene expression first occurs between the four- and eight-cell stages of preimplantation development. *Nature* **332**, 459-461.
- Bruner, S.D., Norman, D.P., and Verdine, G.L. (2000) Structural basis for recognition and repair of the endogenous mutagen 8-oxoguanine in DNA. *Nature* **403**, 859-866.
- Burgeiro, A., Cerqueira, M.G., Varela-Rodriguez, B.M., Nunes, S., Neto, P., Pereira, F.C., Reis, F., and Carvalho, E. (2017) Glucose and Lipid Dysmetabolism in a Rat Model of Prediabetes Induced by a High-Sucrose Diet. *Nutrients* **9**.
- Ceribasi, A.O., Turk, G., Sonmez, M., Sakin, F., and Atessahin, A. (2010) Toxic effect of cyclophosphamide on sperm morphology, testicular histology and blood oxidant-antioxidant balance, and protective roles of lycopene and ellagic acid. *Basic Clin Pharmacol Toxicol* **107**, 730-736.
- Chabory, E., Damon, C., Lenoir, A., Kauselmann, G., Kern, H., Zevnik, B., Garrel, C., Saez, F., Cadet, R., Henry-Berger, J., *et al.* (2009) Epididymis seleno-independent glutathione peroxidase 5 maintains sperm DNA integrity in mice. *J Clin Invest* **119**, 2074-2085.
- Cutillas-Tolin, A., Minguez-Alarcon, L., Mendiola, J., Lopez-Espin, J.J., Jorgensen, N., Navarrete-Munoz, E.M., Torres-Cantero, A.M., and Chavarro, J.E. (2015) Mediterranean and western dietary patterns are related to markers of testicular function among healthy men. *Hum Reprod* **30**, 2945-2955.
- De Iuliis, G.N., Thomson, L.K., Mitchell, L.A., Finnie, J.M., Koppers, A.J., Hedges, A., Nixon, B., and Aitken, R.J. (2009) DNA Damage in Human Spermatozoa Is Highly Correlated with the Efficiency of Chromatin Remodeling and the Formation of 8-Hydroxy-2'-Deoxyguanosine, a Marker of Oxidative Stress. *Biology of Reproduction* **81**, 517-524. [In English]
- Desai, N., AbdelHafez, F., Sabanegh, E., and Goldfarb, J. (2009) Paternal effect on genomic activation, clinical pregnancy and live birth rate after ICSI with cryopreserved epididymal versus testicular spermatozoa. *Reprod Biol Endocrinol* **7**, 142.
- Fan, Y., Liu, Y., Xue, K., Gu, G., Fan, W., Xu, Y., and Ding, Z. (2015) Diet-induced obesity in male C57BL/6 mice decreases fertility as a consequence of disrupted blood-testis barrier. *PLoS One* **10**, e0120775.
- Fullston, T., Ohlsson Teague, E.M., Palmer, N.O., DeBlasio, M.J., Mitchell, M., Corbett, M., Print, C.G., Owens, J.A., and Lane, M. (2013) Paternal obesity initiates metabolic disturbances in two generations of mice with incomplete penetrance to the F2



generation and alters the transcriptional profile of testis and sperm microRNA content. *FASEB J* **27**, 4226-4243.

Fullston, T., Palmer, N.O., Owens, J.A., Mitchell, M., Bakos, H.W., and Lane, M. (2012) Diet-induced paternal obesity in the absence of diabetes diminishes the reproductive health of two subsequent generations of mice. *Hum Reprod* **27**, 1391-1400.

Gharagozloo, P., and Aitken, R.J. (2011) The role of sperm oxidative stress in male infertility and the significance of oral antioxidant therapy. *Hum Reprod* **26**, 1628-1640.

Gomez, E., Buckingham, D.W., Brindle, J., Lanzafame, F., Irvine, D.S., and Aitken, R.J. (1996) Development of an image analysis system to monitor the retention of residual cytoplasm by human spermatozoa: Correlation with biochemical markers of the cytoplasmic space, oxidative stress, and sperm function. *Journal of Andrology* **17**, 276-287. [In English]

Haverfield, J.T., Meachem, S.J., Nicholls, P.K., Rainczuk, K.E., Simpson, E.R., and Stanton, P.G. (2014) Differential permeability of the blood-testis barrier during reinitiation of spermatogenesis in adult male rats. *Endocrinology* **155**, 1131-1144.

