# ROLE OF *Heterotrigona itama* BEE BREAD ON REPRODUCTIVE SYSTEM IN MALE RATS FED WITH HIGH-FAT DIET

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# ROLE OF *Heterotrigona itama* BEE BREAD ON REPRODUCTIVE SYSTEM IN MALE RATS FED WITH HIGH-FAT DIET

by

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## LIST OF SYMBOLS

| | |
|---|---|
| α | Alpha |
| β | Beta |
| μ | Micro |
| < | Less than |
| ↑ | Increase |
| ↓ | Decrease |
| ± | Plus-minus sign |

## LIST OF ABBREVIATIONS

| | |
|---|---|
| ANOVA | analysis of variance |
| AR | androgen receptor |
| Bax | BCl-2-associated -x-protein |
| BB | bee bread |
| BCl-2 | beta cell lymphoma-2 |
| BMI | body mass index |
| BW | body weight |
| cAMP | cyclic adenosine monophosphate |
| Caspase | cysteine-aspartic proteases |
| CAT | Catalase |
| CDNB | 1-chloro-2,4-dinitrobenzene |
| cGMP | cyclic guanidine monophosphate |
| CYP11A1 | cytochrome P450 11A1 |
| CYP17A1 | cytochrome P450 17A1 |
| DNA | deoxyribonucleic acid |
| DPPH | 1,1-diphenyl-2-picrylhydrazyl |
| E2 | Estradiol |
| ED | erectile dysfunction |
| ELISA | enzyme-linked immunosorbent |
| eNOS | endothelial nitric oxide synthase |
| FBG | fasting blood glucose |
| $FeCl_3$ | iron III chloride |
| $FeSO_4.7H_2O$ | ferrous sulphate heptahydrate |

| | |
|---|---|
| FI | food intake |
| FRAP | ferric reducing antioxidant power |
| FSH | follicle stimulating hormone |
| GAPDH | glyceraldehyde-3-phosphate dehydrogenase |
| GC-MS | gas chromatography mass spectrometry |
| GLUT1 | glucose transporter 1 |
| GLUT3 | glucose transporter 3 |
| GnRH | gonadotropic releasing hormone |
| GPx | glutathione peroxidase |
| GR | glutathione reductase |
| GSH | glutathione |
| GSSG | glutathione disulphide |
| GST | gluthanione-s-transferase |
| $H_2O_2$ | hydrogen peroxide |
| HCl | hydrochloric acid |
| HDL-C | high-density lipoprotein cholesterol |
| HFD group | high-fat diet group |
| HFD+B group | high-fat diet plus bee bread group |
| HFD+O group | high-fat diet plus orlistat group |
| HPG | hypothalamic pituitary gonadal |
| HPLC | high performance liquid chromatography |
| HRP | horseradish peroxidase |
| HSD | hydroxysteroid dehydrogenase |
| ICD-9 | international classification of diseases ninth revision |
| IHC | immunohistochemistry |

| | |
|---|---|
| IL-1 | Interleukin-1 |
| IL-10 | interleukin-10 |
| IL-6 | Interleukin-6 |
| iNOS | inducible nitric oxide synthase |
| Keap1 | kelch-like ECH-associated protein 1 |
| $KH_2PO_4$ | potassium dihydrogen phosphate |
| LDL-C | low-density lipoprotein cholesterol |
| LH | luteinizing hormone |
| LHR | luteinizing hormone receptor |
| MCT2 | monocarboxylate transporter 2 |
| MCT4 | monocarboxylate transporter 4 |
| MDA | malondialdehyde |
| MHC II | major histocompatibility complex class II |
| MIF | macrophage migration inhibitory factor |
| mRNA | messenger ribonucleic acid |
| $Na_2HPO_4$ | disodium hydrogen phosphate |
| $NADP^+$ | nicotinamide adenine dinucleotide |
| NADPH | nicotinamide adenine dinucleotide phosphate |
| $NaH_2HPO_4.2H_2O$ | sodium dihydrogen phosphate dihydrate |
| NaOH | sodium hydroxide |
| NC | normal control |
| NF-kB | nuclear factor-kappa b |
| nNOS | neuronal nitric oxide synthase |
| NO | nitric oxide |
| NQO1 | NAD(P)H dehydrogenase [quinone]1 |

| | |
|---|---|
| Nrf2 | nuclear factor erythroid 2-related factor 2 |
| $O_2^-$ | superoxide anion |
| OECD | organization for economic co-operation and development |
| OGTT | oral glucose tolerance test |
| $OH^-$ | hydroxyl |
| $ONOO^-$ | peroxynitrite |
| p53 | tumor protein |
| PCNA | proliferating cell nuclear antigen |
| QoL | quality of life |
| $ROO^-$ | Peroxyl |
| ROS | reactive oxygen species |
| RT-qPCR | reverse transcription quantitative polymerase chain reaction |
| SDS | sodium dodecyl sulphate |
| SOD | superoxide dismutase |
| StAR | steroidogenic acute regulatory protein |
| TAC | total antioxidant capacity |
| TBA | 2-thiobarbituric acid |
| TBARS | thiobarbituric acid reactive substances |
| TC | total cholesterol |
| TEP | 1,1,3,3-tetraethoxypropane |
| TPC | total phenolic content |
| TG | Triglyceride |
| TNB | 5-thio-2-nitobenzoic acid |

| | |
|---|---|
| TNF- α | tumor necrosis factor-alpha |
| TPTZ | 2,4,6-tripyridyl-s-triazine |
| Tris-HCl | tris(hydroxymethyl)aminomethane-HCl |
| TSPO | translocator protein |
| USM | Universiti Sains Malaysia |
| w/v | weight/ volume |
| WC | waist circumference |
| WHO | World Health Organisation |
| WHR | waist hip ratio |