Keltz, J., Zapantis, A., Jindal, S.K., Lieman, H.J., Santoro, N., and Polotsky, A.J. (2010) Overweight men: clinical pregnancy after ART is decreased in IVF but not in ICSI cycles. *J Assist Reprod Genet* **27**, 539-544. [In eng]

Koppers, A.J., De Iuliis, G.N., Finnie, J.M., McLaughlin, E.A., and Aitken, R.J. (2008) Significance of mitochondrial reactive oxygen species in the generation of oxidative stress in spermatozoa. *Journal of Clinical Endocrinology & Metabolism* **93**, 3199-3207.

La Vignera, S., Condorelli, R.A., Vicari, E., and Calogero, A.E. (2012) Negative effect of increased body weight on sperm conventional and nonconventional flow cytometric sperm parameters. *J Androl* **33**, 53-58.

Lane, M., McPherson, N.O., Fullston, T., Spillane, M., Sandeman, L., Kang, W.X., and Zander-Fox, D.L. (2014) Oxidative stress in mouse sperm impairs embryo development, fetal growth and alters adiposity and glucose regulation in female offspring. *PLoS One* **9**, e100832.

Lee, K.M., Ward, M.H., Han, S., Ahn, H.S., Kang, H.J., Choi, H.S., Shin, H.Y., Koo, H.H., Seo, J.J., Choi, J.E., *et al.* (2009) Paternal smoking, genetic polymorphisms in CYP1A1 and childhood leukemia risk. *Leuk Res* **33**, 250-258.

Lewis, S.E., and Aitken, R.J. (2005) DNA damage to spermatozoa has impacts on fertilization and pregnancy. *Cell Tissue Res* **322**, 33-41.

Liu, C.Y., Chou, Y.C., Chao, J.C., Hsu, C.Y., Cha, T.L., and Tsao, C.W. (2015) The Association between Dietary Patterns and Semen Quality in a General Asian Population of 7282 Males. *PLoS One* **10**, e0134224.

- Liu, D.Y., and Baker, H.W. (2000) Defective sperm-zona pellucida interaction: a major cause of failure of fertilization in clinical in-vitro fertilization. *Hum Reprod* **15**, 702-708.
- Loft, S., Kold-Jensen, T., Hjollund, N.H., Giwercman, A., Gyllemborg, J., Ernst, E., Olsen, J., Scheike, T., Poulsen, H.E., and Bonde, J.P. (2003) Oxidative DNA damage in human sperm influences time to pregnancy. *Hum Reprod* **18**, 1265-1272.
- Lord, T., and Aitken, R.J. (2015) Fertilization stimulates 8-hydroxy-2'-deoxyguanosine repair and antioxidant activity to prevent mutagenesis in the embryo. *Dev Biol* **406**, 1-13.
- Mardby, A.C., Schioler, L., Sundell, K.A., Bjerkeli, P., Lesen, E., and Jonsson, A.K. (2016) Adherence to antidepressants among women and men described with trajectory models: a Swedish longitudinal study. *Eur J Clin Pharmacol* **72**, 1381-1389.
- McPherson, N.O., Bakos, H.W., Owens, J.A., Setchell, B.P., and Lane, M. (2013) Improving metabolic health in obese male mice via diet and exercise restores embryo development and fetal growth. *PLoS One* **8**, e71459.
- McPherson, N.O., Fullston, T., Kang, W.X., Sandeman, L.Y., Corbett, M.A., Owens, J.A., and Lane, M. (2016) Paternal under-nutrition programs metabolic syndrome in offspring which can be reversed by antioxidant/vitamin food fortification in fathers. *Sci Rep* **6**, 27010.
- McPherson, N.O., and Lane, M. (2015) Male obesity and subfertility, is it really about increased adiposity? *Asian J Androl* **17**, 450-458.
- Meseguer, M., de los Santos, M.J., Simon, C., Pellicer, A., Remohi, J., and Garrido, N. (2006) Effect of sperm glutathione peroxidases 1 and 4 on embryo asymmetry and blastocyst quality in oocyte donation cycles. *Fertil Steril* **86**, 1376-1385.
- Meseguer, M., Martinez-Conejero, J.A., O'Connor, J.E., Pellicer, A., Remohi, J., and Garrido, N. (2008) The significance of sperm DNA oxidation in embryo development and reproductive outcome in an oocyte donation program: a new model to study a male infertility prognostic factor. *Fertil Steril* **89**, 1191-1199.
- Mosbah, R., Yousef, M.I., and Mantovani, A. (2015) Nicotine-induced reproductive toxicity, oxidative damage, histological changes and haematotoxicity in male rats: the protective effects of green tea extract. *Exp Toxicol Pathol* **67**, 253-259.
- Ng, S.-F., Lin, R.C.Y., Laybutt, D.R., Barres, R., Owens, J.A., and Morris, M.J. (2010) Chronic high-fat diet in fathers programs [bgr]-cell dysfunction in female rat offspring. *Nature* **467**, 963-966.
- Nobranc, A., Damon-Soubeyrand, C., Karrich, B., Henry-Berger, J., Cadet, R., Saez, F., Guiton, R., Janny, L., Pons-Rejraji, H., Alvarez, J.G., *et al.* (2013) DNA oxidative damage in mammalian spermatozoa: where and why is the male nucleus affected? *Free Radic Biol Med* **65**, 719-723.