**PERANAN ROTI LEBAH *Heterotrigona itama* KE ATAS SISTEM REPRODUKTIF DALAM TIKUS JANTAN YANG DIBERI DIET TINGGI LEMAK**

## ABSTRAK

Obesiti dilaporkan telah menyebabkan stres oksidatif testikular, inflamasi dan apoptosis di mana ia menyebabkan kegagalan fungsi reproduktif lelaki manakala roti lebah menunjukkan kesan antioksidan, antiradang dan antiapoptosis sepertimana dilihat dalam tisu-tisu lain. Walau bagaimanapun, sehingga kini, peranan roti lebah *Heterotrigona itama* ke atas sistem reproduktif lelaki obes belum dilaporkan. Oleh sebab itu, objektif kajian ini adalah untuk (i) menilai pH, komposisi dan sifat antioksidan roti lebah *Heterotrigona itama* dan (ii) menentukan peranan roti lebah *Heterotrigona itama* ke atas parameter antropometrik, parameter sperma, stres oksidatif testis, keradangan, apoptosis, tingkahlaku seksual dan juga prestasi reproduktif dalam tikus jantan yang diberi diet tinggi lemak (HFD). Tiga puluh dua tikus *Sprague Dawley* yang beratnya di antara 250-300 g diletakkan secara dirawak dalam empat kumpulan (n=8/kumpulan) yang dinamakan kumpulan kawalan (NC), HFD, HFD dengan roti lebah (HFD+B) dan kumpulan HFD dengan ubat anti-obesiti orlistat (HFD+O). Roti lebah (0.5g/kg/hari) dan orlistat (10 mg/kg/hari) dibancuh dalam air suling dan diberi secara paksaan oral selama 12 minggu. Pada minggu ke sepuluh, setiap tikus jantan diletakkan dengan tikus betina yang subur untuk menilai tingkahlaku seksual dan prestasi reproduktif tikus jantan. Pada akhir minggu ke -12, tikus jantan dibius dan darah serta organ reproduktif diambil untuk menentukan fungsi reproduktif. Roti lebah adalah berasid, mempunyai sifat antioksidan *in vitro* dan sembilan sebatian fenol. Roti lebah secara signifikan memperbaiki parameter antropometri dan profil lipid dalam tikus yang diberi HFD. Begitu juga dengan roti lebah di mana ianya secara signifikan memperbaiki stres oksidatif testis, keradangan,

apoptosis dan proliferasi sel germa yang diberi HFD. Seterusnya, roti lebah secara signifikan membaikpulih bilangan sperma, viabiliti, motiliti dan mengurangkan morfologi sperma tidak normal dan juga nDNA yang terpecah. Tambahan lagi, roti lebah secara signifikan meningkatkan hormon reproduktif dan kitaran monofosfat guanosin zakar, dan menurunkan kadar leptin, oleh itu meningkatkan jumlah tikus dengan intromisi dan ejakulasi yang lebih baik. Kesimpulannya, roti lebah secara signifikan meningkatkan sistem reproduktif dalam kalangan tikus jantan yang diberi HFD dengan mengurangkan stres oksidatif testis, keradangan, apoptosis dan meningkatkan proliferasi sel germa testis. Walau bagaimanapun, kajian lebih lanjut diperlukan untuk mengkaji tindakan mekanisma molekul roti lebah dan menentukan keselamatannya melalui kajian ketoksikan sebelum ianya digunakan sebagai rawatan komplementari dalam kalangan pesakit obes.

**ROLE OF *Heterotrigona itama* BEE BREAD ON REPRODUCTIVE SYSTEM IN MALE RATS FED WITH HIGH-FAT DIET**


## ABSTRACT

Obesity has been reported to cause testicular oxidative stress, inflammation and apoptosis thereby resulting in impaired male reproductive function while bee bread, on the other hand, exhibits antioxidant, anti-inflammatory and anti-apoptotic properties as seen in other tissues. However, to date, the role of *Heterotrigona itama* bee bread on male reproductive system in obesity has not been reported. Therefore, the objectives of this study were (i) to assess pH, composition and antioxidant properties of *Heterotrigona itama* bee bread and (ii) to determine the role of *Heterotrigona itama* bee bread on anthropometric parameters, sperm parameters, testicular oxidative stress, inflammation, apoptosis, sexual behaviour as well as reproductive performance in male rats fed with high-fat diet (HFD). Thirty-two adult male *Sprague Dawley* rats weighing between 250-300 g were randomised into four groups (n=8/group), namely normal control (NC), HFD, HFD plus bee bread (HFD+B) and HFD plus an anti-obesity drug orlistat (HFD+O) groups. Bee bread (0.5g/kg/day) and orlistat (10 mg/kg/day) were suspended in distilled water and given by oral gavage for 12 weeks. During the tenth week, each male rat was cohabited with a fertile female rat to assess male sexual behaviour and reproductive performance. At the end of 12 weeks, male rats were anaesthetized and blood, as well as the reproductive organs, were removed for the determination of reproductive functions. Bee bread was acidic, had *in vitro* antioxidant properties and nine phenolic compounds. Bee bread significantly improved the anthropometric parameters and lipid profile in rats fed with HFD. Similarly, bee bread also significantly ameliorated testicular oxidative stress, inflammation, apoptosis and germ cell proliferation in rats fed with HFD. Furthermore,

bee bread significantly enhanced sperm count, viability, motility, and reduced abnormal sperm morphology as well as fragmented nDNA. In addition, bee bread significantly increased the levels of reproductive hormones and penile cyclic guanosine monophosphate, and decreased leptin level, thereby increasing the number of rats with improved intromission and ejaculation as well as mating and fertility indices. In conclusion, bee bread significantly improved the reproductive system in male rats fed with HFD by attenuating testicular oxidative stress, inflammation, apoptosis and improving testicular germ cell proliferation. However, further studies are needed to further investigate the molecular mechanism of action of bee bread and to determine its safety via toxicity study before it is used as a complementary treatment among obese patients.

# CHAPTER 1

# INTRODUCTION

## 1.1 Background of study

Obesity is a multiplex disease comprising of several factors leading to the progression of metabolic syndrome (Furukawa *et al.*, 2017). It has recently been regarded as an overwhelming worldwide concern which is usually defined as excess body weight for a given height, with its accompanying severe health problems and having detrimental effects on the various systems in the body (Jayagopal *et al.* 2005; Eleazu *et al*., 2020; Suleiman *et al.* 2020a). The risk factors include; abdominal adiposity, hypertension, hypercholesteremia, sleep apnea, pulmonary hypertension, cardiovascular disease and type 2 diabetes (Gadde *et al.*, 2018), reduction in high-density lipoprotein (HDL), increased triglycerides and glucose intolerance (Leisegang *et al.*, 2019). Although the physiology and pathophysiology of obesity seem complex, it is caused by increased intake of calories combined with a concomitant reduction in energy expenditures (Levine *et al.*, 2018).

Obesity results from the accumulation of fats in the body with the individuals being excessively overweight (Zapata *et al.* 2020). One of the major contributory factors to obesity is the intake of excessive dietary fat, encouraged by its deliciousness and low satiety effect. Several diets including high-fat diet (HFD) amongst others have been implicated in the etiology of obesity (Kang *et al.* 2017).

Obesity has since been linked to infertility in couples globally; male obesity accounts for 20-70% of the problems and is associated with several adverse reproductive outcomes (Amjad *et al.*, 2019). Ramaraju *et al.* (2018) report that there is evidence of disturbances in the hypothalamic-pituitary-gonadal (HPG) axis that eventually affect testosterone and oestrogen levels. These decreased levels of

testosterone and oestrogen have a detrimental effect on spermatogenesis. The FSH and LH secretions are suppressed as a result of the negative effects of estrogen on the hypothalamus by altering the gonadotropin-releasing hormone (GnRH) level (Katib, 2015).

Numerous pathological conditions have been attributed to obesity, but the corroboration relating to impaired fertility in males with obesity are vague (Bellastella *et al.*, 2019). Epidemiological and experimental studies have suggested the implication of oxidative stress in the mechanism of abnormal spermatogenesis and impaired fertilizing potential in obesity (Sallmén *et al.*, 2006; Nguyen *et al.*, 2007; Ramlau-Hansen *et al.*, 2007). Oxidative stress is also reported as the major causative factor for reproductive abnormalities in obese men, the occurrence of which is demonstrated to activate pro-cytotoxic pathways like inflammation and apoptosis (Agarwal *et al.*, 2012). Oxidative stress is the condition related to harmful cellular damage caused by oxygen and oxygen-derived oxidants generally known as reactive oxygen species (ROS). The testicular tissues and spermatozoa are very delicate to ROS assault and lipid peroxidation which are attributed to the highly rich polyunsaturated fatty acid content of sperm membranes. Various studies have reported a negative relationship between sperm concentration, motility and sperm-oocyte interaction, and male obesity (Codoñer-Franch *et al.*, 2011; Mortazavi *et al.*, 2014), while others reported otherwise (Erdemir *et al.*, 2012; Galaly *et al.*, 2014).

In obese men, reduced sperm concentrations, sperm count and sperm motility were compared to lean men (Jensen *et al.*, 2004; Kort *et al.*, 2006; Hammoud *et al.*, 2008; Paasch *et al.*, 2010), sperm chromatin integrity was also compared in obese and lean men subjected to semen analysis at fertility clinics (Kort *et al.*, 2006; Chavarro *et al.*, 2010; Rybar *et al.*, 2011; Tunc *et al.*, 2011; Dupont *et al.*, 2013). Increased

apoptosis has been reported in spermatozoa of male patients with infertility, infertile mice and other rodents, although apoptosis is necessary for cellular homeostasis and male germ cell development (Miao *et al.*, 2018).

Obesity was reported as the leading cause of male (Katib 2015) and female (Talmor & Dunphy 2015) subfertility in the last three decades. The relationship between HFD and spermatozoa fertilizing capacity was accentuated in a previous study which reported decreases in sperm motility and quality, increased sperm DNA fragmentation and decreased lactate availability (Ferramosca *et al.* 2016, Suleiman *et al.* 2020a), which may ultimately result in decreased fertility potential. Sertoli cells provide enabling environment for the developing germ cells in the seminiferous tubules by ensuring a steady supply of lactate to the germ cells (Heinrich *et al.* 2020). Studies have revealed that metabolized glucose within the Sertoli cells is converted into lactate which serves as the substrate for adenosine triphosphate (ATP) production in the germ cells, to support spermatogenesis (Sengupta *et al.* 2020). Therefore, decreased germ cell ATP production may negatively affect fertility rates.

The integrity of sperm chromatin is a possible marker of the quality of sperm, decreased fertility and defective embryo development (Oleszczuk *et al.*, 2013). In several studies carried out on sperm chromatin integrity showed a positive association between sperm nuclear DNA fragmentation and body mass index. Furthermore, intracytoplasmic sperm injection success, pregnancy rate and live birth rate are negatively influenced by increased paternal body mass index (Umul *et al.*, 2015). However, other studies revealed a weak or no association between obesity in males and semen parameters (MacDonald *et al.*, 2009; Duits *et al.*, 2010; M Al-Ali *et al.*, 2014). Sermondade *et al.*, (2012) found that paternal overweight and obesity are linked

to a higher incidence of abnormal sperm parameters such as azoospermia or oligozoospermia.

Some possible mechanisms have been suggested to explain the impact of obesity on sperm parameters and male fertility. These include alteration in the hypothalamic-pituitary-testis axis, higher levels of leptin (Du Plessis *et al.*, 2010), hyperinsulinaemia and increased oxidative stress in the testicular microenvironment (Michalakis *et al.*, 2013). A negative association exists between the accumulation of fat, concentrations of sex hormone-binding globulin and total testosterone (Mah and Wittert, 2010). Consequently, increased inflammation, oxidative stress and lipid peroxidation are associated with obesity (Ozata *et al.*, 2002; Vincent *et al.*, 2007; Johnson *et al.*, 2012; Furukawa *et al.*, 2017). Increased apoptosis and oxidative stress in the sperm of obese male mice have shown the relevance of oxidative stress to sperm quality (Palmer *et al.*, 2012).

The relaxation of the penile vascular smooth muscles and retention of blood in the penile spongy tissues orchestrated by nitric oxide (NO) – cyclic guanosine monophosphate (cGMP) pathway, is required for the initiation and sustenance of penile erection. In erectile dysfunction, besides testosterone decline, penile oxidative stress also occurs in obesity through superoxide and reactive oxygen species generation (Minhas *et al.* 2002; Suleiman *et al*., 2019a). This is supported by reports which showed that obese men were 2.5 times likely to have erectile dysfunction (BMJ 2010), and overweight male juvenile rats fed with hypercaloric diet had impaired sexual behaviour (Macrini *et al.* 2016).