- Oger, I., Da Cruz, C., Panteix, G., and Menezo, Y. (2003) Evaluating human sperm DNA integrity: relationship between 8-hydroxydeoxyguanosine quantification and the sperm chromatin structure assay. *Zygote* **11**, 367-371.
- Ohno, M., Sakumi, K., Fukumura, R., Furuichi, M., Iwasaki, Y., Hokama, M., Ikemura, T., Tsuzuki, T., Gondo, Y., and Nakabeppu, Y. (2014) 8-oxoguanine causes spontaneous de novo germline mutations in mice. *Sci Rep* **4**, 4689.
- Oostingh, E.C., Steegers-Theunissen, R.P., de Vries, J.H., Laven, J.S., and Koster, M.P. (2017) Strong adherence to a healthy dietary pattern is associated with better semen quality, especially in men with poor semen quality. *Fertil Steril* **107**, 916-923 e912.
- Palmer, N.O., Bakos, H.W., Owens, J.A., Setchell, B.P., and Lane, M. (2012) Diet and exercise in an obese mouse fed a high-fat diet improve metabolic health and reverse perturbed sperm function. *Am J Physiol Endocrinol Metab* **302**, E768-780.
- Rengan, A.K., Agarwal, A., van der Linde, M., and du Plessis, S.S. (2012) An investigation of excess residual cytoplasm in human spermatozoa and its distinction from the cytoplasmic droplet. *Reprod Biol Endocrinol* **10**, 92.
- Robaire, B., Hinton, B.T., and Orgebin-Crist, M. (2006) The Epididymis. In 'Knobil and Neill's Physiology of Reproduction.' (Ed. JD Neill). (Elsevier)
- Ross, C., Morriss, A., Khairy, M., Khalaf, Y., Braude, P., Coomarasamy, A., and El-Toukhy, T. (2010) A systematic review of the effect of oral antioxidants on male infertility. *Reprod Biomed Online* **20**, 711-723.
- Sakkas, D., Seli, E., Bizzaro, D., Tarozzi, N., and Manicardi, G.C. (2003) Abnormal spermatozoa in the ejaculate: abortive apoptosis and faulty nuclear remodelling during spermatogenesis. *Reprod Biomed Online* **7**, 428-432.
- Sallmen, M., Sandler, D.P., Hoppin, J.A., Blair, A., and Baird, D.D. (2006) Reduced fertility among overweight and obese men. *Epidemiology* **17**, 520-523. [In eng]
- Shen, H.M., Chia, S.E., and Ong, C.N. (1999) Evaluation of oxidative DNA damage in human sperm and its association with male infertility. *J Androl* **20**, 718-723.
- Showell, M.G., Brown, J., Yazdani, A., Stankiewicz, M.T., and Hart, R.J. (2011) Antioxidants for male subfertility. *Cochrane Database Syst Rev*, CD007411. [In eng]
- Showell, M.G., Mackenzie-Proctor, R., Brown, J., Yazdani, A., Stankiewicz, M.T., and Hart, R.J. (2014) Antioxidants for male subfertility. *Cochrane Database Syst Rev*, CD007411.
- Smith, T.B., Dun, M.D., Smith, N.D., Curry, B.J., Connaughton, H.S., and Aitken, R.J. (2013) The presence of a truncated base excision repair pathway in human spermatozoa that is mediated by OGG1. *J Cell Sci* **126**, 1488-1497.