Bee bread is different from bee pollen. Bee pollen is gathered by scavenging bees and conveyed on their hind legs in a pollen bin back to the hive (Disayathanoowat *et al.*, 2020). Bee bread is made from pollen which is gathered by bees, but it is further

mixed, preserved and fermented with honey and bee digestive enzymes. Bee bread or fermented bee pollen has a higher content of lactic acid when compared with bee pollen. It has a high protein and phenolic contents as well as antioxidant activity (Nagai *et al.*, 2004). Previous reports have shown that bee bread possesses antioxidant (Bakour *et al.*, 2017), antitumor (Sobral *et al.*, 2017), antihypertensive (Khalifa *et al.*, 2020), antimicrobial (Kowalski and Makarewicz, 2017), neuroprotective (Khalifa *et al.*, 2020), anti-inflammatory (Bakour *et al.*, 2017) and anticancer (Zakaria and Haron, 2018) activities in most organs of the human body.

## 1.2 Research problem statement

Excessive food intake by men has resulted in being overweight and obese which could affect various organs including the male reproductive system leading to increased subfertility/ infertility. Traditionally, obesity is managed by adjusting one's lifestyle, administration of medications such as orlistat and metformin as well as by surgical methods. For decades now, these medications have been known to cause some detrimental side effects such as diarrhea, stomach cramp, vomiting and muscle wasting thereby necessitating the use of alternative medicine.

## 1.3 Justification of the study

Bee bread has been traditionally consumed for enhancement of male fertility. The previous study has reported that date palm pollen (bee pollen) at 120 and 240 mg/kg/day for 35 days significantly improves sperm parameters (counting, motility, morphology and DNA quality) with a concurrent gain in the weights of testis and epididymis in normal male rats (Bahmanpour *et al.*, 2015). Furthermore, bee pollen at 5 to 10 g/kg for 28 days significantly reduces body weight, Lee obesity index, the weight of fat in the groin, liver, abdominal cavity, total cholesterol and triglyceride

levels in obese mice induced by high cholesterol diet and subcutaneous L-monosodium glutamate (Komosinska-Vassev *et al.*, 2015).

However, it has not been established to date whether bee bread can improve male reproductive function in obesity or hyperlipidaemic condition. Therefore, this study aimed to determine the role of Malaysian bee bread on the reproductive system in male rats fed with a high-fat diet (HFD).

## 1.4 General objective

To determine the role of *Heterotrigona itama* bee bread on the reproductive system in male rats fed with HFD.

## 1.5 Specific objectives

1. To investigate pH, composition and *in vitro* antioxidant properties of *Heterotrigona itama* bee bread (phase 1 study)
2. To determine the effects of *Heterotrigona itama* bee bread on food consumption, body weight gain, and body mass index (BMI) and Lee obesity index in male rats fed with HFD (phase 2 study).
3. To investigate the effects of *Heterotrigona itama* bee bread on biochemical parameters (blood glucose, lipid profile, leptin and adiponectin) in male rats fed with HFD (phase 2 study).
4. To determine the effects of *Heterotrigona itama* bee bread on sperm parameters and reproductive hormones (phase 2 study).
5. To determine the effects of *Heterotrigona itama* bee bread on sexual behaviour and reproductive performance in male rats fed with HFD (phase 2 study).

6. To investigate the effects of *Heterotrigona itama* bee bread on histology of epididymal fat and reproductive organs (testis and epididymis) in male rats fed with HFD (phase 2 study).
7. To determine the effects of *Heterotrigona itama* bee bread on testicular mRNA and protein levels of parameters of steroidogenesis, energy metabolism in male rats fed with HFD (phase 2 study).
8. To determine the effects of *Heterotrigona itama* bee bread on testicular mRNA and protein levels of parameters of oxidant-antioxidant status, inflammation, apoptosis and germ cell proliferation in male rats fed with HFD (phase 2 study).
9. To determine the effects of *Heterotrigona itama* bee bread on sperm proteomics in male rats fed with HFD (phase 2 study).
10. To determine the effects of *Heterotrigona itama* bee bread on epididymal oxidant-antioxidant status in male rats fed with HFD (phase 2 study).

## 1.6 Hypothesis

*Heterotrigona itama* bee bread significantly improves testicular functions and fertility in rats fed with HFD by reducing oxidative stress, inflammation and apoptosis, and by improving steroidogenesis, energy metabolism and germ cell proliferation in the testis.

# CHAPTER 2

# LITERATURE REVIEW

## 2.1 Obesity

Obesity is a disease condition associated with significant disturbance in biochemical and hormonal levels that can affect various systems leading to several diseases such as diabetes mellitus, hypertension, dyslipidemia, cardiovascular disease, lung diseases, osteoarthritis, some types of cancer, and certain reproductive and metabolic disorders (Hammoud *et al.*, 2008). It can be caused by a combination of factors such as excessive food intake, lack of physical activity, medications, endocrine disorders, mental disorders, genes and genetic susceptibility (Guyenet and Schwartz, 2012).

WHO has reported that more than 1.9 billion adults aged 18 years and above were overweight, with over 650 million adults obese in 2016. Similarly, an estimated 38.2 million children under the age of 5 years were overweight or obese (WHO, 2019). The prevalence of obesity has also reached an alarming rate in many developing countries including Malaysia in which 29.1% were overweight while 14% were obese based on previous National Health and Morbidity Surveys (NHMSs) carried out in Malaysia (Nor *et al.*, 2008; Mohamed, 2012; Chan *et al.*, 2017), in comparison with some South-East Asian countries such as Vietnam (2.1%), Cambodia (3.9%), Lao (5.3%), Myanmar (5.8%), Singapore (6.1%), Philipines (6.4%), Indonesia (6.9%), Thailand (10%), and Brunei (14%) (Nor *et al.*, 2008; Mohamed, 2012; Chan *et al.*, 2017).

In men, the relationship between the male reproductive system and obesity is poorly understood (Fernandez *et al.*, 2011; Fernández *et al.*, 2015). Some reports have shown that obesity in men is associated with a decrease in serum levels of total and

free testosterone leading to low sperm count (Du Plessis *et al.*, 2010; Fernandez *et al.*, 2011). On the other hand, there is a negative correlation between obesity and various semen parameters (Oliveira *et al.*, 2017), while a recent study has suggested that there is no relationship between increased body mass index (BMI) and sperm DNA (Bandel *et al.*, 2015).