- Soubry, A., Hoyo, C., Jirtle, R.L., and Murphy, S.K. (2014) A paternal environmental legacy: evidence for epigenetic inheritance through the male germ line. *Bioessays* **36**, 359-371.
- Soubry, A., Schildkraut, J.M., Murtha, A., Wang, F., Huang, Z., Bernal, A., Kurtzberg, J., Jirtle, R.L., Murphy, S.K., and Hoyo, C. (2013) Paternal obesity is associated with IGF2 hypomethylation in newborns: results from a Newborn Epigenetics Study (NEST) cohort. *BMC Med* **11**, 29.
- Sukcharoen, N., Keith, J., Irvine, D.S., and Aitken, R.J. (1995) Predicting the fertilizing potential of human sperm suspensions in vitro: importance of sperm morphology and leukocyte contamination. *Fertil Steril* **63**, 1293-1300.
- Thomson, L.K., Zieschang, J.A., and Clark, A.M. (2011) Oxidative deoxyribonucleic acid damage in sperm has a negative impact on clinical pregnancy rate in intrauterine insemination but not intracytoplasmic sperm injection cycles. *Fertil Steril* **96**, 843-847.
- Tunc, O., Bakos, H.W., and Tremellen, K. (2011) Impact of body mass index on seminal oxidative stress. *Andrologia* **43**, 121-128.
- Valavanidis, A., Vlachogianni, T., and Fiotakis, C. (2009) 8-hydroxy-2'-deoxyguanosine (8-OHdG): A critical biomarker of oxidative stress and carcinogenesis. *J Environ Sci Health C Environ Carcinog Ecotoxicol Rev* **27**, 120-139.
- Vernet, P., Fulton, N., Wallace, C., and Aitken, R.J. (2001) Analysis of reactive oxygen species generating systems in rat epididymal spermatozoa. *Biol Reprod* **65**, 1102-1113.
- Vitale, M., Masulli, M., Cocozza, S., Anichini, R., Babini, A.C., Boemi, M., Bonora, E., Buzzetti, R., Carpinteri, R., Caselli, C., *et al.* (2016) Sex differences in food choices, adherence to dietary recommendations and plasma lipid profile in type 2 diabetes - The TOSCA.IT study. *Nutr Metab Cardiovasc Dis* **26**, 879-885.
- Walczak-Jedrzejowska, R., Wolski, J.K., and Slowikowska-Hilczer, J. (2013) The role of oxidative stress and antioxidants in male fertility. *Cent European J Urol* **66**, 60-67.
- Yang, Q., Zhao, F., Hu, L., Bai, R., Zhang, N., Yao, G., and Sun, Y. (2016) Effect of paternal overweight or obesity on IVF treatment outcomes and the possible mechanisms involved. *Sci Rep* **6**, 29787.
- Yeung, E.H., Sundaram, R., Ghassabian, A., Xie, Y., and Buck Louis, G. (2017) Parental Obesity and Early Childhood Development. *Pediatrics* **139**.
- Zareba, P., Colaci, D.S., Afeiche, M., Gaskins, A.J., Jorgensen, N., Mendiola, J., Swan, S.H., and Chavarro, J.E. (2013) Semen quality in relation to antioxidant intake in a healthy male population. *Fertil Steril* **100**, 1572-1579.
- Zini, A., San Gabriel, M., and Baazeem, A. (2009) Antioxidants and sperm DNA damage: a clinical perspective. *J Assist Reprod Genet* **26**, 427-432.

# | APPENDIX

**A1:** *Saline for injection (0.9% sodium chloride)*

0.9g of sodium chloride (Sigma-Aldrich, St. Louis, MO, USA) was added to 100ml of MilliQ water (Merck Millipore, Darmstadt, Germany) filtered through a 0.25µm nylon filter and stored at 4°C.

**A2:** *Preparation of 25% glucose solution*

2.5g of glucose (Sigma-Aldrich) was dissolved in 10ml of saline, filtered with 2µM filter and stored at 4°C for up to a week.