Natural products are chemical compounds or substances produced by living organisms which could be derived from plants, animal, microorganisms and marine sources. For many years, natural products have been used in the prevention of diseases and have also played a very important role in health. The ancient civilizations of the North Africans, Indians and Chinese provide written evidence for the use of natural sources for treating various diseases (Moudgil and Khalil, 2016). In those early times, mandrake was prescribed for pain relief, turmeric possessed blood clotting properties, roots of the endive plant were used for the treatment of gall bladder disorders, and raw garlic was prescribed for circulatory disorders (Moudgil and Khalil, 2016). These natural products are still being used in several countries as alternative medicines (Arafat and Rahman, 2017). The role of these products in the treatment of obesity and fertility has received increased attention owing to the recent and rapid increase in the prevalence of obesity in the developed world (Hruby and Hu, 2015).

## 2.2 Definition and classification of obesity

Obesity is usually caused by an immoderate build-up of fat deposits in the body culminating in detrimental consequences on the total wellbeing of an individual (Russo *et al.*, 2016). The main factors required for the diagnosis of obesity include a waist-to-hip ratio (WHR), waist circumference (WC) and BMI. WHR is also a measure for some other serious health conditions, however, World Health Organisation (WHO)

defined obesity as WHR that is more than 0.90 for males and 0.85 for females (Mohamed *et al.,* 2014). Both men and women with WHR greater than 0.8 and 1.0, respectively, are prone to health risk due to increased fat distribution and have proven to be a more reliable predictor of cardiovascular disease than BMI and WC. Recently, there is evidence of WHR as an indicator of health risks (Anusruti *et al.*, 2020). The fat distribution around the waist can be calculated as follows:

WHR = Waist Circumference x Hip circumference (Anusruti *et al.*, 2020)

### 2.2.1 Diagnosis of obesity

In obesity clinics, patients are grouped into co-morbidity risk status based on the guidelines provided by clinicians for the recognition, evaluation, and medical care of obesity. The International Classification of Diseases Ninth Revision (ICD-9) diagnostic code is used to identify patient co-morbidities (Wagner *et al.*, 2016a). Up to 50% of the adult population in Organisation for Economic Co-operation and Development (OECD) countries have a BMI $\geq$ 25 Kg/m$^2$) (Pierre, 2016; Booth *et al.*, 2017). Table 2.1 outlines the classification of overweight, obesity and waist circumference. The various types of obesity are categorized based on their origin which is stated in Table 2.2. Overweight results when an individual consumes food above his level of activity, leading to a rise in the level of fat storage which is precipitated by impairment of the hormonal or metabolic system.

BMI levels are categorised as follows: Underweight (< 18.5 kg/m$^2$), Normal (18.5 – 24.9 kg/m$^2$), and Overweight. Obesity is further divided into Class I Obesity (25.0 – 29.9 kg/m$^2$), Class II Obesity (30.0 – 34.9 kg/m$^2$), Class III obesity (35.0 – 39.9 kg/m$^2$), and Extreme obesity (40 kg/m$^2$) (De Lorenzo *et al.*, 2016). Different obesity types have been identified which include central/ abdominal, android or apple

peripheral/ visceral, gynaecoid or pear, diffuse, localised, formerly obese, childhood, morbid and sarcopenic obesity (Mazidi and Kengne, 2017).

Table 2.1: Classification of overweight and obesity by BMI, waist circumference and associated disease risk

| | | | Disease risk* (relative to normal weight and waist circumference | |
|---|---|---|---|---|
| | BMI ($Kg/m^2$) | Obesity class | Men≥40 inch (102cm) Women ≥35 inch (88cm) | >40 inch (102cm) >35 inch (88cm) |
| Underweight | <18.5 | | | |
| Normal | 18.5 - 24.9 | | | |
| Overweight | 25.0 - 29.9 | | Increased | High |
| Obesity | 30.0 – 34.9 | I | High | Very high |
| | 35.0 – 39.9 | II | Very high | Very high |
| Extreme obesity | 40.0 | III | Extremely high | Extremely high |

Adapted from World Health Organization (Suleiman *et al*., 2019). BMI, Body Mass Index, *Disease risk for type 2 diabetes, hypertension, and coronary heart disease.

Table 2.2: Types of obesity

| **Types of obesity** | **Sex** | **Body region affected** | **Diseases associated with** | **References** |
|---|---|---|---|---|
| Central/ abdominal, android or apple | Male | Abdomen | Metabolic disorders | (Sahakyan *et al.*, 2015) |
| Peripheral/ visceral, gynaecoid or pear | Female | Buttocks, hips, and thighs | Infrequently associated with metabolic disorders | (Rosso *et al.*, 2016) |
| Diffuse | Both | Whole body | - | (Józsa, 2011) |
| Localized | Both | | Barraquer-Simons Syndrome, lipodystrophic disorders | (Datta *et al.*, 2006) |
| Formerly obese | Both | Skin (redundant cutaneous mantle) | - | (Rossi *et al.*, 2016) |
| Childhood | Children, adolescent | Whole body | Type 2 diabetes, asthma, fatty liver disease, cardiovascular disease | (Ogden *et al.*, 2014) |
| Morbid | Both | Whole body (BMI of more than 40) | high blood pressure or diabetes | (Kagan *et al.*, 2006) |
| Sarcopenic | Both | Low muscle mass, muscle strength, and high fat | geriatric syndromes | (Goisser *et al.*, 2015) |

Source: Suleiman *et al.*, 2019

### 2.2.2 Management of obesity

Obesity can be managed by a modification of lifestyle, medications, or bariatric surgery (Misra *et al.*, 2009). The modification of life's style consists of healthy dieting, indulging in physical activities and avoidance of high intake of alcohol. On the other hand, anti-obesity drugs like orlistat (Suleiman *et al*., 2020b) and metformin (Nna *et al*., 2019) are universally available and endorsed for long term use, in any case, these medications may have longer-term complexities of obesity. Their utilization is related to high rates of side effects such as vomiting and abdominal cramping (Waring *et al.*, 2009). Over the past 50 years, patients with morbid obesity have received bariatric surgery as a treatment option (Bardia *et al.*, 2007). It comprises the adjustment of the digestive tract by either restricting the gastric volume or changing the route of the food bolus creating an element of malabsorption. Bardia et al. (2007) reported that several barriers militate against the management of obesity which includes, patient, physician, allied health care, health system and socio-economic factors.