**A3:** *Preparation of 1 IU insulin*

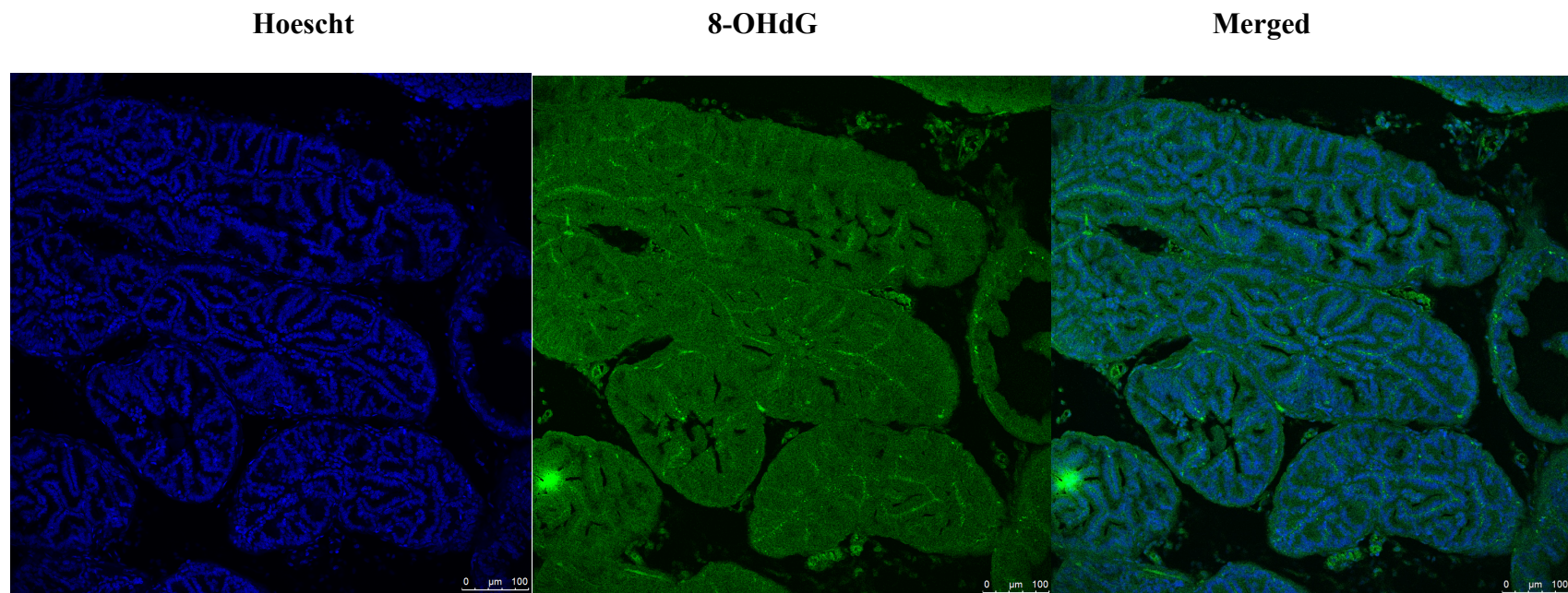
10ul of insulin (Actapid; Novo Nordisk, Bagsvaerd, Denmark) was added to 10ml of saline and stored at 4°C.

**A4:** *Avertin anesthetic stock solution*

0.5g of 2-2-2 tribromethanol (Avertin; Sigma-Aldrich, St. Louis, MO, USA) was dissolved into 0.5ml of 2-methyl-2-butanol (tet-amyl-alcohol, Sigma-Aldrich) and stored at 4°C.

*Avertin anesthetic working solution*

0.2ml of Avertin stock solution was added to 10ml of saline for injection. The solution was dissolved at 37°C for 4 hours. 0.015ml of 0.02% Avertin was injected per 1g of mouse weight.



**Figure A5:** Immunofluorescence of 8-OHdG in malignant lesions from mouse ventral prostate tissue as a positive control for the mouse anti-8-OHdG antibody (ab48508; Abcam).



# Patient Information Sheet

## The impact of dietary antioxidant consumption on sperm quality

Scientific Title: The impact of being overweight and obesity in men with antioxidant consumption on sperm quality, embryo development, pregnancy rates and live birth outcome

You are invited to participate in a research study to investigate the effects of antioxidant intake on sperm quality and pregnancy after IVF. This study will compare sperm quality between men that consume antioxidant supplements and men that do not consume antioxidant supplements.

The study is being conducted by a team of scientists, including a PhD student, from The University of Adelaide and Repromed with the approval from the Women's and Children's Health Network Research Ethics Committee.

Before you decide whether or not you wish to participate in this study, it is important for you to understand why the research is being done and what it will involve. Please take the time to read the following information carefully and ask questions if anything is not clear.

Please forward any questions or concerns you may have to the Principal Investigator, Helana Shehadeh  
T (BH) (AH): 8333 8111 e: [helana.shehadeh@adelaide.edu.au](mailto:helana.shehadeh@adelaide.edu.au)



## A6: Patient information sheet (Page 2 of 2)

### What is the purpose of this study?

A body mass index (BMI) is a measure of body shape based on weight and height. Many studies have shown that sperm quality is lower in men with a high BMI. One of these effects on sperm quality is oxidative stress.

Oxidative stress in sperm can damage sperm health and potentially prevent embryo development.

Antioxidants are compounds that are capable of protecting cells from a damaging environment, including oxidative stress.

This study will investigate whether antioxidant supplements can improve sperm quality and protect against oxidative stress in men with an elevated BMI.

### Why have I been invited to participate in this study?

You have been invited to participate in this study because you and your partner meet the inclusion criteria set for this study. This includes you and your partner's BMI, age and if you're undergoing a fresh single embryo transfer after IVF or ICSI.

### What happens during the study?

As standard procedure during your IVF/ ICSI cycle, males produce a semen sample to fertilize the eggs obtained. The lab staff prepare the sperm for fertilization and the leftover sample that is not used for fertilization is usually thrown away.

### If you and your partner consent to this study, the leftover sperm from your IVF/ ICSI cycle will be measured for levels of oxidative stress and DNA damage before being discarded.

The collection of leftover sperm in this study will not interfere with your cycle e.g. the development of your embryos or embryo transfer.

Your case notes including the questionnaire that you complete after producing your semen sample will be accessed to get information about your lifestyle, your cycle and your cycle's outcomes.

### What are the risks associated with this procedure?

There are no additional risks involved in participating in this study. The semen sample used for the study is the leftover sample that is not used for your IVF/ ICSI cycle.

### Will I benefit from this study?

Participation in this study may not be of any direct benefit to you. However, this study may provide valuable information to improve the management and treatment of sperm quality in men with an increased BMI.

### What happens if I don't want to take part in the study?

Participation is voluntary and there is no payment for participation. It is completely up to you whether or not you participate. Whatever your decision, it will not affect the treatment you receive now or in the future.

### What happens to the results?

Although your sample will be linked to information identifying your BMI, age and IVF/ICSI cycle details, all aspects of this study will be kept confidential and only those conducting the study will have access to your results.

You are able to contact your primary clinician and request in writing for your results to be released.

When the study is complete, all results will be pooled, analyzed and published in a scientific journal and presented at meetings. Your details will not be identifiable. All information obtained is treated as strictly confidential.

**Thank you for your time to consider this study. Please sign the attached consent form if you wish to participate.**

This study has been approved by the WCHN Human Research Ethic Committee.

If you wish to discuss the approval process, or have any concern or complaint, please contact the Executive Officer of the Human Research Committee at the Women's and Children's Health Network.

Ms Brenda Penny, Research Secretariat, T: 8161 6521

**A7: Patient consent form**

**CONSENT FORM**

**The impact of being overweight and obesity in men with antioxidant consumption on sperm quality, embryo development, pregnancy rates and live birth outcome**

1. I understand the nature and purposes of the study described in the Patient Information Sheet and agree to participate.
2. I understand that I may not directly benefit by participating in this study.
3. I understand that there are no additional risks and/or side effects involved in participating in the study.
4. I understand that while information gained in the study may be published, I will not be identified and information will be confidential.
5. I consent to information regarding my cycle at Repromed being accessed by investigators as part of this study. I understand I will not be identified and all information will be kept strictly confidential.
6. I understand that I am free to withdraw from the study at any stage and that my withdrawal will not affect my medical care or any other aspects of my relationship with Repromed.
7. I understand that there will be no payment to me for participating in this study.
8. I understand that the study will be conducted in accordance with the latest versions of the *National Statement on Ethical Conduct in Human Research 2007* and applicable privacy laws.
9. I am aware that I should retain a copy of the Consent Form, when completed, and the Information Sheet.
10. I consent to my case notes with Repromed to be disclosed to the above titled study, approved by the Women's & Children's Hospital Research Ethics Committee.