## 2.3 Male reproductive system

### 2.3.1 Structures and functions of male reproductive system

The main sex organ in males is the testes which are located in the scrotum hanging between the upper thighs (Peate, 2016). During the intrauterine life, they grow around the adjoining kidneys but, just before birth, they descend into the scrotum. Each testis is oval and about ½ inches long by 1 inch wide (Darda, 2017). The Leydig cells are responsible for the production of testosterone which in turn stimulates the production of sperm and initiates secondary sex characteristics beginning at puberty

(Martin, 2016). Spermatogenesis occurs in the seminiferous tubules which are usually referred to as the functional units of the testis. Once the sperm cells are produced, they move from the seminiferous tubules into the rete testis for further maturation (Sharma *et al.*, 2019).

Sertoli cells present in the seminiferous tubule are known as "mother cells" because they nurture sperm cells and provide secretory and structural support during the process of spermatogenesis. They also possess follicle stimulating hormone (FSH) receptors and are usually stimulated by FSH (Chojnacka *et al.*, 2016).

The sperm complete their maturation with their flagella becoming motile in the epididymis. Furthermore, they are also stored there until the ejaculation. The epididymis comprises of three parts: the proximal part known as head or caput epididymis which conveys the sperms from the testis, the middle part called the body or corpus epididymis and the tail or cauda epididymis which partakes in conveying the sperms to the vas deferens (Neto *et al.*, 2016).

Vas deferens is otherwise called the sperm duct, it moves on its side into the abdominal cavity through the inguinal canal and then emerges from the epididymis in the scrotum (Jiménez-Reina *et al.*, 2016).

Consequently, the seminal vesicles are found posterior to the urinary bladder. Their main function is to secrete fructose which provides energy for sperm and alkalinity to enhance sperm motility (Kalthur and Kalthur, 2017). On the other hand, ejaculatory ducts are responsible for receiving sperm from the vas deferens and the secretions from the seminal vesicle. The two ejaculatory ducts, therefore, open into the urethra (Kalthur and Kalthur, 2017). During ejaculation, the contraction of the smooth muscle of the prostate gland helps in the expulsion of semen from the urethra (Jiménez-

Reina *et al.*, 2016). Urethra then opens into the glans penis and allows for the movement of urine and ejaculation of semen (Neto *et al.*, 2016).

### 2.3.2 Steroidogenesis in male reproductive system

Steroidogenesis is responsible for the production of steroid hormones in various tissues such as the testis, ovaries and adrenal gland. However, of these hormones secreted, testosterone and FSH are the main regulators of spermatogenesis (Sanderson, 2006). The Leydig cells under the influence of luteinizing hormone (LH) are responsible for the synthesis of androgens referred to as testosterone. Similarly, LH is essential for the induction and advancement of spermatogenesis. (Pasquali, 2006) reported that obese men are usually characterized by decreased levels of testosterone due to increasing body weight. On the other hand, the gonadotrophs situated in the anterior pituitary synthesize glycoproteins known as FSH which are usually controlled by pulses of gonadotropin-releasing hormone (GnRH) (Ilacqua *et al.*, 2018).

FSH acts in conjunction with LH in stimulating the maturation of germ cells and induces the formation of Sertoli-Sertoli intracellular tight junctions (Edelsztein and Rey, 2019). The combination of FSH and testosterone binds to receptors on the Sertoli cells which stimulate the production of cAMP thereby mobilizing and transporting cholesterol into the steroidogenic pathway, the cAMP-dependent protein kinase (PKA) activates cholesterol from intracellular cholesterol pools. It is then transferred into the inner-mitochondria membrane by the action of the steroidogenic acute regulatory protein (StAR) and converted to pregnenolone by P450 side chain cleave (Bhadula, 2019).

Pregnenolone circulates into the smooth endoplasmic reticulum where it is converted to progesterone via the action of 3-hydroxysteroid dehydrogenase-4-5 isomerase (3-HSD) (Wang *et al.*, 2019). Meanwhile, the progesterone is converted to 17-hydroxy progesterone, then into androstenedione with the aid of 17-hydroxylase lyase (P450c17). Following this, the androstenedione is then converted to testosterone via the aid of 17-hydroxysteroid dehydrogenase (17-HSD) found on the inner-face of mitochondrial inner matrix membrane (O'Donnell *et al.*, 2017).

### 2.3.3 Spermatogenesis in male reproductive system

Spermatogenesis is referred to as an aggregate of processes that take place within the seminiferous tubules and comes to a climax in the production of male gamete (Griswold, 2015). Spermatogenesis comprises a pool of stem cell, duration of the proliferation of spermatogonia, meiosis, spermiogenesis and the release of spermatozoa into the testicular tubule lumen (Desai *et al.*, 2017). The process of spermatogenesis takes approximately 74 days in humans but more recent studies have revealed that it may vary between 42 to 76 days. The daily range of sperm production in humans is between 150 and 275 million spermatozoa (Neto *et al.*, 2016)

### 2.3.4 Testicular energy metabolism in male reproductive system

Sertoli cells are the largest reservoirs of lactate in the testis which they prefer as the most preferred energy metabolites. These metabolites are produced principally from glucose. The glucose transporters (GLUTs) serve as the rate-limiting step for the movement of glucose from the extracellular space into germ cells (Oonk *et al.*, 1989). The Sertoli cells have at least four GLUTs (GLUT1, 2, 3 and 8) identified till date.

However, GLUT8 is not used for the transportation of glucose from the extracellular space because it is not present in the plasma layer of Sertoli cells but instead, the endoplasmic reticulum layer takes up glucose from the extracellular space into the germ cells (Beattie *et al.*, 1984; Yu *et al.*, 2019). Additionally, the germ cells are constantly nourished by lactate dehydrogenase (LDH) (Fan *et al.*, 2018). On the other hand, the monocarboxylate transporters (MCTs) help in the transportation of lactate from Sertoli cells to the germ cells (Figure 2.1).

Regardless of being a vitality substrate, glucose is not the fundamental metabolite utilized for the production of ATP in the Sertoli cells (Bernardino *et al.*, 2019). Therefore, Sertoli cells can still proceed with their functions irrespective of inadequate glucose, as ATP and lactate are produced from the digestion of amino acids, glycogen and lipids. Accordingly, lipid β oxidation is by all accounts the fundamental metabolic pathway utilized by Sertoli cells to create vitality (Antognelli *et al.*, 2018).