Signed by: ..... (Signature) Dated: .... / .... / .....  
..... (Print full name)

Signed by: ..... (Signature) Dated: .... / .... / .....  
..... (Print full name)

I certify that I have explained the study to the patients and consider that he/she understands what is involved in the study.

Name of investigator: .....

Signature: ..... Date:.....

## A8: Semen collection questionnaire



### SEMEN COLLECTION QUESTIONNAIRE

Patient Label

Patient Label

**Please answer the following questions immediately following the production of the sample and return this form with your sample to an Andrology Staff Member.**

1. When was your sample produced? Time: ..... am/pm Date: .....
2. Did you collect the entire sample when you ejaculated?  YES  NO
3. Were there difficulties in producing the sample?  YES  NO
4. Approximately how many days is it since you last ejaculated? ..... days
5. Where was the sample produced?  Repromed  Brought in
6. Production of sample:  Masturbation  Repromed Condom  Interrupted intercourse (not recommended)
7. Was a lubricant used?  YES  NO  
If YES, what? .....
8. Have you been ill during the last two months?  YES  NO  
If YES, what? .....
9. Have you taken drugs, medication, antioxidant supplements or any other supplements in the last three months?  YES  NO  
If YES, what? .....  
If YES, how often? .....
10. Do you smoke?  YES  NO  
If YES, what? .....  
If YES, how often? .....

I confirm that I/my partner produced this semen sample into the collection container which is labelled with my name/his name and other identifying information.

Name: .....

Sign: .....

**Laboratory use only:** Custody of Gametes  
 Sample received/identity confirmed by: .....  
 Time: ..... am/pm Date: .....

**Table A9:** Composition of micronutrients supplements consumed by men undergoing ART treatment.

<b>Menevit</b>	<b>Swisse Men's multivitamin</b>	<b>Nature's Own multivitamin</b>
Vitamin C 100 mg	Vitamin C 165 mg	Vitamin C 175 mg
Zinc 25 mg	Zinc 6 mg	Zinc 15 mg
Vitamin E 400 IU	Vitamin E 50 IU	Vitamin E 50 IU
Folic acid 500 µg	Folic acid 500 µg	Folic acid 300 µg
Lycopene 6 mg	Lycopene 120 µg	Lycopene 120 µg
Garlic oil 333 µg		
Selenium 26 µg	Selenium 26 µg	Selenium 26 µg
	Betacarotene 5 mg	Betacarotene 6 mg
	Vitamin D 200 IU	Vitamin D 200 IU
	Vitamin B 60 mg	Vitamin B 70 mg
	Nicotinamide 30 mg	Nicotinamide 40 mg
	Biotin 50 µg	Biotin 50 µg
	Calcium 21 mg	Calcium 25 mg
	Magnesium 58 mg	Magnesium 75 mg
	Potassium 4 mg	Potassium 4 mg
	Iron 3 mg	Iron 5 mg
	Copper 28 µg	Copper 28 µg
	Iodine 50 µg	Iodine 50 µg
	Co enzyme Q10 1 mg	
	Lutein 200 µg	

**Table A10:** Sperm intracellular esterase activity in normal weight, overweight and obese men measured using 5-(and 6-) carboxy-2',7'-dichlorofluorescein diacetate (carboxy-DCFDA; Molecular Probes).

	<b>Normal weight</b>	<b>Overweight</b>	<b>Obese</b>
carboxy-DCFDA (fluorescent units) <sup>1</sup>	50.38 ± 1.87	49.85 ± 1.36	55.09 ± 1.82

Data are expressed as mean ± SEM. N=30 live sperm per male. Carboxy-DCFDA fluorescent units were not different (P>0.05) between BMI categories as analyzed by a one-way ANOVA.

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<sup>1</sup> Sperm were incubated in 1µM carboxy-DCFDA in the dark for 1 hour at 37°C before being washed twice in PBS/PVP at 500g. Sperm were then incubated in 1µM propidium iodide (PI; Molecular Probes), a vitality stain, in the dark for 5 minutes at room temperature. Sperm were washed again in PBS/PVP for 5 minutes at 500g, loaded onto slides in 10µl drops and imaged under fluorescent microscopy. Thirty live sperm (negative for PI) were imaged per male patient. Sperm fluorescence was quantified using ImageJ software (Version 1.48, NIH) and results were expressed as a mean of fluorescence for carboxy-DCFDA (minus background).