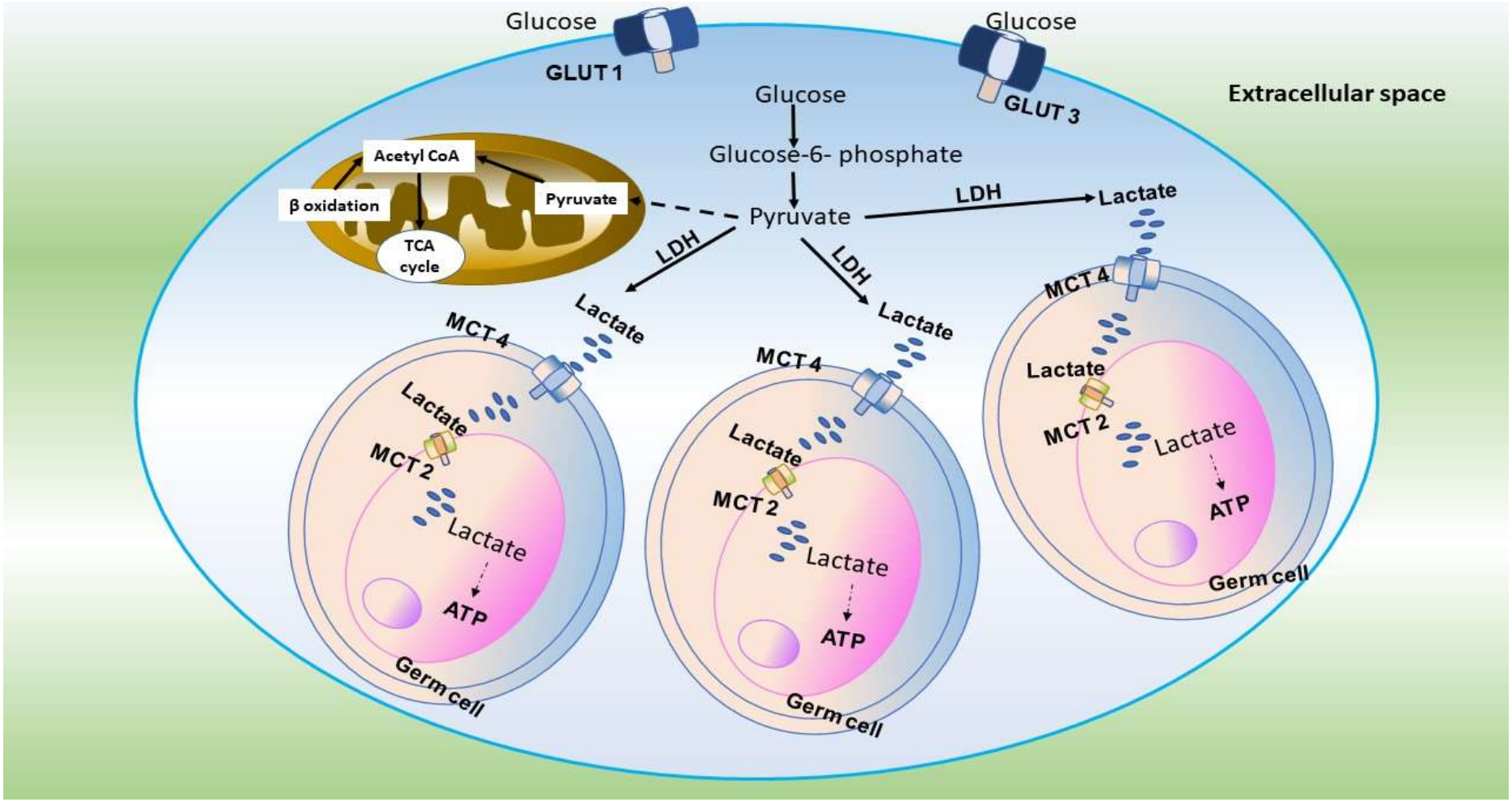


Figure 2.1: Diagram of Sertoli cell energy metabolism

Acetyl CoA: acetyl coenzyme A, ATP: adenine triphosphate, GLUT: glucose transporter, LDH: lactate dehydrogenase, MCT: monocarboxylic transporter, TCA: tricarboxylic acid cycle

### 2.3.5 Male reproductive hormones

Leydig cells are major sites for the production of circulating androgens known as testosterone. In males, for instance, it is vital for the development and maintenance of reproductive tissues such as seminal vesicles, epididymis, penis, testis, prostate, increase in muscle mass and hair growth in the axilla and pubic region (Cunningham *et al.*, 2016). About 95 % of circulating androgenic hormones are produced and secreted from the Leydig cells, which is about 6-7 mg per day, and also adrenal cortex.

Testosterone is synthesized from cholesterol which is often transported via translocator protein (TSPO) from the outer mitochondrial membrane to the inner mitochondrial membrane and the StAR. Saad *et al.* (2017) reported that testosterone deficiency is usually identified with increased sarcopenia and obesity but treatment with testosterone in advanced aged men with testosterone deficiency increases insulin resistance, glucose metabolism, and body composition. Similarly, in a study carried out on 470 men aged 65 years with low libido to assess the responses to specific sexual activities, treatment with testosterone showed increases in testosterone and estradiol levels associated with significant changes in sexual activity and desire (Cunningham *et al.*, 2016).

Estradiol is a type of estrogen which plays a basic role in male sexual behaviour and function. Microsomal P450 aromatase enzyme is responsible for the metabolism of testicular estrogen from testosterone, which converts testosterone to estrogen and testosterone/estradiol ratio. In men, it is very vital for modulating libido, erectile function, and spermatogenesis. There is an abundance of estrogen receptors and aromatase in testis, brain and penis, which are very important for sexual function (Jardí *et al.*, 2017). Previous researches have shown that estradiol is present not only in the

reproductive tract of the adult male but also in the brain. In animals and humans, there are higher concentrations of estradiol in the male reproductive tract and semen than in the serum, especially in Leydig and Sertoli cells. Recent research has shown that estradiol synthesized by germ cells within the seminiferous tubules contributes significantly to the hormonal milieu within the tubules (Schulster *et al.*, 2016).

Follicle stimulating hormone (FSH) also known as gonadotropins is an important manager of gonadal and fertility functions. It is produced by gonadotrophs situated in the anterior pituitary (Grinspon *et al.*, 2018). In men, spermatogenesis is initiated when FSH binds to the Sertoli cells inside the seminiferous tubules (Zhang *et al.*, 2017). Another important function of FSH is the stimulation of the Sertoli cells to produce inhibin, whose functions is to suppress the release of FSH from the pituitary, thereby decreasing testosterone secretion. This hormone shows a direct correlation with Sertoli cell function and sperm number, similarly, inhibin B may be used as a marker of spermatogenic activity (Campo *et al.*, 2019).

Luteinizing hormone (LH) or interstitial cell stimulating hormone secreted by gonadotrophs situated in the anterior pituitary (Uemura *et al.*, 2018). It acts in conjunction with FSH. Low blood testosterone levels cause the release of the hypothalamic GnRH which stimulates the release of LH into the circulation system (Shah *et al.*, 2019). In the testis, the increase in the secretion of testosterone is caused by the binding of LH to the receptors situated on the Leydig cells (Karacaoğlu and Selmanoğlu, 2018; Khalid *et al.*, 2018). Similarly, whenever the testosterone level within the blood circulation gets to its basic limit, it will bind to androgen receptors on the nerve center and anterior pituitary, thereby suppressing the synthesis and release of GnRH and LH, separately. In any event of a decrease in testosterone level, the hormone does not bind with the receptors to a similar manner as before, therefore

GnRH and LH are again released as well as stimulating more testosterone generation (Egba *et al.*, 2017).

### 2.3.6 Role of inducible nitric oxide synthase in male reproduction

Recently, the role of inducible nitric oxide synthase (iNOS) an isoform of nitric oxide synthase (NOS) in the male reproductive system has been elucidated and the direct relationship of dietary factors such as cholesterol, fatty acids and vitamins with constitutive and inducible NO production in mammalian cells have also been established in previous studies (Lee and Yan, 2003; Sohrabi *et al*., 2017).

One of the known mechanisms of immune defence that triggers cytokines against inflammation, infection and cancer is the induction of iNOS, this may occur without any underlying pathological condition especially in the brain, testis and penis leading to an increased level of NO in these tissues (Vernet *et al*., 1995; El-Sweedy *et al*., 2007). The increased NO, as a result, leads to the formation of peroxynitrite which causes apoptosis and subsequently proteolysis. In the penis of an obese person, this process could cause the loss of smooth muscle and nerve tissue involved in the erectile response (Cheng *et al*., 2004).

It is worthy to note that the effects of neuronal nitric oxide synthase (nNOS) and endothelial nitric oxide synthase (eNOS) in the synthesis of nitric oxide as a mediator of penile erection, are well established. In penis, two main sources for the release of NO have been identified; these are the non-adrenergic noncholinergic nerve terminals and endothelium lining of the cavernosal cisternae as well as blood vessels, together they stimulate the guanylate cyclase and cGMP synthesis in the penile smooth muscle. The reduction of intracellular $Ca^{2+}$ causes relaxation of the penile tissue, leading to increased blood flow into the corporal sinusoids and culminating into

swelling of the penis. In rat penis, for example, nNOS and eNOS enzymes catalyze NO synthesis by the conversion of L-arginine to L-citrulline (Garban *et al.*, 1997).

The male sexual act is divided into four phases: excitement, plateau, orgasm, and resolution which collectively is termed as "sexual response cycle" (Boily *et al.*, 2009). In man, several changes occur during this act, for instance, there are visible vascular responses such as skin flush, penile erection, and rise in blood pressure; glandular secretions (Cowper's glands, seminal vesicles, prostate); and stimulation of penile muscles, culminating into seminal emission and ejaculation (Estrada-Reyes *et al.*, 2019). This is achieved by nitric oxide (NO) which is the main intermediary of penile erection. (Champion *et al.*, 1999; Magee *et al.*, 2002).

### 2.3.7 Oxidative stress in the testis

The imbalance between oxidation and reduction reactions in a particular system which leads to increase generation oxidants is referred to oxidative stress (Wagner *et al.*, 2018). In other words, oxidative stress occurs when the generation of reactive oxygen species (ROS) supersedes the antioxidants generated by the body, culminating into cellular damage. One of the main derivatives of oxygen ($O_2$) is ROS. It is worthy to note that some ROS are not true free radicals, for instance, $H_2O_2$. Peroxyl ($ROO\cdot$) and hydroxyl ($OH^-$) radicals, superoxide ($O_2^-$) anion, and hydrogen peroxide ($H_2O_2$) are all ROS. Nitric oxide ($NO^-$) and peroxynitrite anion ($ONOO^-$) though not regarded as ROS play an important role in reactions in infertility (Bhattacharya, 2015).

Most infertile men have oxidative stress as the main causative factor of infertility and testicular oxidative stress have been linked to some environmental pollutants. During metabolism in the body, energy is released as a result of oxidative

phosphorylation which occurs in the mitochondria where free radicals are formed. This is defined as having one or more unpaired electrons in the outermost orbital of an oxygen atom. ROS induces cyclic adenosine monophosphate (cAMP) in spermatozoa that inhibit tyrosine phosphatase leading to tyrosine phosphorylation.

$H_2O_2$ stimulates capacitation via tyrosine phosphorylation triggering a cell signalling cascade. Capacitation not only requires ROS, but it can be inhibited by catalase (CAT) (Bardaweel *et al.*, 2018). It has been described that high levels of ROS promote the acrosome reaction, whereas the presence of CAT or superoxide dismutase (SOD) inhibits the acrosome reaction. Measurement of MDA appears to be of some clinical relevance since its concentration within both seminal plasma and sperm is elevated in infertile men with excess ROS production, compared with fertile controls or normozoospermic individuals (Deshpande *et al.*, 2018).

As oxidative stress is the result of an imbalance between ROS production and total antioxidant capacity (TAC), direct tests reflect the net biological effect between these two opposing forces. The most widely used method of assessing sperm membrane peroxidation is the measurement of MDA levels in sperm or seminal plasma with the thiobarbituric acid assay (Meitern, 2016).

### 2.3.8 Inflammation in the testis

The testis has been reported to play an important role in the immune system while performing its traditional role of maintaining spermatogenesis, it also has the immune-testicular barrier which explains the increased in the proportion of CD8+/CD4+ within the testis in relation to the systemic circulation (Li *et al.*, 2012). The following types of cells are found within the testis; they are macrophages, immune, natural killer and mast cells however, the presence of lymphocytes in the